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COLLEGE OF BASIC AND APPLIED SCIENCES

HEPATITIS DELTA VIRUS (HDV) IN HIV/HBV CO-INFECTED PATIENTS IN GHANA

BY

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A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE AWARD OF DEGREE OF MASTER OF PHILOSOPHY IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES

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DECLARATION

I, Keren Okyerebea Attiku, do hereby declare that, with the exception of cited references, this thesis is my original research work and no part has previously been submitted for the award of a degree or any other qualification at this or any other institution. The work was done under the supervision of Dr. Osbourne Quaye at the Department Biochemistry, Cell and Molecular Biology and Dr. Joseph Kofi Bonney, Department of Virology at Noguchi Memorial Institute for Medical Research, University of Ghana.

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ABSTRACT

Within persons infected with HIV worldwide, almost 10% have chronic HBV infection, and about 6% of all HBV infected patients are co-/superinfected with HDV, resulting in accelerated progression of hepatitis towards end stage liver disease. Even though within HIV/HBV infected patients, an increase in HDV infection has been observed, there is inadequate information on HDV prevalence as well as virologic profile in Ghana. This study sought to determine the presence of HDV in HIV/HBV co-infected patients in Ghana.

This was a longitudinal purposive study which enrolled 113 patients, who have been co-infected with HIV and HBV, attending clinic at Korle-Bu Teaching Hospital (KBTH) in Ghana. Patient demographic information was obtained using a questionnaire, and 5 mL whole blood collected at two-time points (baseline and 4-6 months afterwards). The sera obtained were tested to confirm the presence of HIV, HBV antibodies/antigens and HBV DNA. Antibodies and viral RNA were also determined for HDV with respective serological and molecular assays. Amplified HBV DNA and HDV RNA were sequenced with the Sanger method and sequence reads analyzed using Sequencher software version 2.3. Phylogenetic analysis was carried out with references from the GenBank to establish the genotypes using the MEGA X software.

Out of 113 samples tested, 63 (55.7%) were females and 50 (44.25%) were males. Patients enrolled in this study were within the age range of 24 to 73 years, with a median age of 45 years. A total of 100 (88.5%) samples had detectable HBV surface antigen (HBsAg), and 32/113 had detectable HBV DNA. Nucleotide sequences were obtained for 15 samples, and phylogenetic analysis showed the circulating strains to be HBV genotype E. Of 13 samples that were HBsAg unreactive, 4 (30.8%) had detectable HBV DNA and suggest the presence of an occult infection. HDV was

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detected in four samples and giving a prevalence of 3.54% in the HIV/HBV cohort used for the study. Two of the HDV positive samples sequenced were HDV genotype 1.

In summary, the data suggest the presence and circulation of HDV and occult HBV infection in HIV patients co-infected with HBV in Ghana. The occurrence of occult HBV infection and HDV in the study population is supported by other research studies, and makes it imperative to look out for the presence of HDV and occult HBV in patients co-infected with HIV/HBV.

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DEDICATION

I dedicate this work to God Almighty for seeing me through this two academic year course with His grace and mercy. I dedicate this work also to my mum (Erica Minta-Asare), mother-in-law (Gladys Attiku), my husband (Bryan Edwin Attiku) and my sons (McJason Attiku and Ehud Attiku)

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University of Ghana http://ugspace.ug.edu.gh

TABLE OF CONTENTS

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

No table of figures entries found.

University of Ghana http://ugspace.ug.edu.gh

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LIST OF TABLES

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LIST OF ABBREVIATIONS

HDV	Hepatitis delta virus
HIV	Human immunodeficiency virus
HBV	Hepatitis B virus
KBTH	Korle-Bu Teaching Hospital
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
WHO	world health organisation
HBcAg	Hepatitis B core antigen
HDAg	Hepatitis delta antigen
L-HBsAg	Large hepatitis B surface antigen
S-HBsAg	Small hepatitis B surface antigen
M-HBsAg	Medium hepatitis B surface antigen
mRNA	Messenger ribonucleic acid
ORF	Open reading frame
RNP	Ribonucleoprotein
hNTCP	human sodium taurocholate co-transporting polypeptide
POL	Polymerase
ADAR1	Adenosine deaminase acting on RNA 1
L-ADAR1	Large adenosine deaminase acting on RNA 1
S-ADAR1	Small adenosine deaminase acting on RNA 1
IFN	Interferon
cccDNA	Covalently closed circular deoxyribonucleic acid
CHD	Chronic Hepatitis delta

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IVDU	Intravenous drug user
IgG	Immunoglobulin G
ELISA	Enzyme linked Immunosorbent Assay
IU	International units
NMIMR	Noguchi memorial institute for Medical Research
AIDs	Acquired immune disease syndrome
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
PCR	Polymerase chain reaction
CDC	Centre for Disease Control
TAE	Tris acetate ethylenediamine tetraacetic acid
TBE	Tris borate ethylenediamine tetraacetic acid
VL	Viral load

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CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Background

Viral hepatitis has emerged as a public health concern affecting hundreds of millions of people globally and resulting in significant morbidity and deaths through the establishment of acute and/or chronic infections, as well as development of Liver cancer (Thomas et al., 2015). Chronic hepatitis remains asymptomatic for years in some patients whilst in others, progresses rapidly to cirrhosis and finally to hepatocellular cancer (HCC) (Villeneuve, 2005). Viral hepatitis is mostly caused by hepatitis A virus, hepatitis C virus (HCV), hepatitis B virus (HBV), hepatitis E virus and hepatitis D virus (HDV), (Lee et al., 2008). All five hepatitis (i.e. A, B, C, D and E) viruses cause acute hepatitis while HCV, HBV and HDV cause chronic hepatitis leading to cirrhosis and HCC (WHO, 2017a). In 2015 alone, 1.34 million deaths resulting from viral hepatitis were recorded; a high proportion (96%) being attributed to HBV (66%) and HCV (30%), with the remaining 4% caused by Hepatitis E Virus (3.3%) and Hepatitis A Virus (0.8%) (WHO, 2017a).

Hepatitis B disease incidence is mostly found in the African (6.1%) and the Western Pacific (Asia 6.2%) Regions as defined by WHO (WHO, 2017a). Hepatitis B virus causes chronic infection in about 257 million people globally (Thio et al., 2013), and the likelihood of developing a chronic infection being highest in infected people below 5 years of age (WHO, 2017b). Eventually, people with chronic HBV infection develop liver cirrhosis and HCC leading to 15-25% deaths (Shepard et al., 2006). Transmission of Hepatitis B virus is mainly through exposure to infectious blood or body fluids (Schillie, 2018), including the use of intravenous drug (WHO, 2009), sexual intercourse (Urbanus et al., 2009), as well as other risk factors such as through patient care

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management, blood transfusion, dialysis. In Ghana, studies conducted in 2016 reported an HBV seroprevalence of 12.3% (Ofori-Asenso & Agyeman, 2016), and another annual report for the same year recorded 1,144 cases of chronic viral hepatitis B infection with 24 deaths (Ghana Health Service, 2016).

The advent of antiretrovirals for the management of Human immunodeficiency virus (HIV), have increased the lifespan of persons living with the infection. However, HBV infection has been linked with higher liver related deaths in persons living with HIV (Rockstroh, 2006). Human immunodeficiency virus and HBV are blood borne viruses that share common routes of transmission (WHO, 2017b), with the presence of HIV, having been shown to promote chronicity, liver fibrosis and malignancy in HBV infection (Ibrahim et al., 2004; Kourtis et al., 2012; Matthews et al., 2011; Mphahlele, 2008; Stabinski et al., 2011). The probability of developing chronic HBV in HIV/HBV co-infected patients have been shown to be 6 times more, than their mono HBV associates (Gatanaga et al., 2000; Singh & Wong, 2009). Of the global estimated number of 36.7 million people living with HIV, 2.7 million (7.4%) are known to be co-infected with chronic HBV (Ranjbar et al., 2011), with a higher proportion of the HIV/HBV co-infected persons in sub-Saharan Africa (71%) (Easterbrook et al., 2015), in Ghana, a high HBV prevalence of 13.6% has been reported in people with HIV (Agyeman & Ofori-Asenso, 2016).

Hepatitis delta virus (HDV) infects only persons with hepatitis B infection (WHO, 2017a), and the D virus infections are shown to be acquired through sexual and parenteral routes as well as through blood transfusions and injections, which are also vital routes for HBV and HIV transmission (Farci, 2003). It has been shown that Co-infection of HDV and HBV worsen the severity of acute hepatitis and hepatic decompensation, with an associated increase in liver related mortality. Super-infections of HDV add extensively to the high burden of chronic liver diseases (Smedile et al.,

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1981). A previously stable chronic carrier of HBV can rapidly progress to cirrhosis within two years after a super-infection with HDV (Rizzetto M., 2010). Globally, about 20 million people who are infected with HBV also are infected with HDV (Sy et al., 2015), and in West Africa, a 10.0% prevalence of HBV/HDV has been reported (Jaquet et al., 2017). Meanwhile, a study in Mongolia has reported that 60% of hepatitis B infected patients may also be infected with HDV (Chen et al., 2016). Thus, there is an extensive ambiguity in the report of HBV/HDV prevalence in many countries, since HBV-infected patients are not usually tested for HDV (WHO, 2017a).

Hepatitis D Virus in HIV/HBV leads to accelerated liver disease progression with higher proportions of liver cirrhosis and liver related deaths as well as complications in treatment possibilities compared with HDV in HBV mono-infected patients (Hung et al., 2014). In addition, current infection with HDV complicates viral treatment since the regimen used in the management of HBV does not have an effect on HDV (WHO, 2017a). Though, prevention of HBV results in prevention of HDV, treatment of HBV/HDV co-infected patients differs from that of HBV mono infected patients (WHO, 2017a).

1.1.2 Problem Statement

Hepatitis D virus infection has developed to be a significant public health concern throughout the world, with continuous increase in the rate of HDV infection in HIV/HBV co-infected patients (Soriano et al., 2011). With the dearth of information on deaths due to HBV with HDV as a cofactor (WHO, 2017), as well as limited information on HDV prevalence and virologic profile among HIV patients (Thio et al., 2013) both in Ghana and globally, it has become necessary to further investigate the prevalence, biochemical indices and disease severity of HBV- HDV co-infection in HIV patients.

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1.1.3 Justification

Studies on HDV in Ghana have been conducted mainly in HBV mono-infected patients, leaving the already immunocompromised HIV patients who are co-infected with HBV not catered for. With the majority of deaths occurring in HIV infected patients being attributed to the presence of HBV whose presence equally renders the patient compromised to HDV infection, the disease burden as well as circulating genotypes needs to be studied.

1.1.4 Aim

To identify and characterize HDV and HBV in HIV patients co-infected with HBV using serological and molecular tools.

1.1.4.1 Specific Objectives of study

1. To determine the prevalence of HDV in HIV/HBV co-infected patients
2. To quantify HDV and HBV viral load in HIV/HBV co-infected patients
3. To determine the risk of increase in hepatic decompensation due to HDV in HIV/HBV co-infected patients
4. To determine the genotypes of HDV and HBV in HIV/HBV co-infected patients.

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1.2 LITERATURE REVIEW

1.2.1 Virus Classification

Hepatitis delta virus (HDV) was discovered in 1977 by Rizzetto and colleague scientists whilst investigating why some Hepatitis B infected patients had more serious disease compared to others. In the course of the investigation, a novel antigen, later called the hepatitis delta antigen (HDV) was discovered in the hepatic cells of only hepatitis B patients whose hepatitis B surface antigen (HBsAg) test were positive but whose hepatitis B core antigen (HBcAg) test was negative (Rizzetto et al., 1977). This novel infectious particle now known as HDV consists of an RNA genome coated with HBV envelope proteins (source: Rizzetto et al., 1980).

Classified within the new Deltavirus genus, (Lai, 1995), HDV is the only animal virus to possess a circular single strand negative sense RNA genome with structural characteristics and mechanism of replication which was only previously seen in plant viroids and virusoids (Chen et al., 1986). Dissimilar to viroids, HDV has a lot more nucleotides in its genome compared to the 200 to 400 nucleotides a viroid RNA contains. Also, although HDV codes for its own hepatitis D antigen (HDAg), viroids lack ability to code for proteins. (Abbas & Afzal, 2013). The genome of HDV encodes a ribozyme domain of about 80 to 100 nucleotides long which exhibits a self-cleavage activity. The high GC nucleotide content of HDV as well as its replication through the rolling circulation seems to make the virus unique among animal viruses (Flores et al., 2011a). Currently, HDV is assigned the genus delta virus and is still awaiting a complete taxonomic classification into order and family.

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1.2.1 Virology of HDV

1.2.1.1 Nature of HDV

Hepatitis D virus is the smallest amongst all viruses known to cause an infection in humans. With HDV being the smallest virus, it has a diameter of about 35-37 nm and is spherical. HDV features a spherical, negative sense, single stranded, RNA genome comprising of about 1700 nucleotides (Alfaiate et al., 2015; Abbas & Afzal, 2013; Lin et al., 1990). The RNA genome is an enveloped by proteins derived from HBV surface proteins and single virus particle is composed of approximately 200 hepatitis delta antigen (HDAg) molecules (Gudima et al., 2002). The RNA molecule forms an unbranched rod-like structure resembling viriods through extensive base pairing of approximately 74% (Flores et al., 2012; Taylor & Pelchat, 2010). However, HDV possess more nucleotides compared to viriods. In addition, HDV has the ability to code for its antigens HDAg whereas viriods lack the capability to code for proteins (Flores et al., 2012).

Though HDV encodes its own antigens, it requires HBV for replication (Flores et al., 2012). All three HBV envelopes including the large (L-), medium (M-), as well as the small (S-) HBsAgs are required to surround the HDV genome. The HDV genome encloses a domain that is about 85 nucleotides long encoding a ribozyme that is used during transcription to cleave multimeric RNA molecules generated into a long unit of genomic or antigenomic sequences (Alfaiate et al., 2015; Dastgerdi et al., 2012; Flores et al., 2011b; Greco-Stewart & Pelchat, 2010). The ribozyme sequences among HDV genotypes are very well conserved (Alfaiate et al., 2015) As a result of the uniqueness of HDV genomic make up, there are currently two theories considered regarding the origin of HDV – one theory suggests an evolvement of HDV from either splicing of the host cells pre-mRNA or from plant viroids (Taylor, 2014).

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1.2.1.2 Evolution from a virusoid or a retroviroid

Looking at the complexity of genomes in the biological scale, plant viroids are thought of as the smallest units. Over thirty species of plant viroids have been identified which have comparatively single stranded small mostly circular RNA molecules usually of 250 to 400 nucleotides. Similarly, the plant viroids also do not have coding ability thus depends on host cell enzymes to replicate their genomes. Examples of such plant viroids are the potato spindle tuber viroid, *Popsiviriodae* and avocado surblotch viroid, *Avsunviroidae*. The common factors are the rod-like RNA structure in addition to the replication of HDV nuclear which relates to that of *Popsiviriodae* and the presence of a ribozyme combined with the rolling circle replication mechanism of HDV being similar to *Avsunviroidae*. These theories/experiments leave a dearth of knowledge concerning the relationship between HDV and HBV as well as the source/origin of the delta antigen (Flores et al., 2012).

1.2.1.3. Evolution from a host mRNA precursor & associated ribozyme

The second theory suggests the evolution of hepatitis delta virus from transcriptomes of cells from its host. To support this theory, various studies were conducted which showed HDV ribozyme having similar biochemical properties and secondary structures to host ribozyme RNA being found in the cells of humans such as found in the intron of the gene, CPEB3. It was later observed through an after- study that numerous ribozymes in human cells were similar to the HDV ribozymes through a search based on structures of the HDV ribozyme. Further experiments showed that pseudoknot ribozymes were found in all kingdoms of life with the exception of Archae and viruses of insects (Webb et al., 2009; Salehi-Ashtiani et al., 2006). *In-vitro* studies have established some of these identified RNA ribozymes as active and some further demonstrated an *in-vivo* transcription. Furthermore, based on where these RNA ribozymes are located in the genome of the

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host, suggests the significance of retro-transposition and how important biological regulation of host genes are selected (Taylor & Pelchat, 2010). In addition, the delta antigen has also been proposed to have originated from host cells where an HDV-like species might have been replicated within the cell through a host RNA employed as a template (Teixeira et al., 2004). A potential candidate protein, the delta interacting protein A, was initially identified as the origin for the delta antigen. Though this suggestion was later dismissed, delta interacting protein A may still be considered as a partner to the hepatitis delta antigen (Taylor & Pelchat, 2010; Long et al., 1997; Brazas & Ganem, 1996).

Studies involving the processing of nascent human β-globin messenger RNAs show that these mRNAs contain a ribozyme domain that is found downstream of the CA acceptor site and poly (A) signal. This ribozyme domain has been shown to be conserved in other primate globin genes as well as present on the antigenomic RNA of HDV suggesting an evolutionary conservation (Teixeira et al., 2004)

Interestingly, this theory is suggested to complement the first theory hence an integrated model of the two theories suggest that hepatitis delta virus might have been generated from re-combination of a viroid-like element and host cellular pre-mRNA and mRNA (Robertson, 1996).

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1.2.2 Structure of HDV and HBV

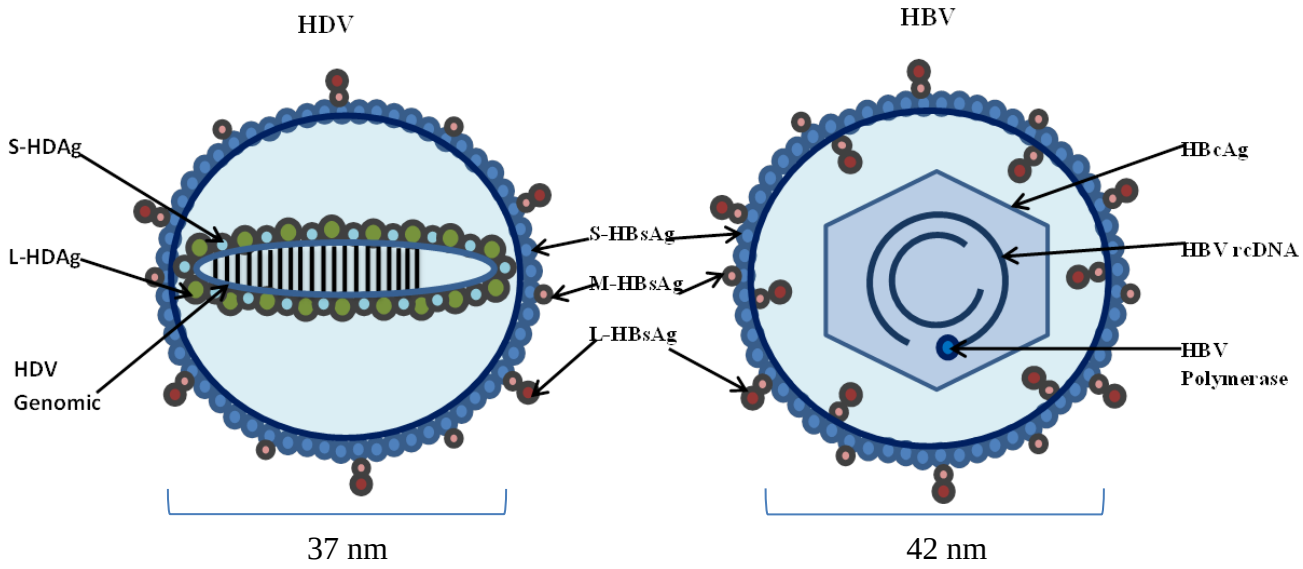

Figure 1.1: Structure of HDV and HBV, showing the shared HBsAg envelope proteins.

S-HDAg =small hepatitis delta antigen, L-HDAg =large hepatitis delta antigen, S-HBsAg =small Hepatitis B surface antigen, M-HBsAg =medium Hepatitis B surface antigen and L-HBsAg =large Hepatitis B surface antigen, HBcAg = Hepatitis B core antigen, HBV rcDNA = Hepatitis B relaxed circular DNA. Adapted from (Mentha et al., 2019).

The first characterized circulating Hepatitis D virion was obtained after infecting a chimpanzee with serum obtained from an Italian who was a chronic carrier of HDV. The 37nm HDV particles comprise a ribonucleoprotein and an envelope. The envelope proteins are made up of the three types of HBV surface antigens as HDV being a defective virus, can't code for its envelope proteins. Three forms of envelope proteins – medium, small and large HBsAg – are used by HDV to envelope its antigens, exit and re-enter into hepatocytes. The three HBsAg have a C-terminus with the S-domain as a common property. The medium HBsAg has a Pre-S2 domain on the hydrophilic domain of the N-terminal whilst the N-terminal of the L-HBsAg has Pre-S2 and an additional domain called the Pre-S1 (Urban et al., 2014). Studies have shown that for HBV replication, the L-HBsAg, though not sufficient is important for HBV to assemble and cause an infection, while

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on the other hand S-HBsAg is required for the budding out of particles from infected cells (Gudima et al., 2007).

1.2.2.1 Viral RNAs

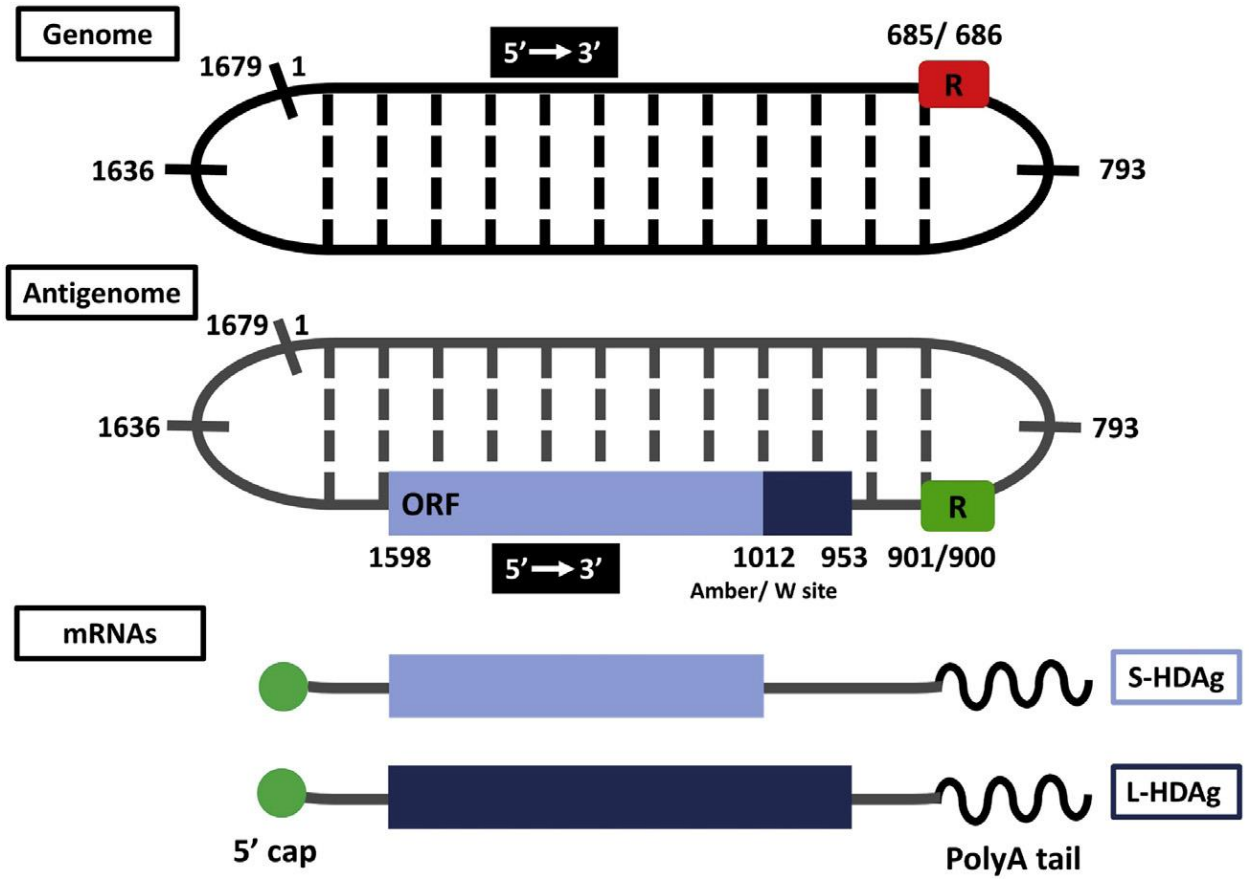

Figure 1.2: Various types of RNA associated with HDV genome and replication

Genomic RNA is first transcribed to antigenomic RNA which serves as a transcript for making more copies of genomic RNA as well as transcription into mRNA. The mRNA is translated into the two isoforms of the delta antigens (HDAg), the large and small hepatitis delta antigens. ORF = Open Reading Frame, R = Self cleaving ribozyme. (Alfaiate et al., 2015)

The HDV has two isoforms of the delta antigens, the large (L-HDAg) and the small (S-HDAg) that associates with the ribonucleoprotein which contains the hepatitis D viral genome to form an important structure nuclear transport of the HDV RNA as well as for assembly of the virus

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(Tavanez et al., 2002). The whole genome of HDV particle was reported in 1986 and was described as a single stranded, covalently closed, circular RNA virus with just about 1,700 nucleotides having about 60% C / G content as well as 74% intramolecular base pairing which makes the genome to pleat into a rod-like structure (Wang et al., 1986). Apart from the genomic RNA, two other types of RNA are found during replication within infected cells (Fig. 2), which are the mRNA which codes for the S-HDAg and L-HDAg (Gudima et al., 2000) and the antigenome RNA, which acts a replicative intermediate by serving as a complementary sequence of the HDV genome and also possess the coding sequence for the HDAg. In addition, the antigenome is 5–22 times less ample than the genomic RNA, and is not packaged into a virion as it is found solely within the nucleus of infected cells (Macnaughton & Lai, 2002; Chen et al., 1986).

Hepatitis D virus ensures a high replication rate in infected cells by generating approximately 3.0 x 10^5 molecules of the HDV genome, within both the cytoplasm and the nucleus (Macnaughton & Lai, 2002; Chen et al., 1986). Hepatitis delta RNA is transcribed by cellular host DNA-dependent/RNA-polymerase II and matures in a similar manner to cellular mRNA by capping and polyA-tailing (Gudima et al., 2000; Hsieh et al., 1990). Hepatitis delta proteins are however, made from a particular 800 nucleotide length of the HDV mRNA (Lo et al., 1998).

1.2.2.2 Ribozyme

Self-cleaving RNA sequences of approximately 85 adjoining nucleotides called ribozymes in the genomic and antigenomic materials of HDV, are accountable for the cleavage of multimeric linear molecules of RNA that emanate during the replication process of HDV (Wu et al., 1989; Kuo et al., 1988). The ribozymes are generated during the transcription of HDV genome or antigenome sequences. The sequences of these ribozymes are also well conserved among all HDV genotypes with different crystal structures of the HDV ribozyme being observed with its cleavage mechanism

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characterized as a pseudoknot-like mechanism. The structural and functional characteristics of HDV ribozyme are unique and distinct from other viroid (Serganov & Patel, 2007).

1.2.2.3 Viral proteins

The HDV RNA is suggested to have 9 open reading frames (ORFs) with 4 ORFs in the genome and 5 ORFs in its antigenome but the virus makes only one viral protein which is the HDAg. However, a small polypeptide has been described to be produced from ORF-K, an alternate ORF (Bichko et al., 1996; Wang et al., 1986). Though the antigenomic molecule contains the coding region for HDAg, translation occurs from a linear mRNA and translation of HDAg leads to the generation of two HDAg isoforms; small, (S)-HDAg of approximately 24 kDA and a large, (L)-HDAg approximately 27 kDA. Both isoforms of HDAg originate from the same ORF. The S-HDAg is the first isoform to be generated in infected cells and can be translated from the transcript obtained from first round transcription of the HDV genome.

The S- and L- HDAgs share a common N-terminus with 195 amino acids enclosing several functional domains which include RNA binding motifs, a helix-loop-helix motif, a coiled-coil domain, and a nuclear localization signal (Fig 1.3). Both HDAg isoforms interact with host factors hence undergoes several post-translational modifications to modulate several host cellular functions that contributes to HDV's replication and pathogenesis.

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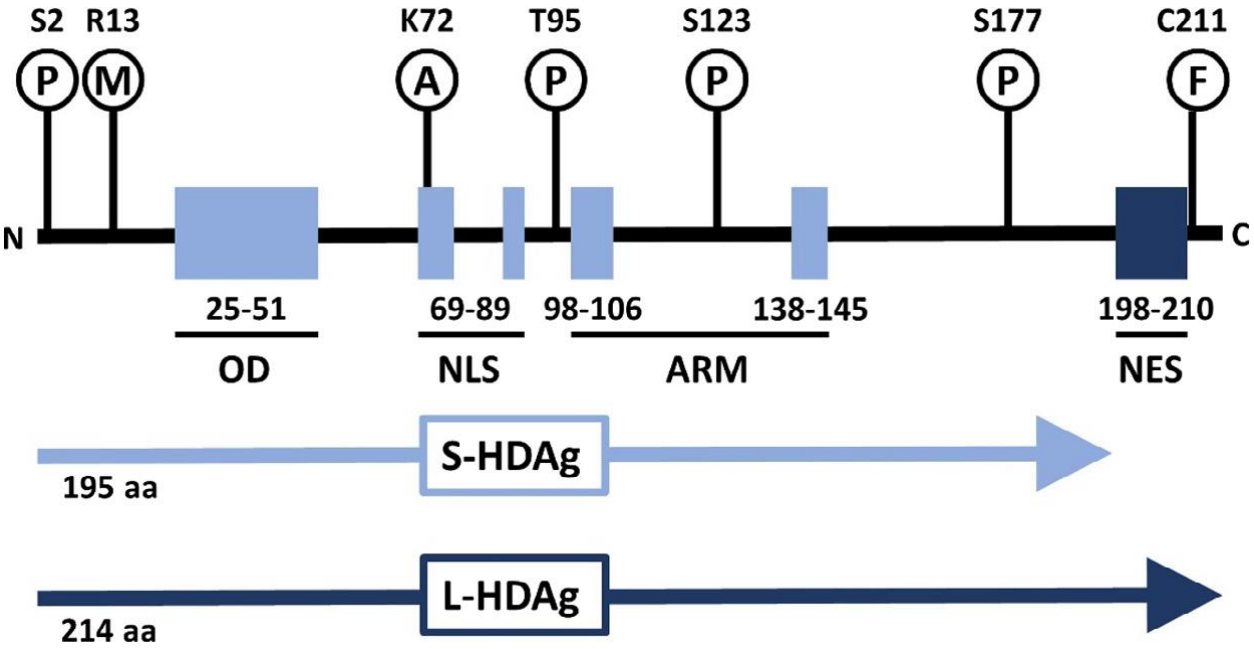

Figure 1.3: Schematic diagram of the functional regions of HDAgs

The S- and L- HDAgs share some common functional domains such as the oligomerisation domain, nuclear localisation signal (NLS), and arginine-rich motif. The additional amino acid of the L-HDAg confers an additional domain which is the nuclear export signal to it. Both forms of HDAg undergo modifications after translation, such as Phosphorylation (P), acetylation (A), methylation (M), and farnesylation (F) (Alfaiate et al., 2015).

Some of these post-translational modifications mainly include addition of moieties such as phosphorylation of serine, acetylation of lysine, arginine methylation and sumoylation of 5lysine (Lai, 2005; Glenn et al., 1992). The additional 19 residues present in the L-HDAg which is proline rich, possess extra domains including a viral assembly signal, a prenylation site, and a nuclear export signal (Lee et al., 2001). For instance, prenylation of the cysteine residue at position 211 of L-HDAg, by host cellular farnesyltransferase has been proven to be involved in particle assembly of the virion (Glenn et al., 1992). This confers extra virus-host interactions for the L-HDAg. Therefore, Small and Large HDAgs play diverse roles during the HDV's replication cycle, hence inducing varying pathogenic mechanisms and effect.

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Though Small HDAg does not have polymerase activity, it is essential for initiating HDV replication, elongating the enzyme RNA Pol II as well as the retaining HDV RNAs during the HDV's life cycle (Kuo, Chao, & Taylor, 1989; Yamaguchi et al., 2001). On the other hand, the L-HDAg operates as a major inhibitor to HDV replication, by distinctively inhibiting the synthesis of the HDV genome, but not that of the antigenome, and is necessary for packaging and assembly of the virion (Chang et al., 1991; Chao et al., 1990; Modahl et al., 2000)

1.2.2.4 Ribonucleoprotein

The HDV protects genomic RNAs by associating with HDAg to form an RNP which is present in both the virion and infected cells. The formation of RNP is critical for the assembly of the virus particle. However, it is also required for the nucleocytoplasmic exchange of the HDV RNAs (Tavanez et al., 2002). The HDAg oligomerizes and associates with the RNA molecules hence the different morphology and stoichiometry of the ribonucleoprotein have been proposed. A study by Ryu et al., proposed a molar ratio of 70 HDAg for every genome molecule in the virion, whereas an average of 30 HDAg per both genome and antigenome molecule in the nucleus of infected cells to form RNP structures (Ryu et al., 1993). A later study suggested an association of 200 molecules of HD antigens per genome molecule in both the virion and cells that have been infected (Gudima et al., 2002). Importantly, rather than its primary sequence, the secondary structure seems to dictate how specific HDAg binds to the genome (Griffin et al., 2014).

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1.2.3 Viral life cycle

An overall illustration of HDV life cycle in a hepatocyte co-infected with HBV is described in Fig. 1.4. The following text details the important steps of the HDV life cycle.

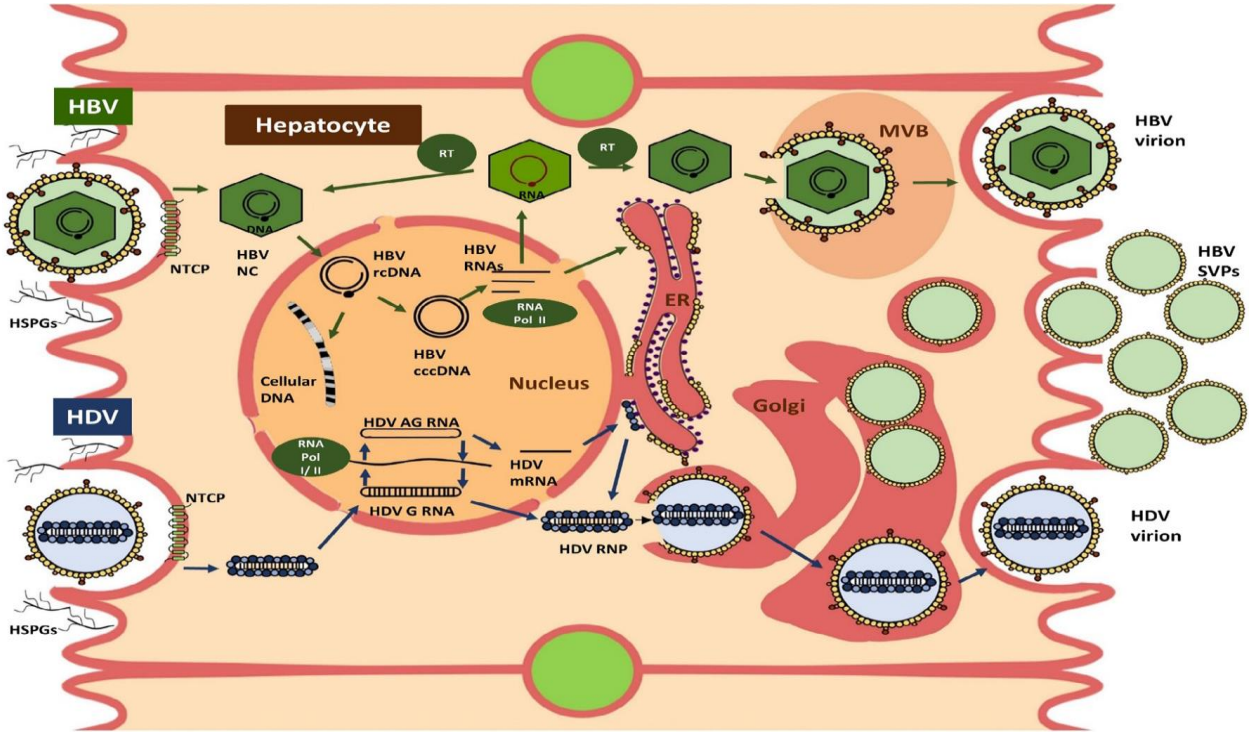

Figure 1.4: A Schematic illustration of the life cycle of HBV and HDV

To cause an infection in a hepatocyte, HDV requires the presence of HBV in the same hepatocyte. With HDV requiring the use of HBsAgs as an envelope, the site and receptors for attachment and entry into the hepatocytes are thought to be the same and mediated by the binding of NTCP with the pre-S1 domain of HBsAg. Apart from entry points and receptors being the same for both viruses in the hepatocyte, replication pathway of HDV genome and assembly are independent of HBV replication pathway (source: Alfaiate et al., 2015).

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1.2.3.1. Attachment and entry

1.2.3.1.1 Hepatotropism.

The tropism and productive replication of HDV is dependent on the entry process and co-infection with HBV. The productive cycle of HDV necessitates the expression of HBV surface glycoproteins within the same infected cell to facilitate assembly of the HDV virions. However, other processes of the replication cycle of the two viruses are independent since no other component or HBV proteins are required to aid HDV replication and neither HDV components nor proteins are needed for HBV replication (Bichko et al., 1996). HDV is thought to primarily target the hepatocytes (Negro et al., 1989) although in contrast to HBV, HDV genome replication has been observed to take place in different mammalian cells if experimentally well delivered into the cell as its genome is not dependent on liver-specific transcription factors (Taylor, 2009). Recent studies by He et al., 2015, demonstrated HDV infectable hNTCP transgenic mice; supporting the statement that HDV replication is not only in human hepatocytes, when HDV genome is delivered rightly into the other cell type.

Considering that HDV and HBV share similar envelope proteins, HDV is thus thought to have the same entry points as HBV (Sureau & Negro, 2016). Meanwhile, to cause an infection HBV and HDV depend on the presence of some 75 amino acids found in the Pre-S1 domain of the N-terminal of the L-HBsAg (Urban et al., 2014) as well as some S-HBsAg specific amino acid residues (Abou-Jaoudé & Sureau, 2007; Sureau et al., 1994).

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1.2.3.1.2 Viral factors

The L-HBsAg envelope protein is important for both HBV and HDV infections. Specific mutations or deletions within the Pre-S1 domain (i.e. the 75 amino acids of the N-terminal) or impairing myristoylation or acetylation activity leads to the inhibition of infectivity (Blanchet & Sureau, 2007; Gripon et al., 1995). The antigenic loop domain and glycosylation pattern of the S-HBsAg with specific mutations in this domain also seem to play a role in inhibiting infection independent of the PreS1 domain (Julithe et al., 2014; Le Duff et al., 2009).

1.2.3.1.3 Host factors

Viral attachment and receptor-specific entry of HDV and HBV have been experimentally shown to be through the binding of both viruses to the carbohydrate side chains, which are the heparin sulfate proteoglycans – HSPGs that are found on the outer surface of the hepatocytes (Longarela et al., 2013; Sureau & Salisse, 2013; Leistner et al., 2008; Schulze et al., 2007).

Though specific type of HSPGs are yet to be determined, Glypican-5 has been described to be involved in this HBV/ HDV viral attachment and entry process (Verrier et al., 2016) although its removal did not completely block infection of HBV and HDV suggesting that is not solely responsible for HBV/HDV infection (Verrier et al., 2016; Han et al., 2011).

Further studies have also suggested that purinergic receptors (P2XRs and P2YRs) potentially have a role to play in attachment and entry of HBV and HDV (Longarela et al., 2013). After attachment, the virus further interacts with a co-receptor to allow entry into hepatocytes and up until 2012, this receptor was unknown.

A study from Yan et al., 2014, led to identification of a functional receptor for HBV and HDV, human sodium taurocholate co-transporting polypeptide (hNTCP), encoded by SLC10A1) that aids HBV and HDV entry into liver cells. The hNTCPs, an integral transmembrane glycoprotein

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have been shown to be involved in the taking up of bile acids into the hepatocyte and is situated at the basolateral membrane of liver cells , suggesting that residues that are involved in bile uptake and not that of sodium are what facilitates HBV/HDV infection (Yan et al., 2014).

An interaction between the myrsitoylated N-terminal viral sequence - 75 amino acids - of the L-HBsAg's Pre-S1 region and the NTCP bile acid binding domain found in the outer leaflet (helix5) of the cell membrane , have been shown to be important and appropriate for HDV and HBV infection (Ni et al., 2014; Yan et al., 2014)

1.2.3.2 Uncoating and transport into the nucleus

Though the L-HDAg of HDV has been identified as clathrin-adaptor-like protein, there is currently no evidence to support clathrin-mediated internalization. However, internalization of HBV has been suggested to be through the clathrin-dependent endocytosis compartments of the endosome without the protease and acidification activities (Huang et al., 2007; Huang et al., 2012; Macovei et al., 2013) mediating nuclear transport of HDV's RNP and uncoating of the viral genome after fusion. Interaction of importins with HDAg is known to be involved in the translocation of HDV RNP between cytoplasm and the nucleus where RNA polymerases I and II facilitate replication of the viral genome. Polymerase I present in the nucleolus initiates the transcription of viral genome into antigenome whilst polymerase II present within the nucleoplasm enhances the replication of the genome from the antigenome as well as the transcription of mRNA (Huang et al., 2008; Tavanez et al., 2002; Chou et al., 1998).

1.2.3.3 Replication

Replication of HDV occurs in the nucleus independently of HBV and through the entire replication process, antigenomic RNA molecules are restricted to the nucleus, whilst genomic RNA can either

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proceed to a new cycle of replication in the nucleus or be transported and assembled into new infectious virions in the cytoplasm (Macnaughton & Lai, 2002).

In summary, the HDV replication uses the rolling circle mechanism where multimeric liver transcripts that are complementary to the HDV genome are synthesized. The synthesized antigenomic RNA is sliced by its own self cleaving ribozyme, separating the monomers from the multimeric transcripts. These monomers are ligated to form circular antigenomic molecules that serve as a template for synthesis of genomic strand of the newly synthesized molecules (Greco-Stewart et al., 2007).

1.2.3.3.1 Double rolling-circle mechanism of replication

The HDV employs an RNA-dependent RNA replication involving the use of a double rolling-circle mechanism that is dependent on two types of rod-like RNA templates with inverse polarity, the genomic and antigenomic RNA templates. The double rolling-circle mechanism is initiated by transcribing the antigenome from the viral genome RNA to produce an antigenomic RNA through the enrolment of host-cell DNA-dependent RNA polymerases and template switching (Sureau & Negro, 2016; Taylor, 2009; Lai, 2005). Antigenome of more than one unit length is produced leading to the production of intermediate multimeric linear transcripts (Sureau & Negro, 2016; Taylor, 2009; Lai, 2005).

The replication of HDV RNA, require three enzymatic activities: 1) a polymerase to generate antigenomic RNA oligomeric strands from circular viral templates, 2) a ribozyme-dependent RNase activity to intrinsically self-cleave the antigenomic strands into strands, and 3) cellular ligases to ligate and circularize the antigenome to form monomers (Alfaiate et al., 2015; Abbas & Afzal, 2013).

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Till date, HDV is not known to have its own polymerases for its RNA replication which is in contrast to some RNA viruses of larger genomes and neither uses the polymerase of HBV thus, HDV is heavily dependent on host-cell factors (Lai, 2005; Taylor, 2009). The DNA dependent RNA polymerase of the host cell have been implicated to mediate HDV replication with several studies supporting the involvement of RNA Pol II :- i. HDV RNA display a 5' capping and a 3' poly-A tail similar to that of cellular mRNA (Gudima et al., 2000; Hsieh et al., 1990). ii. The transcription of HDV RNA has been shown to be inhibited by α-amanitin (Chang et al., 2008). iii. RNA Pol II has been demonstrated to bind both to genomic and anti-genomic polarity of HDV RNA (Chang et al., 2008; Greco-Stewart et al., 2007). Although experiments have shown the binding of RNA Pol I and III to HDV RNA, the Pol I have been associated with transcription of HDV antigenome with some reports suggesting that Pol I has some level of resistance to the inhibition of HDV antigenome transcription by α-amanitin whilst the exact function of Pol III has so far not been reported. The possibility of HDV RNA utilizing or interacting with Pol I and Pol II during replication suggests that the genome and antigenome could be synthesized within different nuclear compartments (Greco-Stewart, et al., 2009; Li et al., 2006; Modahl et al., 2000). Therefore, these studies suggest that HDV has the ability to redirect host cell DNA-dependent RNA-polymerase to its RNA template even though the mechanism of replication of HDV is similar to plant viroids, the means by which HDV uses to capture the host DNA dependent RNA polymerase and use for its own replication is still not known (Rackwitz, et al., 1981). Several hypotheses have been proposed, and the precise role of the different polymerases in HDV replication is yet to be elucidated, one of such is the fact that Pol II of the host cell is thought to be able to recognize the sites found at both poles of the rod-like genome; thus, suggesting the role played by the HDV RNA secondary structure (Greco-Stewart et al., 2007).

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1.2.3.3.2 Role of S-HDAg

The regulation of HDV replication is dependent on both forms of the HDAg, with the L-HDAg being important for assembly as well as inhibit replication of the virus whilst the S-HDAg is important for transcription of the 3 forms of viral RNAs (Chang et al., 1991; Yamaguchi et al., 2001), as well as interact with diverse subunits of Pol I and II leading to the enrolment of chromatin remodelling complexes unto the HDV RNA (Abeywickrama-Samarakoon et al., 2018).

The S-HDAg is a nuclear protein demonstrated to bind to HDV RNA, hence plays a crucial role in the hijacking of RNA Pol II. The binding of S-HDAg to HDV RNA, represents structural resemblances to transcription factors such e.g. NELF-A, and as transcriptional regulatory protein which can undergo modifications such as acetylation and (Lai, 2005). In addition S-HDAg contributes to the enhancement of transcription by stimulating elongation or by reverting inhibitory factors through its binding to RNA Pol II (Yamaguchi et al., 2001). Furthermore, in a screening for proteomic-RNA interference, S-HDAg was demonstrated to interact biochemically with 9 out of the 12 subunits of RNA Pol II (Cao et al., 2009). The interaction of S-HDAg is not limited to RNA Pol II, as it has also been demonstrated to interact and/or co-localize with nucleolar proteins including B23 and nucleolin, hence supporting the role of Pol I in HDV replication (Huang et al., 2001).

1.2.3.4 Cleavage

Unit-length linear RNA templates serve as precursors for processing genome and antigenome molecules through the catalytic cleavage by ribozyme. This ribozyme activity makes use of the auto-cleaving ribozyme sequences that is found in both genomic and antigenomic RNAs which are transcribed at least twice from circular templates to create a unit length, extenuating the presence of multimers (Alfaiate et al., 2015).

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1.2.3.5 Ligation

Ligation is employed to circularize the linear transcript monomers into genomic and antigenomic molecules during HDV replication. The ribozyme sequences of HDV have been shown to possess self-ligating properties (Sharmeen et al., 1989). Meanwhile, other studies suggest that ligation of HDV RNA molecules occurs mainly in mammalian cells, thus recruitment of a host-cell ligase is assumed to be involved in the ligase activity of the linear monomers into circular forms (Reid & Lazinski, 2000).

1.2.3.6 RNA Editing and Large HDAg Synthesis

Hepatitis Delta virus is known to have 9 ORFs of which one, located in the viral antigenome, directs the synthesis of the S-HDAg and L-HDAg. The difference between the two isoforms is the editing of the antigenome by cellular adenosine deaminase acting on RNA 1 (ADAR1) which adds a further 19 amino acids to the C- terminal of the S-HDAg to form the L-HDAg (Casey, 2011; Glenn et al., 1992) where both isoforms ensures a balance between replication of the genome and assembly into virion particles (Casey, 2011). The ADAR1 also exist in two isoforms – small ADAR1 (S-ADAR1) which is always expressed and large ADAR1 (L-ADAR1) whose expression is dependent on stimulation by type 1 interferon (IFN 1) (Mentha et al., 2019). Editing of ADAR1 has been studied to take place on the amber/w site which normally contains a stop codon (UAG) during translation and leading to production of the S-HDAg. Thus, when ADAR1 acts on this amber/w site, it deaminates the adenosine (A) in the stop codon to inosine which during transcription leads to the production of tryptophan (UGG) instead of a stop codon, UAG, in the mRNA eventually leading to the translation of the extra 19 amino acids which results in the synthesis of the L-HDAg (Casey, 2011). A pictorial description is found in figure 1.5.

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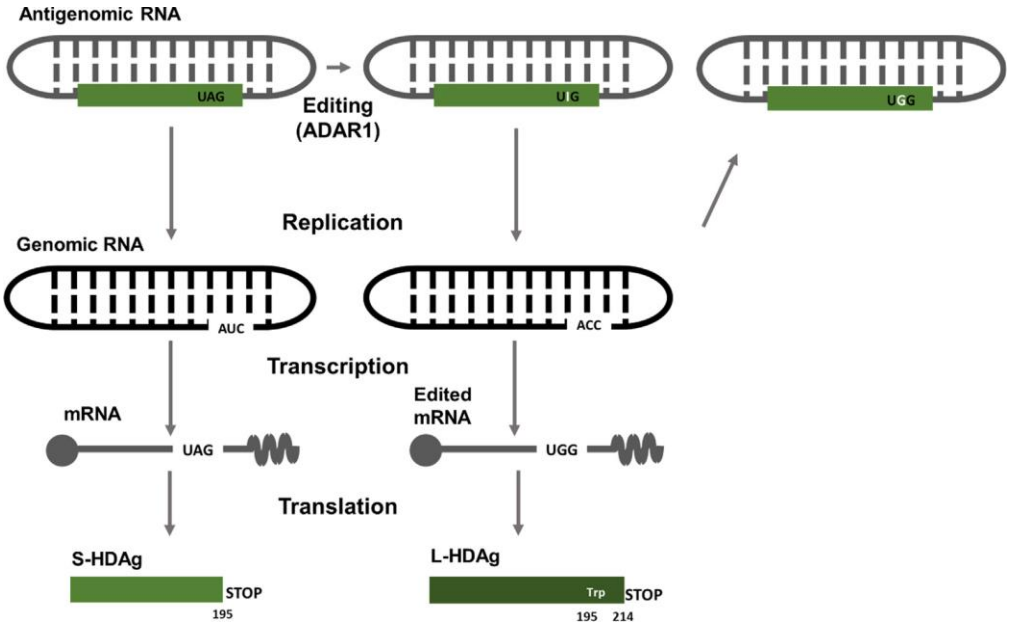

Figure 1.5: Synthesis of hepatitis delta antigens

The synthesis of small hepatitis D antigen (S-HDAg) and the birth of the large hepatitis D antigen (L-HDAg) after post translational modification by adenosine deaminase acting on RNA 1 (ADAR1). mRNA = messenger ribonucleic acid (source: Alfaiate et al., 2015).

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1.2.3.7 Post Translational Modifications of HDAg Proteins

After production of the two isoforms of HDAg, they undergo post translational modifications to determine the roles played by each HDAg during replication. When the two serine residues of HDAg are phosphorylated, the S-HDAg is able to interact with the host cells Pol II, thus enabling HDV RNA to be replicated (Abbas & Afzal, 2013). Meanwhile, the additional 19 amino acids encodes a farnesylation signal which enables the addition of a farnesyl lipid group covalently at position 211 to cysteine by the enzyme farnesyltransferase which is important for inhibition of HDV RNA replication and as well as virion assembly. Experiments in-vitro and in-vivo have proven that when farnesyl lipid group is inhibited from being added to the L-HDAg's C-terminus,

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the interaction between L-HDAg and HBsAg is consequently inhibited preventing secretion of the HDV virion (Bordier et al., 2003; Casey, 2011; Mentha et al., 2019).

1.2.3.8 Viral Assembly and Release

In a typical infection to form HDV virions and be released from the cells, HDV has to be co-infected with HBV in the same cell and in as much as HDV replication is independent of HBV, for HDV particles to be released from the hepatocytes, HDV requires an interaction of the N-terminus of L-HDAg with the S-HBsAg to form an infectious virion (Hwang & Lai, 1993; Sureau et al., 1993).

During HBV infection, the S, M, and L HBsAgs, produced in larger quantities than needed for HBV complete virions, can assemble together and be released as empty subviral particles. These un-infectious subviral particles are released more than the infectious HBV virions and are thought to play a role in immune escape of the infectious HBV particles.

Given that the infectious HDV particles and the subviral particles are similar in terms of composition and the subviral particles are released through the Golgi apparatus whilst HBV infectious particles are secreted via the multivesicular body pathway, HDV is thought to also be released through the Golgi apparatus (Bonino et al., 1986; Sureau & Negro, 2016). This is because, the cytoplasmic domain of HBsAg located between the PreS1 and PreS2 junction, does not seem critical for the assembly and release of HDV (Taylor, 2012; Watanabe et al., 2007; Zeisel et al., 2015).

Clathrin has also been suggested to be involved in export of viral particles after trafficking of HDV RNP (Huang et al., 2009). Regarding the envelopment of HDV viral components, farnesylation which involves adding a 15 carbon chain to C211XXQ box peptide motif, found on the carboxy-terminal of the Large hepatitis D antigen and which is conserved among all the HDV genotypes,

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have been demonstrated to be crucial as this modification intercedes the interaction with the S region of the HBsAg (Hwang & Lai, 1993).

1.2.4 Pathogenesis

Although the pathogenesis of HDV is not clear, viral replication is known to happen only in the hepatocytes. Thus, damages caused by the HDV are associated mainly with the liver cells. There are several schools of thoughts on how this damage is caused. According to Niro G. A. and Smedile A., 2012, liver damage is thought to be caused by immune response in HDV infection. Other studies suggest a direct cytopathic effect on liver cells principally in the acute hepatitis phase by HDV (Ponzetto et al., 1987; Govindarajan et al., 1986; Canese et al., 1984; Kamimura et al., 1983) and is categorized by eosinophilic cytoplasm, pyknotic nuclei and the presence of slight inflammatory cells in the liver parenchyma. The described cytopathic effects are evident from cell culture system (Cole et al., 1993) and human studies (Lefkowitch et al., 1987; Popper et al., 1983). The S-HDAg is assumed to be liable for this direct cytopathic effect (Popper et al., 1983), with L-HDAg promoting HDV chronicity making liver cells susceptible to immune-mediated injury.

Experimental models such as cell culture, human studies and woodchuck animal models are instrumental for the testing of efficacy, protective role of novel treatments and vaccine candidates for HDV. The use of these experimental models has shown that DNA and protein immunizations are ineffective against HDV super-infection (Fiedler et al., 2001; Fiedler & Roggendorf, 2001).

During acute and chronic HDV super infection, differences in immune-mediated responses such as delayed and insufficient immune response as well as recognition of limited viral epitopes lead to failure in HDV infection clearance. This may explain persistence and chronicity of HDV super-infections (Casey et al., 2006). Interaction of HDV with HBV is thought to influence the pathogenesis of HDV (Hourioux et al., 1998; Niro et al., 2005).

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1.2.5 HBV/HDV Interaction

Work done in experimental models has shown that an infection with HDV causes a decrease in the replication of HBV although the expression of HBsAg is not affected much. This phenomenon has been proven by the observation of an increased HBsAg/HBV DNA ratio when cells were co-infected with HDV (Alfaiate et al., 2016). Experimental studies conducted using samples obtained from patients infected with HDV, revealed a decline in HBV replication although dominance of HDV after infection has so far not been established due to variations in HBV/HDV levels over time (Pollicino et al., 2011).

To inhibit HBV replication but ensuring constant availability of HBsAg for HDV assembly i.e. infectious particles, several mechanisms have been suggested: - 1) HDV antigens have been proposed to regulate the transcriptional activity of cccDNA by transcribing more PreS or S mRNA than the pregenomic RNA. 2) HBV enhancer sequences have been shown to be repressed by S-HDAg and L-HDAg thereby having an effect on the replication of HBV. 3) HDAg may bind to the mRNA of HBV selectively affecting HBV mRNA stability (Chen et al., 2018; Mentha et al., 2019). 4) Increasing experimental evidence indicates that HBsAg is abundantly produced even in the absence of HBV replication by the integrated HBV DNA and has been shown to assist in the assembly and release of infectious HD virions. Although not demonstrated in-vivo, HDV has been hypothesized to complete its life cycle by using HBsAgs produced by the integrated HBV DNA when in the same hepatocyte without depending on HBV replication (Mentha et al., 2019; Freitas et al., 2014).

Hepatitis D virus has been proven to induce type-1 interferon response strongly (Giersch et al., 2015) as the viral RNAs of HDV are recognized by the melanoma differentiation antigen 5 (MDA5) which increases the expression of antiviral interferon stimulated genes such as the MxA

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thus contributing to inhibition of HBV replication although HBV is considered not to be directly recognized by the innate immune system (Zhang et al., 2018; Giersch et al., 2015; Wieland et al., 2004). Although the effect of stimulated host cell IFN on HDV is not clear, HDV replication has been shown to be inhibited when an external source of IFN alpha (IFN-α) is introduced. The inhibition of HDV replication has been shown to be as a result of ADAR1 expression due to L-HDAg (Giersch et al., 2017; Hartwig et al., 2004).

In comparison to HBV mono-infection, HDV/HBV chronic infection results in a more rapidly progressive severe hepatic disease leading to early development of cirrhosis, decompensation HCC and a shorter survival (Romeo et al., 2009; Sheldon et al., 2008; Taylor, 2006).

Three stages of HDV chronicity have been proposed – a primary active stage where HDV is actively replicating with suppressed HBV; the second stage involves moderately active HDV replication and reactivation of HBV. The last stage is characterized by the development of cirrhosis and HCC resulting from either HBV or HDV replication. Remission may result from a noticeable reduction of both HBV and HDV viruses (Hadziyannis, 1997).

1.2.6 HIV/HBV/HDV Co-infection

Transmission and infection with HBV and HDV is common in individuals infected with HIV as a result of shared transmission routes (Wang et al., 1987). The wide use and success of HIV antiretroviral therapy has revealed that clinical complications and death in HIV/HBV/HDV co-infected patients was mostly due to liver diseases (Chen et al., 1986). Studies conducted before highly active antiretroviral therapy (HAART) was introduced for the management of HIV infection as regards to the effect of HDV infection on patients with HBV/HIV co-infection have shown inconsistent results (Sheldon et al., 2008). Some authors suggest that co-infection with HIV leads to a more severe cause of chronic hepatitis delta (CHD) (De Pouplana et al., 1995; Housset

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et al., 1992) where as others have shown that long-term HDV infection is not swayed by concomitant HIV infection (Buti et al., 1996; Pol et al., 1994).

To this same end, HDV/HBV/HIV trio infection might lead to exacerbation and rapid progression of chronic liver disease, liver failure and death (Novick et al., 1988; Saravanan et al., 2014; Stroffolini et al., 1994). Some studies estimate 1.9% to 5% of HIV infected patients to be co-infected with HBV and HDV (Puoti et al., 1998; Shukla & Poles, 2004) whilst another study observed a high prevalence of HDV (36%) among HIV/HBV co-infected patients (Saravanan et al., 2014). Though HDV co-infection bestowed a virologic benefit by suppressing HBV replication, this is not the same in patients co-infected with HIV (Buti et al., 1996).

Treatment guidelines on HIV/HBV co-infection management outlined the significance of early treatment of chronic HBV even though efficient antivirals are readily available to treat patients co-infected with HBV and HIV (Puoti et al., 1998). With the absence of an effective treatment for HDV infection (Branch & Robertson, 1984; Hughes et al., 2011) still being an issue, various studies have shown the value of potent anti-HBV nucleos(t)ides analogue therapy for CHD (Sheldon et al., 2008). To this effect, some studies observed no effect of anti-HIV antivirals such as lamivudine a potential HBV replication inhibitor (Lau et al., 1999; G. Niro, et al., 2005) and famiclovir (Yurdaydin et al., 2002) on the replication of HDV. Another study has shown that clevudine reduces HBsAg and cccDNA levels more potently (Peek et al., 2001). In woodchuck animal models, clevudine was observed to inhibit HDV RNA (Casey et al., 2005), emphasizing the assertion that only potent anti-HBV therapies that successfully reduce HBsAg and cccDNA might reduce HDV replication (Sheldon et al., 2008).

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1.2.7 Epidemiology of HDV

HDV infection mainly spread through parenteral exposure and globally distributed (Niro et al., 2010; Rizzetto et al., 1990) with an estimated co-infection or super-infection of 15-20 million with HBV. Hence, the virus is endemic to regions with a high prevalence of HBV including Middle East, Eastern and Mediterranean Europe, Sub-Sahara Africa and northern part of South America (Hughes et al., 2011; Wedemeyer et al., 2007).

1.2.8 Geographical Distribution of HDV and Genotypes

Fifteen (15) million out of 350 million chronic HBV carriers have serological indication of exposure to HDV (Farci, 2003). The virus is prevalent in West Africa, the Amazon Basin, the Horn of Africa, eastern and Mediterranean Europe, parts of Asia and the Middle East (Rizzetto, Ponzetto, & Forzani, 1990). High rates of HDV infections are observed in HBV endemic regions with exceptions to Indonesia (Depamede et al., 2009) and Vietnam (Nguyen et al., 2007). Hepatitis Delta virus genotype 1 is worldwide (Hughes et al., 2011; Shakil et al., 1997), whilst genotype 2 is found in Japan (Imazeki et al., 1990; Pascarella & Negro, 2011), Taiwan (Wu et al., 1998) and Yakutia-Russia (Ivaniushina et al., 2001). Genotype 3 is common in the Amazon Basin (Alvarado-Mora et al., 2011; Paraná et al., 2006) with genotype 4 found in Japan (Lin et al., 2015; Sakugawa et al., 1999) and Taiwan (Wu et al., 1998). HDV genotypes 5 to 8 are found in individuals with African origin (Hughes et al., 2011; Le Gal et al., 2006; Pascarella & Negro, 2011; Radjef et al., 2004). Like any successful virus causing infection, HDV has evolved to sustain it spread within the human population. HDV employs 3 evolutional mechanisms including mutation, editing and recombination (Chao, 2007).

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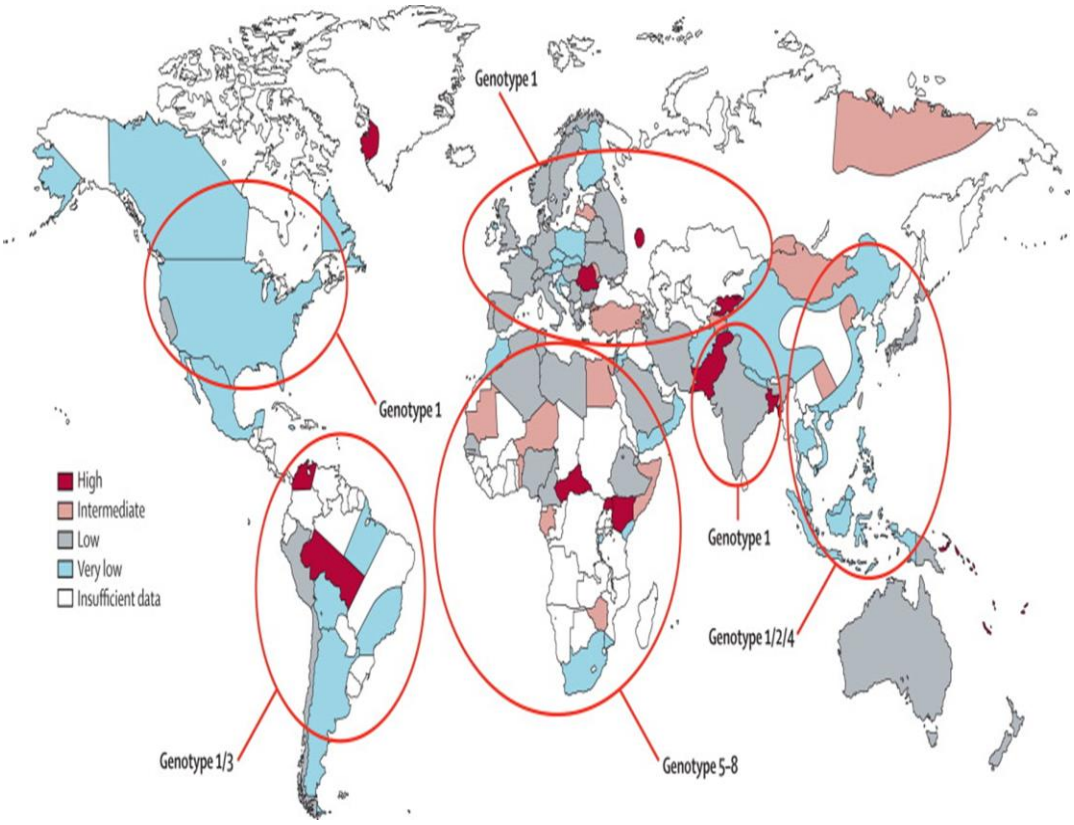

Figure 1.6: Geographical distribution of the Hepatitis D virus genotypes. Source:

1.2.9 Transmission

Transmission of HDV occurs mainly through parenteral routes. Firstly, it is considered to occur through contact with infected blood especially through Intravenous drug with a high frequency in HCV and HIV infected individuals (Braga et al., 2012). Intrafamilial exchange of body fluids also contributes to the transmission of HDV. In addition, studies have demonstrated HDV transmission in both homosexuals and heterosexuals particularly within the endemic regions (Alfaiate et al., 2015; Niro et al., 1999). However, mother-to-child transmission has not be demonstrated and currently attributed to HDV's suppression of HBV replication (Alfaiate et al., 2015).

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1.2.9 Diagnosis

Screening of all HBsAg positive individuals for HDV is important and should be considered since recent evidence suggest an increase in global HDV disease burden and also allow for the establishment of the actual prevalence of HDV infection as well as a wider coverage and early therapeutic mediation which will decrease the disease complications. There are several markers that can be used in the diagnosis of HDV infection such as anti-HDV antibodies – immunoglobulin M, immunoglobulin G and total antibody detection. Enzyme linked immunosorbent assay (ELISA), radioimmuno assay, and quantitative microarray antibody capture assays have been developed to detect anti-HDV antibodies Currently, ELISA is the first assay used in detecting HDV total antibodies. This approach has limitations such as non-detection of total anti-HDV antibodies within the early weeks of acute infection as well as non- persistence of anti-HDV IgG after HDV infection. In the United States and Mongolia, quantitative microarray antibody capture has been validated to be used for quantifying anti-HDV IgG antibody with an association between the quantitative microarray antibody capture results and HDV RNA detection being established (Chen et al., 2016). Anti-HDV immunoglobulin M which appears earlier in acute HDV infection stage has been shown to relate with active chronic infection, although it is not frequently detectable and does not allow a discrepancy between acute and chronic HDV infections. In the meantime, detection of HDAg in serum is short-lived in the acute stage of infection and also limits its quantification ability (Mentha et al., 2019) thus confirmation of an infection depends on the ability detect HDV RNA via quantitative RT-PCR approach in addition to a positive results for anti-HDV antibody. This helps to differentiate chronic from past infection and allows for the determination treatment response (Castelnau et al., 2006).

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In recent times, steps have been taken to obtain a standard by the WHO which allows results to be reported in international units (IU) and allowing for the development of commercial kits whose detection span across all genotypes (Le Gal et al., 2017). Recent advances made towards the detection of HDV have yielded the development of a point of care rapid diagnostic kit for detection of HDV (Lempp et al., 2019). Immunohistochemistry may also be used in the detection in the detection of HDAg from liver biopsy samples as well as the use of in-situ hybridization to identify RNA from liver biopsy samples. Meanwhile, genotyping of HDV is constrained to research institutes (Mentha et al., 2019).

1.2.10 Treatment/Management

At present, there has not been any antiviral treatment that has proven useful in the controlling of chronic hepatitis delta patients thus the management of patients with acute HDV infection relies on the use of broad-spectrum measures of support or liver transplantation in cases of acute liver failure (Niro et al., 2005). Currently only pegylated interferon alpha is recommended for treatment of CHD since there are no specific antivirals that acts directly on HDV. In the meantime, several molecules that target the host are currently being developed. Clearance of hepatitis B and D infections from the liver is the ideal treatment response to CHD infection, eventually leading to the prevention of disease progression in the liver.

Studies have revealed that the kinetics of HDV serum RNA during and after treatment cannot be used to envisage long term virologic response to treatment. In CHD cases, relapses have been observed in more than 50% of patients being treated with peg-IFN-α after 24 weeks of treatment hence making it difficult to use undetectable viremia levels 24 weeks post treatment as a marker of sustained virologic response (Heidrich et al., 2014). Various research have therefore been

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undertaken to evaluate the effectiveness of peg-IFN-α with a summary of such studies presented in Table 1.1

Table 1.1: Summary of studies evaluating the efficacy of IFN-alpha treatment against chronic hepatitis D

Treatment	Treatment Duration (months)	Number of patients	Sustained HDV RNA suppression at 24-weeks of follow-up
3 to 18 Mio units of IFN-α three times a week	3–12	201	17%
180 mg/kg Peg IFN- α 2a once a week	18	16	25%
	18 + 1-1.2 g Ribavirin daily for 12 months	22	18%
	12	14	43%
	12	12	17%
	12	48	25%
180 mg/kg Peg IFN- α 2a once a week	12	29	26%
	12 + 10 mg adefovir daily for 12 months	31	31%
1.5 mg/kg Peg IFN- α 2b once a week or 180 mg/kg Peg IFN- α 2a once a week.	12	104	

(Mentha et al., 2019)

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Other anti-viral treatments such as those used in the treatment of HBV mono-infected individuals (e.g. ribavirin, entecavir, famciclovir, adefovir, and lamivudine, have not shown any effectiveness against HDV (Abbas et al., 2016; Wedemeyer et al., 2011; Yurdaydin et al., 2002). Studies conducted with HBV/HDV co-infected patients who are also infected with HIV and were treated with tenofovir for 58 weeks showed favourable treatment response with undetectable levels of HBV DNA in all studied patients whilst 53 percent of study participants had undetectable levels of HDV RNA. Also, in 60 percent of patients who had undetectable HDV RNA, there was an improvement in the gravity of liver fibrosis (Soriano et al., 2014). Table 1.2 summarises elements in clinical development.

1.2.11 Prevention

As at now, prevention of HDV infection is by vaccination against HBV (WHO, 2017). The numbers of individuals who can be infected with HBV thus becoming potential candidates for HDV infection have reduced since the inception of campaigns for HBV vaccination. This has been proven in various studies. Strategies to develop vaccines against HDV infection in patients who are already infected with HBV have so far has not been successful even in animal models for there to be a translation into clinical trials (Mele et al., 2007; Roggendorf, 2012).

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Table 1.2: A summary of some elements in clinical development

Treatment	Duration of Treatment (weeks)	Number of Patients	Virologic Outcome	Development Phase
120 or 180 mg weekly of Peg IFN-λ	48	33	Four out of ten patients were HDV negative by PCR at week 24 of treatment.	Stage 2
2 mg/Kg daily of Myrcludex B for 24 weeks followed by monotherapy of peg IFN-alpha for 48 weeks	72	24	A decline of 1.67 log10 HDV RNA was observed at week 24 of treatment	Stage 2
2 mg/Kg daily of Myrcludex B + peg IFN for 24 weeks followed by peg IFN-α monotherapy, at 24 weeks	48		A decline of 2.59 log10 HDV RNA was observed at week 24 of treatment	
Peg IFN-alpha - monotherapy	48		A decline in 2.17 log10 HDV RNA was observed at week 24 of treatment	
2, 5 or 10 mg Myrcludex B daily	24	120	A Decline of 1.75 log10 for 2 mg; 1.6 log10 for 10 mg and 2.7 log10 for 5 mg. in HDV RNA was observed at week 24 of treatment.	Stage 2
245 mg daily of Tenofovir	24		A decline in 0.18 log10 HDV RNA was observed at week 24 of treatment	
2 or 5 mg daily of Myrcludex B + Peg IFN-alpha	48	30	A decline of 3.62 log10 and 4.48 log10 HDV RNA was observed for 2 mg and 5 mg of treatment respectively at week 48.	Stage 2
2 mg daily of Myrcludex B	48	15	A decline in 2.48 log10 HDV RNA was observed at week 24 of treatment	

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Treatment	Duration of Treatment (weeks)	Number of Patients	Virologic Outcome	Development Phase
Peg IFN-alpha	48	15	A decline in 1.14 log10 HDV RNA was observed at week 24 of treatment	
Twice daily dose of either 100mg or 200 mg Lonafarnib	4	1 4	A decline of 0.73 log10 and 1.54 log10 HDV RNA was observed for 100 mg and 200 mg of treatment respectively at week 28.	
Twice daily dose of 200 mg Lonafarnib	12	3	Disparity in HDV RNA was observed at 12th week of treatment to be 0.03 log10	Stage 2A
Twice daily dose of 300 mg Lonafarnib	12	3	Reduction in the RNA levels of HDV at the 12th week treatment was 1.78 log10	Stage 2A
100 mg of Lonafarnib twice daily po + weekly dose of peg IFN-α	8	3	Reduction in the RNA levels of HDV at the 8th week treatment was 2.19 log10	
24 weeks of IFN-alpha monotherapy	8	3	Reduction in the RNA levels of HDV at the 8th week treatment was 2.97 log10	
500 mg of 2139-Ca weekly for 15 weeks followed by REP 2139-Ca + peg IFN-α weekly for 15 weeks, then followed by the administration of peg IFN-α for 33 weeks	63	1 2	There was a greater than 5 log reduction in the HDV load in 11 patients with HDV RNA not detected in 10 patients by week 30. By the end of the treatment phase, HBsAg seroconversion was observed in 5 patients with HDV RNA detectable in 9 patients	Stag e 2

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CHAPTER 2

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Study Design

This was a longitudinal study using a purposive sampling method involving HIV/HBV co-infected patients at the Fever's Unit of the Korle-Bu Teaching Hospital (KBTH) between the ages of 18 years and above. This study involved taking blood samples at two time points with 4 months interval from all the HIV/HBV co-infected patients who consented to be part of the study.

2.1.2 Ethical Consent

Ethical approvals were obtained from the Institutional Review Boards of Korle-Bu Teaching Hospital and the Noguchi Memorial Institute for Medical Research (NMIMR). Eligible patients were recruited from KBTH unto the study and venous blood taken into serum separating tubes and the serum dispensed into appropriately labelled vials/microtubes for testing and subsequently stored. A replica of the ethical authorization letters are attached as appendix A1 and A2.

2.1.3 Recruitment of Study Subjects

Folders for HIV positive patients were reviewed to obtain data on their HBV status. Data such as sex, date of diagnosis of HBV and contact information were retrieved from the patients who were identified to be HIV/HBV co-infected. The folder numbers of patients who had participated in a previous study to determine HBV prevalence amongst HIV patients were also obtained from the principal investigator of that study and contact numbers of those patients retrieved. Patients were contacted by a staff of the Fever's Unit and invited to be part of the study through the booking of appointments. Upon arrival at the Fever's Unit, self-introduction of the principal investigator was

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done, a general overview of the research work was described and the aim of the study as well as the possible benefits, and risks were explained to the patients in detail. Where language was a barrier, the service of an interpreter was used to explain the information being given by the principal investigator to the patients. The patients were given the opportunity to ask questions they may have for clarifications. Informed consent was concluded with the signing or thump-printing the consent forms from which the details were read out. Using a structured questionnaire, information on the sex, age, type of antiretroviral therapy, duration on the therapy, vaccination status, and place of residence were obtained from the patients as well as from the patient's hospital folder. A copy of the consent form and questionnaire are attached as appendices B and C respectively.

2.1.4 Study Sites

The patients recruited into this study were from the fever's Unit of the Korle-Bu Teaching hospital (KBTH) in Accra Ghana. Korle-Bu is Ghana's leading tertiary referral teaching hospital which was established on 9th October 1923 with about 2000 bed capacity. It has 17 diagnostic and clinical departments or Units which includes the Fever's Unit. It serves patients from all parts of Ghana having daily, an average turnout of 1500 patients and approximately 250 in-patients. The unit houses the HIV/AIDs clinic that is responsible for HIV prevention and intervention programmes including the provision of free anti-retroviral therapy across the country.

The Fever's Unit of the Korle-Bu Teaching hospital serves not only HIV patients but also attends to other patients who have high fever and serves patients referred from other hospitals or institutions country-wide.

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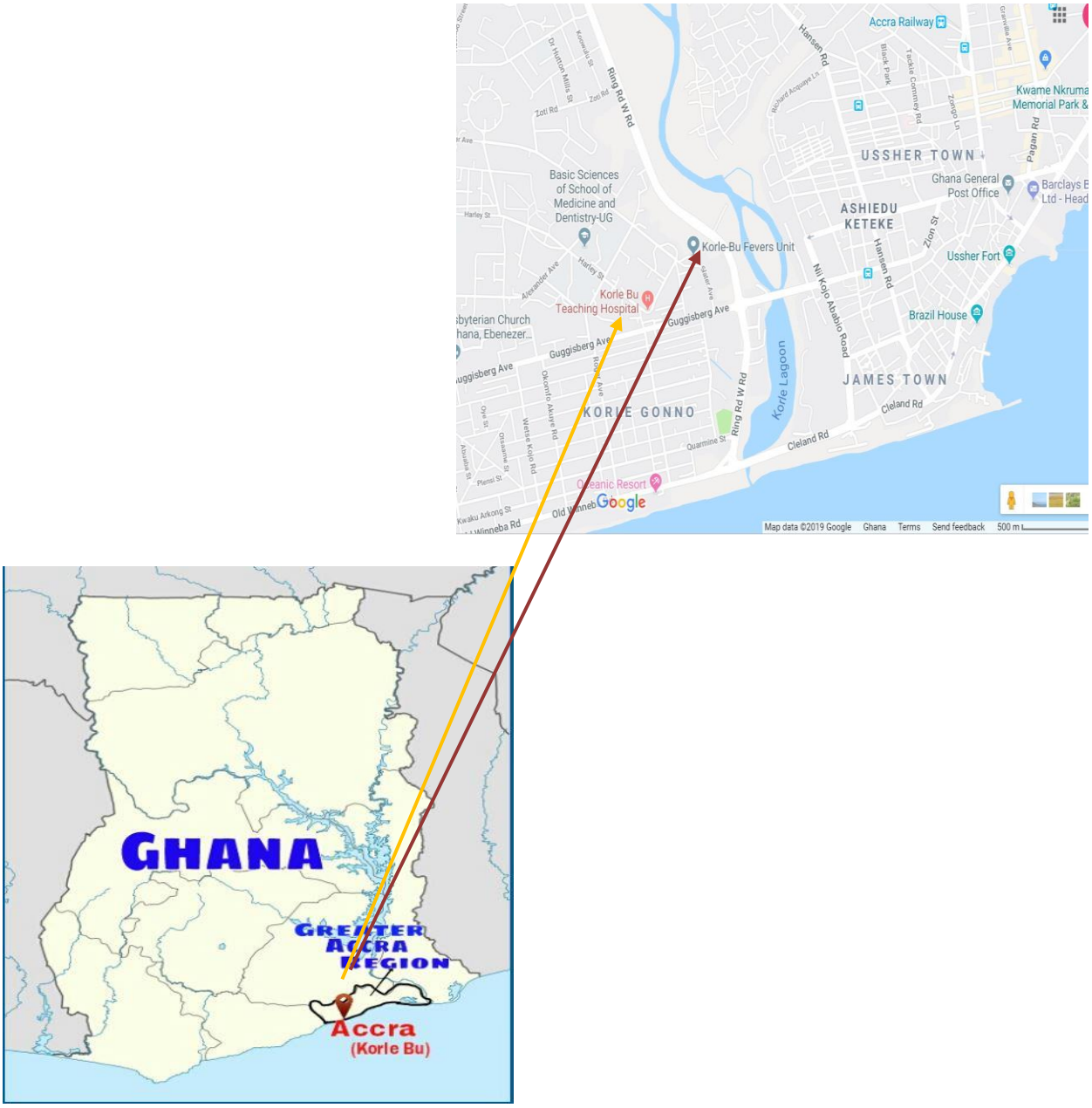

Figure 2.1 Map of Ghana displaying the location of Fever's Unit at the Korle-Bu Teaching Hospital

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2.1.5 Study Population

This research looked at HIV patients who were co-infected with hepatitis B. A patient to be recruited as a participant must not have been diagnosed with alcohol related liver diseases and must not have a previous history of hepatitis B vaccination. Using a prevalence of 10% (Jaquet et al., 2017) and the sample size determination formula below,

$$N = \frac{Z^2[p*q]}{d^2}$$

Where n is the sample size, Z is the statistic that corresponds to the 95% level of confidence, P is the expected prevalence and d is the precision.

The minimum number of samples for the study based on Z (1.96), a proposed prevalence rate of 10.0% and the precision set at 5%, is 138.

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2.2 METHODS

2.2.1 Pre-Data and Sample Collection

2.2.1.1 Pre-Data Collection

A pre-evaluation of HIV patient folders was done to look for those who have been diagnosed as hepatitis B virus positive. Other information such as date of HBV diagnosis, type of ART the patient is receiving; the folder number of the patient, names and contact of the patient or guardian, date of ART initiation, and gender of the patient were also obtained from the patient folder using the aid of a structured questionnaire. Using the same questionnaire, information such as age, nationality, place of residence and vaccination status of the patient is obtained by means of an interview.

2.2.1.2 Sample Collection

Patients contacts were obtained from the pre-evaluation data and patients were called and requested to come to the Fever's Unit to partake in the study. Upon arrival, the general overview, purpose and objectives of the study were explained to the patients in a language they understand. It was effectively communicated that, the study was purely voluntary and there would neither be any direct benefit to them, nor would their involvement or non-involvement bring any changes to their current therapy management plan. The patients were made aware of the possible risks such as dizziness, pain or discomfort from needle stick, bruise, swelling or bleeding and asked to contact the principal investigator should any of these happen. Patients were made aware that every information given will be kept confidential and that they could choose not to partake in the study. The patients were given the opportunity to ask for clarifications if any and the consenting process were concluded when they sign or thump-print. With the help of a trained phlebotomist from the Fever's Unit lab, 5 mL venous blood was collected into a serum separator tube (yellow cap), given

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lab identification numbers and left to stand for at least 30 minutes prior to centrifuging at 4000 g for a minute. Three 2 mL vials were labelled with the lab identification numbers on the serum tube as well as specimen type and date of collection. The separated serum was dispensed into the 3 vials, each containing 1 mL of serum. The dispensed specimens were then placed into zip lock bags, placed in a cool box that contained ice packs and then transported to NMIMR for storage in a -80 ^{o}C freezer awaiting laboratory investigations.

2.2.2 Confirmatory Assays

The OraQuick HIV-1/2 rapid diagnostic test was used in confirming the HIV status of the participants enrolled in this study. The principle behind this test is an antigen-antibody detection system where specific positions on the cassette of the test kit have the HIV antigen embedded in the membrane. Ten microliters of serum were pipetted into the labelled bottle containing the buffer of the OraQuick HIV-1/2 test kit and swirled to mix the contents. The cassettes which have the antigen coated strips were then placed into the buffer and results read after 20 minutes. When bands were observed on the control (C) and test (T) lines, the test is said to be positive. If a band is only seen on the C line, the test is valid and called negative. However, if a band is observed on the T line and not on the C line, the test is invalid and must be repeated.

2.2.2.1 Confirmation of HBV status of patients.

Confirmatory test for HBV was done with the Alere Determine (Alere Medical Co. Ltd., Japan) rapid test kits. For the Alere determine rapid test kit, 50 µL of serum were pipetted onto the HBsAg antibody coated test strip labelled with the sample ID. The results were then read after 20 minutes. When bands were observed on the control (C) and test (T) lines, the test was said to be positive. If a band was only seen on the C line, the test was valid and called negative. However, if a band was observed on the T line and not on the C line, the test was invalid and was repeated. A second line

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confirmatory assay, Electrochemiluminescence assay using the Elecsys HBsAg II assay (Roche Diagnostics, Germany) was also used to check the HBV status of all 113 HIV positive samples.

2.2.2.1 Electrochemiluminiscence Assay

Elecsys HBsAg II assay employs the use of polyclonal and monoclonal anti-HBs (mouse and sheep) antibodies to detect and quantify HBsAg. The assay principle was based on the sandwich principle where 50 µl of patient serum samples, two biotynylated anti-HBsAg monoclonal antibodies, and a mixture of anti-HBsAg monoclonal and polyclonal antibodies labelled with ruthenium complex were incubated to form a sandwich compound. Microparticles coated with streptavidin were added to form a complex which attached to the solid phase through an interaction between streptavidin and biotin. The reaction mixture was extracted into the measuring cell where microparticles were captured magnetically onto an electrode's surface. The substances which were not captured were removed with Procell M. Chemiluminescent emission by the induction of a voltage to the electrode. The chemiluminescent radiation was measured using a photomultiplier.

The test results were calculated in the form of cut-off index equal to test signal/cut-off. Samples with cut-off index less than 0.90 were considered as non-reactive thus HBsAg negative. Samples with cut-off index greater than or equal to 1.0 were considered as reactive and called HBsAG positive.

2.2.3 ELISA for HDV Detection

Using an enzyme link immunosorbent assay (ELISA), Hepatitis D Virus Antigen Antibody (IgG) ELISA Kit (Colorimetric), (Bio-Techne, Abingdon, UK), the presence or absence of exposure to HDV was determined. The principle behind the method for qualitative anti-HD determination is a sandwich-based ELISA technique.

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2.2.3.1 Principle of the Hepatitis D Virus Antigen Antibody (IgG) ELISA Kit (Colorimetric), (Bio-Techne, Abingdon, UK), assay

The Hepatitis D Virus Antigen Antibody (IgG) ELISA Kit (Colorimetric) makes use of the qualitative enzyme immunoassay system. The microtiter plate provided in this kit was pre-coated with the antigens of HDV. Samples were dispensed into the wells with the mouse anti-human Immunoglobulin G conjugated Horseradish Peroxidase (IgG-HRP). Any antibody that is specific for the HDV antigen present, will attach to the antigen pre-coated in the wells. The plate was washed to remove any unattached substance, and a substrate solution added to the wells with the amount of colour developed being proportional to the quantity of human HDV IgG antibody bound in the primary step. The colour development was halted and intensity of the colour was measured.

2.2.3.2 Assay procedure

The serum samples were tested for the presence of HDV using a sandwich ELISA assay, Human Hepatitis D Virus Antigen Antibody (IgG) ELISA Kit (Colorimetric), (Bio-Techne, Abingdon, UK), to ascertain the prevalence of HDV in HIV/HBV co-infected patients.

The frozen sera were allowed to completely thaw and mixed well before testing. The ELISA reagents were also allowed to attain room temperature before used for testing in accordance with the manufacturer's instructions; the procedure for testing was strictly followed. A working stock of wash solution was prepared by pouring the required amount of concentrated wash solution in a graduated measuring cylinder and topping it up with distilled water to make 1X concentration. Well A1 was left as the blank well after which 100 µL Reagent control was pipetted into wells B1 and C1 followed by addition of 100 µL Positive control in well D1. Hundred microliters of each participant’s specimen was assigned wells starting at F1 to H12. An incubator was set to the temperature of 37 ^{o}C. A 100 µL sample diluent was dispensed into the sample wells and 10 µL of

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samples added to their corresponding wells. The assay plate was then sealed to prevent evaporation during the 30 minute incubation at 37 ^{o}C. After incubation, the plate sealer was discarded, and the strips were rinsed five times using 200 µL of 1 X wash buffer with soaking for 45 seconds for each wash. After rinsing, the plate was turned upside down on a tissue paper and tapped gently to drain any liquid residue. A 100 µL of HRP-conjugate was pipetted into all the wells with the exception of the blank well, the plate sealed, and incubated at 37 ^{o}C for 30 minutes. After incubating for 30 minutes, the 5 times wash cycle was repeated and 50 µL of both substrate A and substrate B pipetted into all the wells including the blank well to develop a blue colour in wells containing reactive specimen while the non-reactive specimen remained clear. The plate is sealed and incubated away from light in an incubator for 10 minutes at 37 ^{o}C. Fifty microliter of stop solution was dispensed into all the wells after the 10 minute incubation to stop the reaction indicated by the development of a yellow colour in wells having reactive samples. The absorbance was then read at 450/630 nm for each well using a photometer (BioTek ELX808, USA) within 30 minutes.

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For a test to be valid,

1. The blank well should not have an optical density of more than 0.08
2. The mean optical density for the negative controls must be less than 0.10
3. The positive control optical density must be more than 0.30

To call a sample result positive, the optical density value of that sample must be more than/equal to the cut-off value, whilst a negative sample has its optical density value to be less than that of the cut-off value.

A cut-off value was well-defined as the mean negative control value plus 0.1.

OD value of negative control that has a value less than 0.05, was calculated as 0.05.

2.2.4 Biochemistry and HIV Viral load Determination

Biochemistries on the patient samples including ALT, AST and HIV viral load tests were performed at the Central laboratory of KBTH with established in-house protocols.

2.2.5 Detection and Quantification of HBV DNA

2.2.5.1 Deoxyribonucleic acid extraction

Deoxyribonucleic acid (DNA) was obtained from patient sera using the QIAamp DNA viral mini kit (Qiagen, Hilden, Germany) with the following processes.

In a Biosafety cabinet (Airtech ClassIIA/B3, AirTech, Japan), two hundred microliters (200 µL) of the serum were pipetted into labelled 1.5 mL microtubes. Twenty microliters (20 µL) of proteinase K plus 200 µL of lysis buffer were added to the samples. The lysate was then mixed by pulse vortexing for 15 seconds and placed on a heat block to incubate for10 minutes at a temperature of 56 ^{o}C. After the heat incubation, 200 µL of absolute ethanol (molecular grade) was added and the lysate mixed by pulse vortexing to attain a homogeneous mixture. The lysate was then pipetted into a corresponding labelled spin column (from the kit). The spin column was then

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centrifuged using the Eppendorf centrifuge (Eppendorf AG 22331 Hamburg) at 8000 revolutions per minute (rpm) for a minute. The collection tube along with the filtrate was thrown away and replaced with a new collection tube. In the spin column, 500 µL of wash buffer 1 (AW1) was dispensed and then centrifuged at 8000 rpm for a minute. A second wash buffer (AW2) of the same volume was then added and centrifuged for 3 minutes at 14000 rpm. The flow through was thrown out and the spin column put back on the same collection tube. This was centrifuged again at 14000 rpm for 1 minute to remove any traces of the wash buffer and ethanol. The collection tube was thrown away, and the spin column placed in a clean labelled microtube with 50 µL of elution buffer being dispensed into the spin column and let to sit on the bench for a minute before centrifuging at 8000 rpm for 1 minute. The spin column was then thrown away, and the labelled microtube capped and kept at 4 ^{o}C temporarily prior to HBV DNA detection using real time PCR assay.

2.2.5.2 Qualitative and Quantitative detection of HBV DNA by real time PCR

To quantify or detect the HBV DNA, a commercial kit (Techne QPCR Kit, DNA Hepatitis B virus, Bibby Scientific, Cyprus.) was purchased from Bibby Scientific in Cyprus and reaction mix set up with modifications to manufacturer's protocol. The thermal conditions and interpretation of results were according to the manufacturer's instructions.

Reaction mixes used in the detection of HBV DNA contained 5.0 µL 2 x qPCR master mix (Quanta Tough mix), 0.5 µL of HBV primer/probe mix (Techne QPCR Kit, DNA Hepatitis B virus, Bibby Scientific, Cyprus.), 2.0 µL of RNase/DNase free water and 5 µL of template DNA for a total volume of 12.5 µL per sample. The amplification conditions were 95 °C for 2 minutes, and 50 cycles (95 °C for 10 seconds and a minute at 60 °C). To validate the results, a separate reaction mix was prepared as an endogenous control using an endogenous primer and probe mix to identify

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nucleic acid material in the obtained samples. The ABI 7500 PCR system, (Life Technologies) was used in the detection of the HBV DNA.

2.2.6 Amplification of HBV S gene by Conventional PCR

Patients' samples that tested positive for HBV surface antigen after the confirmation were selected and amplified using reagents by Roche diagnostics international limited. The cocktail of buffers for the PCR included the 10 X PCR buffer with magnesium, Taq DNA polymerase, GmP grade 5 U/µL, and 10mM dNTPs. Nested PCR was used in amplifying the S gene using 2 sets of primers obtained from the US CDC, Atlanta, Georgia. The amplification conditions were adapted from the US CDC protocol used in amplifying the HBV S gene. Below is a table showing the sequences of the primers used in amplifying and sequencing the HBV DNA.

Table 2.1: Primer Sequences used in amplifying and sequencing the S-gene of HBV

Region	Primer name	Nucleotide sequence of the primers (5´–3´)
SI	SIF	CTAGGACCCCTGCTGGTGTT
	SIR	TCGAACCACTGAACAAATGGCACT
SN	SNF	GTTGACAAGAATCCTCACAATACC
	SNR	GGCTGAGGCCCACTCCCATA

2.2.6.1 First round PCR

The reaction mixture for 1st round PCR was prepared by mixing 2.5 µL 10 X PCR buffer with magnesium, 0.2 µL Taq polymerase 250 U (5 U/µL), 0.35 µL of sense primer (15 µM SIF), 0.35 µL of anti-sense primer (15 µM SIR), 1 µL of 10 mM dNTP, 11.6 µL of PCR water and 9 µL of template DNA, making an total volume of 25 µL. The ESCO thermal cycler (ESCO microplate Ltd, Singapore) was used in the amplification process with the following thermal settings: initial

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denaturation and activation of the Taq DNA polymerase enzyme for 5 minutes at 94 ^{o}C, 35 cycles of denaturation for 45 seconds at 94 ^{o}C, annealing at 55 ^{o}C for 20 seconds, extension for 1 minute at 72 ^{o}C and extended finally for 7 minutes at 72 ^{o}C. The amplicons were kept at 4 ^{o}C ahead of second round amplification.

2.2.6.2 Second Round PCR

The reaction mix for the 2nd round PCR was prepared by mixing 5.0 µL of 10X PCR buffer with magnesium, 0.4 µL Taq polymerase 250 IU (5 IU/µL), 0.70 µL of 15 µM sense primer (SNF), 0.7 µL of 15 µM anti-sense primer (SNR), 2 µL of 10 mM dNTP, 39.2 µL of PCR water and 2 µL of template DNA, making a final volume of 50 µL. Amplification was done using the ESCO thermal cycler (ESCO microplate Ltd, Singapore) with these thermal conditions: initial denaturation and activation of the Taq DNA polymerase enzyme for 5 minutes at 94 ^{o}C, followed by 35 cycles (denaturation for 45 seconds at 94 ^{o}C, annealing for 30 seconds at 55 ^{o}C and extended for 1 minute at 72 ^{o}C) with a final of 7 minutes at 72 ^{o}C. The amplicons were kept at 4 ^{o}C prior to gel electrophoresis and Cycle Sequencing of successfully amplified samples.

2.2.7 Detection and Quantification of HDV RNA

2.2.7.1 Ribonucleic Acid Extraction

Ribonucleic acid (RNA) was extracted from the serum of patient samples using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) with the following procedures.

One hundred and forty microliters (140 µL) of the serum samples were pipetted into labelled 1.5 mL microtubes. Five hundred and sixty microliters of lysis buffer plus carrier RNA was added to the samples. The lysate mixture was then mixed by pulse vortexing for 15 seconds and let to sit on the bench at room temperature for 10 minutes. After the incubation, 560 µL of cold absolute ethanol (molecular grade) was added and the lysate pulse vortexed to obtain a homogeneous

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mixture. Six hundred and thirty microliters of the lysate was then pipetted into a corresponding labelled spin column (from the kit). The spin column was then centrifuged using the Eppendorf centrifuge (Eppendorf AG 22331 Hamburg) for a minute at 8000 revolutions per minute (rpm). The filtrate and collection tube were thrown away and the spin column placed in a new collection tube. The remaining lysate mixture was pipetted into the corresponding labelled spin column and centrifuged for 1 minute at 8000 revolutions per minute (rpm). The collection tube with the filtrate was thrown away and the spin column was placed in a new collection tube. Five hundred microliters (500 µL) of wash buffer 1 (AW1) was pipetted into the spin column and then centrifuged for 1 minute at 8000 rpm. A second wash buffer (AW2) of the same volume was then added and spun at 14000 rpm for 3 minutes. The filtrate was thrown out from the collection tube and the spin column put back on the same collection tube. This was centrifuged again at 14000 rpm for 1 minute to remove any traces of the wash buffer and ethanol. The collection tube was then thrown away, and the spin column placed in a clean labelled microtube with 50 µL of elution buffer dispensed onto the membrane of the spin column and let sit in the bench for 1 minute before centrifuging at 8000 rpm for 1 minute. The spin column was then thrown away, and the labelled micro tube, capped and kept at 4 ^{o}C temporarily for detection of HDV RNA using real time RT-PCR.

2.2.7.2 Qualitative and Quantitative Detection of HDV by Real Time RT-PCR

To quantify or detect the HDV RNA, a commercial kit was purchased from Bibby Scientific in Cyprus and reaction mix set up with modifications to manufacturer's protocol. The thermal conditions and interpretation of results were according to the manufacturer's instructions. Reaction mixes for detection of HDV contained 5.0 µL 2 x qPCR master mix (Quanta Tough mix), 2.0 µL of Nuclease free water, 0.5 µL of HDV primer/probe mix and 5 µL of template DNA for a total

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volume of 12.5 µL per sample. The amplification conditions were 10 minutes at 55 °C reverse transcription stage, 2 minutes at 95 °C to stimulate the Taq polymerase enzyme, and 50 cycles (10 seconds at 95 °C and 1 minute at 60 °C). To validate the results, a separate reaction mix was prepared as an endogenous control using an endogenous primer/probe mix to detect presence of nucleic material in the extracted sample. The ABI 7500 PCR system, (Life technologies) was used in the detection of the HDV RNA.

2.2.8 Amplification of HDV 3' region (R0) by Conventional PCR

2.2.8.1 cDNA Synthesis

After RNA extraction, cDNA was synthesised using the Invitrogen superscript IV kit with the following reaction mix and thermal conditions: - Random hexamers were first annealed to the RNA extracts by preparing a reaction mix containing 2 µL of random hexamers, 2 µL of 10 mM dNTP mix, 4 µL of Nuclease free water and 12 µL of RNA extract. This was then incubated at 65 ^{o}C for 5 minutes, placed on ice for a minute and reverse transcribed by adding a mixture of 4 µL of 10 X RT buffer, 8 µL of 25 mM $MgCl_2$, 4 µL of 0.1 M DTT, 2 µL of 40 U/µL RNase out, 2 µL of 200 U/µL Superscript III RT. The mixture was then placed on a thermal cycler and run using the following thermal conditions: - 10 minutes at 25 °C, 50 minutes at 50 °C, and 5 minutes at 85 °C. After the run ended, 1 µL of RNase H was added to each tube and incubate at 37 °C for 20 minutes. After the 20 minutes incubation, the cDNA was stored at -20 °C till ready for use.

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2.2.8.2 First round PCR

The reaction mix for 1st round of PCR was prepared by putting together in a clean PCR tube, 15 µL of Platinum PCR super mix, 0.42 µL of sense primer (10 µM R0F), 0.42 µL of anti-sense primer (10 µM R0R), and 9 µL of template cDNA, making a total volume of 25 µL. Amplification was then carried out in the ESCO thermal cycler (ESCO microplate Ltd, Singapore) with the following thermal conditions: initial denaturation and activation of the Taq DNA polymerase enzyme for 5 minutes at 94 ^{o}C, followed by 35 cycles of denaturation at 94^{o}C for 45 seconds, annealing at 55 ^{o}C for 20 seconds, extension at 72 ^{o}C for 1 minute and a final extension after the 35 cycles at 72 ^{o}C for 7 minutes. The amplicons were kept at 4 ^{o}C prior to second round amplification.

2.2.8.3 Second Round PCR

The reaction mix for 2nd round PCR was prepared by mixing 45 µL of Platinum PCR supermix, 0.42 µL of sense primer (10 µM R0F), 0.42 µL of anti-sense primer (10 µM R0R), and 4µL of template first round PCR product, making a total volume of 25 µL. Amplification was carried out in the ESCO thermal cycler (ESCO microplate Ltd, Singapore) using the following thermal conditions: initial denaturation and activation of the Taq DNA polymerase enzyme for 5 minutes at 94 ^{o}C, followed by 35 cycles of denaturation at 94 ^{o}C for 45 seconds, annealing at 55 ^{o}C for 20 seconds, extension at 72 ^{o}C for 1 minute and a final extension after the 35 cycles at 72 ^{o}C for 7 minutes. The amplicons were kept at 4 ^{o}C prior to gel electrophoresis, Purification and Sequencing. Below is a table showing the primer sequences used in the amplifying and sequencing the HDV RNA.

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Table 2.2: Primer Sequences used in amplifying and sequencing the 3' R0 region of HDV RNA

Region	Primer name	Nucleotide sequence of the primers (5´–3´)
R0	889s (R0F)	CATGCCGACCCGAAGAGGAAAG
(889–1289)	1289as (R0R)	GAAGGAAAGGCCCTCGAGAACAAGA

2.2.9 Agarose Gel Electrophoresis

To view the results obtained from the amplification of the HBV S gene and HDV on a 2% agarose in tris borate ethylenediamine tetraacetic acid (TBE) buffer, 1 X TBE was first prepared from a 10 X stock solution by using a measuring cylinder to measure 100 mL of the stock TBE buffer and adding 900 mL of distilled water in a 1 L reagent bottle, mixing the contents well. 100 mL of the 1 X TBE buffer was measured into a conical flask and using a weighing balance, 2 grams of agarose was weighed into the conical flask containing the 1 X TBE buffer. The mixture was swirled to mix the contents and microwaved to melt the agarose, which was allowed to cool down to about 45 ^{o}C, gel red, a DNA staining dye added and swirled to mix contents. The gel was the poured in a casting tray which had a desirable comb size and number in place and allowed to set for around 15 minutes. The set gel was placed in a gel electrophoresis tank containing 1 X TBE buffer as running buffer. Five microliters of the amplified PCR products were mixed with 1 µL of 6 X Blue/orange loading dye and loaded into the wells on the gel alongside a 1 kb DNA ladder (GeneRuler, ThermoFisher Scientific, USA) and run at 100 volts (V), 500 milliamps (mA) for 45 minutes. The gel was visualized on a UV-trans illuminator. The positive amplicons were reloaded onto a 2% agarose in tris acetate ethylenediamine tetraacetic acid (TAE) buffer by mixing 40 µL of amplicons with 8 µL of 6 X Blue/orange loading dye alongside a 1 kb DNA ladder and run at 100 V for 30 minutes, after which the products were excised for gel purification.

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2.2.10 Post PCR Purification - Gel Extraction and Purification

The positive amplicons were extracted from the gel and purified using the QIAquick Gel extraction kit (Qiagen GmbH, Hilden, Germany), following manufacturers protocol. Briefly, the DNA of the positive samples were cut out from the gel using a sharp scalpel, weighed and then placed into a colourless micro centrifuge tube. Three times the volume of solubilization and binding buffer, QG, was added to the 1 volume of excised gel (where 100 mg of excised gel ~ 100 µL) and incubated at 56 ^{o}C until all the gel melted, vortexing every 2 minutes. A volume of isopropanol was added to the melted gel and mixed thoroughly. All centrifugation steps were done at 13000 rpm for 1 minute. The mixture was then pipetted (maximum 800 µL) into a labelled spin column and centrifuged after which the flow through was thrown away and the spin column placed back into the same collection tube. This process was repeated until all the mixture was applied to the spin column. Five hundred microliters of buffer QG was dispensed into the spin column and centrifuged, discarding the flow through and placing the spin column back into the collection tube. Buffer PE, the washing buffer was pipetted (750 µL) into the spin column, allowed to sit on the bench for 5 minutes, before centrifuging and discarding the flow through and placing the spin column back into the same collection tube. The spin column was centrifuged again to remove any residual traces of ethanol and wash buffer contents. The collection tube was thrown away, and the spin column placed in a clean labelled 1.5 mL micro centrifuge tube. Thirty microliters of elution buffer (EB) was then pipetted directly onto the spin column and allowed to sit for 1 minute before centrifuging to obtain the purified DNA which was kept at 4 ^{o}C prior to cycle sequencing.

2.2.11 Cycle Sequencing of Purified DNA Products

Purified DNA products were sequenced using the applied Biosytems' ABI Big dye Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA). The pipetting protocol for the reagents was

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adapted from the sequencing protocol of the National Polio Reference lab at the Noguchi Memorial Institute for Medical Research (NMIMR). A mixture of 2 µL of Big Dye v3.1, 1 µL of 5 X sequencing buffer, 1 µL primer, 5 µL water and 1 µL 50 ng template DNA was prepared and sequenced on the ESCO thermal cycler with the following thermal conditions 96 ^{o}C for 3 minutes, 96 ^{o}C, 30 seconds; 55 ^{o}C, 30 seconds; 60 ^{o}C, 1 minute and a final extension of 60 ^{o}C, 10 minutes.

2.2.12 Post Cycle Sequencing Purification

After sequencing of the genes of interest, the sequences were cleaned using the Agencourt magnetic Beads, prior to electrophoresis using the ABI 3130XL genetic analyzer. Ten microliters of the magnetic beads as well as 42 µL of 85% ethanol were dispensed into each tube and pipetted up and down about 7 times to mix the contents in the tube. The mixture was then placed on a magnetic field for 3 minutes attract the beads to the walls of the tube after which the solution was pipetted out of the tube and 100 µL of 85% ethanol was pipetted into the tubes and allowed to sit on the magnetic field for another 3 minutes. After the incubation time was up, the ethanol was pipetted out and the tubes allowed to air-dry on the magnetic field for 10 minutes. The tubes were removed from the magnetic field and 40 µL of nuclease free water added to each tube and allowed to incubate on the bench for 5 minutes to elute the sequenced DNA. The tubes were placed back on the magnetic field for three minutes and 30 µL of the elute pipetted into an ABI standard 96 well plate and loaded onto the ABI 3130XL genetic analyzer for base calling of the sequences generated.

2.2.13 Data Analysis

2.2.13.1 Analysis of Sequence Reads

The oligosequence reads were edited using the Sequencher software and consensus sequences generated were ‘blasted’ in National Center for Biotechnology Information (NCBI) blast search to

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confirm the virus type. The consensus sequences were aligned with reference sequences obtained from NCBI using the ClustalW multiple alignment accessory tool in Bioedit software version 7.2.5 (Hall, 1999). The aligned reference sequences and the consensus sequences were then imported into the Molecular Evolutionary Genetics Analysis software version X (MEGA X) (Kumar et al., 2018) phylogenetic and molecular evolutionary analysis.

2.2.13.2 Statistical Analysis

The Skewness/Kurtosis tests, Shapiro-wilk w test and ladder of powers were used to test for the normality of the data. Demographic data were expressed in terms of means and standard deviations. The students'-test was used in testing for the association between liver enzyme levels and viral infection type. A univariate logistic regression analysis was used in testing an association between HBV viral load suppression, as well as other risk factors and liver enzyme activity which is usually indicative liver damage using STATA 15 software.

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CHAPTER THREE

3.0 RESULTS

3.1 STUDY PARTICIPANTS

This study was conducted amongst HIV patients attending clinic at the fever's unit of the KBTH and it recruited 113 eligible patients. After the day of enrolment, patients were followed up after 4 to 6months. Thirty-five of the patients were however lost to follow up as they were not reachable or available for follow up samples to be taken. All patients recruited for the study were confirmed to be HIV/HBV positive, as regards previous test result records for HIV and HBV maintained in their hospital folders. The ages of the recruited patients range between 24-73 years and a median age of 45 years. Majority (85.9%) of the patients however, were within the age range of 31 – 60 years. Females were the higher gender enrolled in the study 63/113 (55.8%). Almost all the patients, 111/113 (98.2%) were on Antiretroviral therapy (ART) management with 2 patients being ART naïve. Thirty-six of the patients have been on ART for less than 2 years. Also, for patients on ART, the vast majority are on first line class of drugs which includes a combination of 2 nucleos(t)ide reverse transcriptase inhibitor (NRTI) such as Lamivudine (3TC), abacavir (ABC), zidovudine (AZT), emtricitabine (FTC), stavudine (d4T) or tenofovir-DF (TDF) and 1 non-nucleos(t)ide reverse transcriptase inhibitor (NNRTI) such as efavirenz (EFV) or nevirapine (NVP), with few of the patients being on the second line class of drug which includes a combination of 2 nucleos(t)ide reverse transcriptase inhibitor (NRTI) and a Protease inhibitor such as atavanavir (ATV) or Lopinavir (LVP) boosted with ritonavir (/r), Figure 3.1. All 113 patients had not received hepatitis B vaccination. A summary of patient demographics are presented in Table 3.1.

Table 3.1: Demographic Characteristics of Study Participants

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Gender	N = 113
Female	63 (55.8)
Male	50 (44.2)
Age (years)	
21-30	8 (7.1)
31-40	35 (31.0)
41-50	40 (35.4)
51-60	22 (19.5)
≥61	8 (7.1)
Median (IQR)	45 (24 - 73)
Duration of ART (months)	
0-24	37 (32.7)
≥25	75 (66.4)
Vaccination	
No	113 (100)
Yes	0 (0)
Class of ART	
First Line (2NRTI+1NNRTI)	102 (92.0)
Second Line (2NRTI + 1 PI	9 (8.0)

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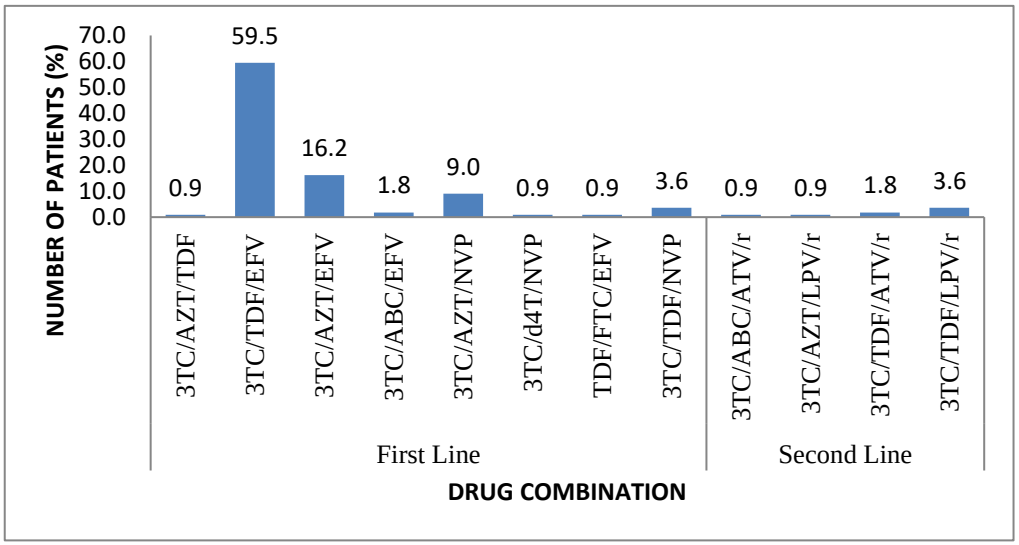

Figure 3.1: Treatment options used in the management of HIV/HBV co-infected patients at the Fever's Unit of the Korle-Bu Teaching Hospital.

First line drugs (Lamivudine (3TC), abacavir (ABC), zidovudine (AZT), emtricitabine (FTC), stavudine (d4T) or tenofovir-DF (TDF), efavirenz (EFV) or nevirapine (NVP), second line class of drugs (atavanavir (ATV) or Lopinavir (LVP) boosted with ritonavir (/r).

3.2 HIV STATUS

The HIV status of the study participants were re-tested using rapid diagnostic test kits on baseline samples. All patients 113 (100%) were reactive on the HIV rapid diagnostic test (RDT) kit (OraQuick HIV 1/2 Rapid Antibody Test, OraSure Technologies, Inc, Thailand). The viral loads for HIV was also measured for all baseline samples and for patients who had samples available from follow up sampling. The median HIV RNA copies for all baseline samples with RNA copies greater than 20 cp/mL was 3054.5 cp/mL, 47 patients had undetectable viral load, 20 patients had viral load less than 20 cp/mL. The follow up samples for the 113 patients enrolled at baseline, whose HIV RNA copy numbers were greater than 20 cp/mL had a median RNA load of 111 cp/mL. Forty-eight out of the 113 patients enrolled at baseline were lost to follow up [Patients not reachable (33 patients)

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and due to time constraint (15)], 26 patients had undetectable viral load; whilst 14 patients had HIV load less than 20 cp/mL. Overall, with the follow up samples, an increase in viral load was observed in 22 patients' samples whilst 16 had their viral loads reduced.

Table 3.2: Characteristics of HIV Status of patients

HIV Status	**Number of Patients**	**Viral Load (cp/mL)**
YES	113	
NO	0	
HIV RNA Copies (cp/mL)		
Baseline Samples	113	
Target not detected	47	
<20	28	
>20	36	
Failed	2	
Median of >20, (IQR)		3054.5 (21 - 1265314)
Follow Up		
Lost to follow up	48	
Target not detected	26	
Sustained VL	20	
Reduced VL	6	From <20 and 62 (BL) to Undetectable limit (FU)
<20 copies	14	
Sustained VL	4	
Reduced VL	3	From 23, 33 & 197 to <20
Increased VL	7	From undetectable VL (BL) to <20
>20 cp/mL	22	
Increased VL	15	To a median of 5635 (22 – 493032)
		From 55997 (41 – 706880) BL to 71 (29 -7441)
Reduced VL	7	FU*
Median of >20 copies, (IQR)		111 (22 – 493032)

* = Median (IQR); VL = Viral load; IQR = Interquartile range, AVE = Average; cp/mL = copies per milliliter

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3.3 HBV STATUS

The HBV status of the enrolled patients was confirmed. A total number of 100 (88.5%) out of the 113 study participants were reactive for hepatitis B surface antigen (HBsAg) on the Alere Determine rapid diagnostic test kit (Alere Medical Co. Ltd., Japan. For patients with enough serum, a second assay was also used in confirming the presence and quantity of HBsAg present in the study participants. Out of 113 patients, seventy-six patients had enough serum for HBsAg quantification using the ECLIA kit (Elecys® HBsAg II, Roche Diagnostics, Germany) with a calculated median of 1934 IU/mL and ranged from 48 – 9118 IU/ml. Using real time PCR assay to assess the presence and quantity of HBV DNA, 31/113 (27.4%) of the study participants enrolled at baseline had detectable HBV DNA with HBV viral loads established for 13 out of the 31 (41.9%) samples. The median HBV DNA load was 709.3 IU/mL with patients' viral loads ranging from 1.66 to 25,538.24 IU/mL. Among the 31 real time PCR positives for the presence of HBV DNA, 4 samples were HBsAg negative. After the 4 to 6 month's follow up sampling, 49 patients were lost to follow up and 50 out of the 64 patients whose blood samples were obtained tested negative on real time PCR for HBV DNA. Out of these 50, 4 had their baseline testing positive for HBV DNA. Among the follow up samples, 14 had detectable DNA with 1 having a negative test results at baseline. Seven out of the 14 (50%) follow up samples with detectable DNA were quantitatively positive with a median VL of 223.62 IU/Ml (IQR = 0.41 – 11890 IU/Ml). Out of the seven samples with quantifiable HBV DNA, 3 had an increased HBV VL whilst four had a decreased HBV VL.

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Table 3. 3: Characteristics of HBV status of patients

HBV Status	Number of Patients	Viral Load (IU/mL)
RDT		
Yes	100	-
No	13	-
ECLIA		
Median, IU/mL (IQR)	76	1934 (48 - 9118)
Real time PCR (Baseline)	113	
Negative	82	-
Positive	31	-
Qualitative	18	-
Quantitative	13	709 (1.66 – 25538.25)
Occult HBV Infection	4	
Real time PCR (Follow up)	64	
Negative	50	-
Positive	14	-
Qualitative	7	-
Quantitative	7	709 (1.66 – 25538.25)
Increased VL	3	(3704.32 BL – 13498.69 FU)*
Decreased VL	4	(3474.58 BL – 2447.979 FU)*

*= Average of the viral load of the number of samples counted; BL = Baseline; FU = Follow up; VL = Viral load; IQR = Interquartile range

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3.4 PREVALENCE OF HDV IN HIV/HBV CO-INFECTED AMONG STUDY POPULATION

To determine the exposure of patients to HDV, the level of immunologic response was checked by measuring the levels of immunoglobulin G (IgG) in the patient serum sample. Of the 113 serum samples tested, 2 (1.8%) had serological indication of exposure to HDV. The presence of HDV RNA was also tested for in all patient samples both baseline and for those whose follow up samples were obtained. Of the 113 samples tested 2 positives were obtained when real time PCR was used whilst 4 positives were obtained by conventional PCR which included both positives obtained from ELISA and real time PCR assay. The overall prevalence of HDV in HIV/HBV co-infected patients was 3.5% in this study population. The observed prevalence may potentially be slightly higher as the sample size obtained is less than the calculated sample size of 138.

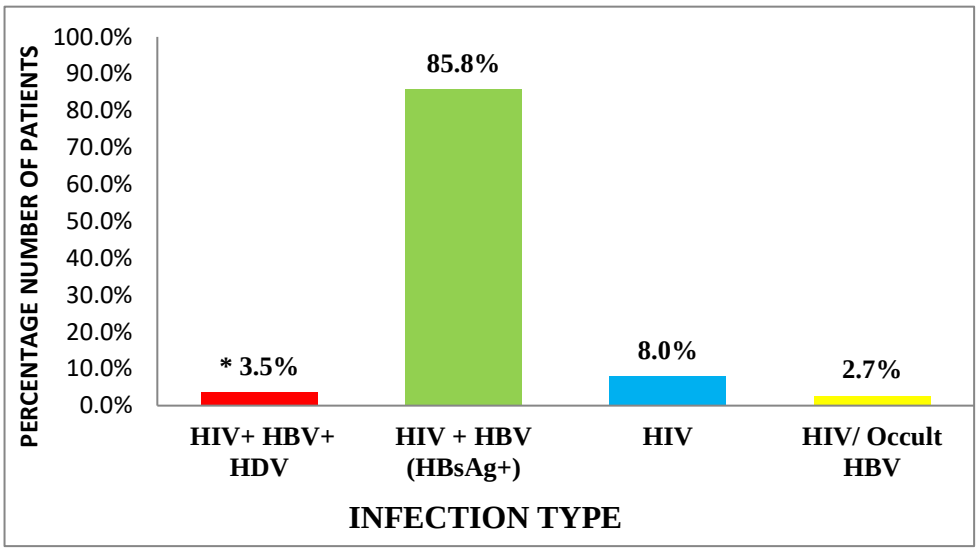

Figure 3.2: Percentage distributions of infection types in enrolled patients

*One patient had HIV/occult HBV infection

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3.5 BIOCHEMICAL ANALYSIS OF ALT AND AST IN HIV INFECTED PATIENTS CO-INFECTED WITH HBV AND HDV.

To assess the biochemical presentation of HBV and HDV in HIV infected individuals, firstly, a comparison of the liver enzyme (ALT and AST) levels between HBsAg negative and positive patients were done. The measured median level of liver enzymes ALT, in HBsAg positive patients was 19 (IQR = 3-93IU/Ml) and that of HBsAg negative patients were 19 (2 – 53) with no statistical significance observed (p-value = 0.44). The median level of AST in both HBsAg positive patients, 25 (IQR = 7-82) and HBsAg negative patients, 26 (IQR = 14-51) was not statistically different (p-value = 0.59). The median levels of ALT and AST in HDV positive patients (19.5, IQR= 8 - 51 and 24.5, IQR=21 – 43) were slightly higher but statistically not significant (p-value = 0.74) than that of HDV negative patients (19.0, IQR= 2 - 93 and 25.5 IQR= 7 – 75).

To test whether the amount of HBV DNA load affects the level of liver enzyme activity, a univariate analysis of the various risk factors such as Age, Sex, HIV VL suppression, infection type and ART regimen affecting the liver enzyme activity was tested using logistic regression analysis. In this study patients who had low HBV DNA were observed to have low levels of ALT and AST compared to those whose HBV DNA load was not suppressed. This observation was statistically significant (p-value = 0.027). Other risk factors did not show any association when assessed (Table 3.4)

All four HDV positive patients have lamivudine (3TC) as part of their ART regimen with one patient on both lamivudine and tenofovir (TDF) based regimen. High ALT (51IU/Ml) and AST (43 IU/Ml) levels was observed in the HDV positive patient receiving TDF as part of their ART regimen as compared to HDV positive patients not receiving TDF (Fig 3.3) but no statistical inference could be drawn.

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Table 3.4: Univariate analysis between liver decomposition and various risk factors

Independent Variables		Univariate Analysis		
Variable	Category	High ALT [n=13(11.50%)]	Low ALT [n=100(89.50)]	Test Statistic (p-value)
ART regimen	With TDF	7 (70.0)	68 (71.58)	
	Without TDF	3 (30.0)	27 (28.42)	X^2=0.01 (0.916)
HBV VL Suppress	Yes	5 (71.43)	69 (94.52)	X^2=4.91
	No	2 (28.57)	4 (5.48)	(0.027)
HBV/HIV co-infect	Reactive	11 (84.62)	89 (89)	X^2=0.22
	Nonreactive	2 (15.38)	11 (11)	(0.641)
Sex	Male	8 (61.54)	42 (42)	X^2=1.78
	Female	5 (38.46)	58 (58)	(0.182)
Age	Mean	49	44	t=1.86(0.06)

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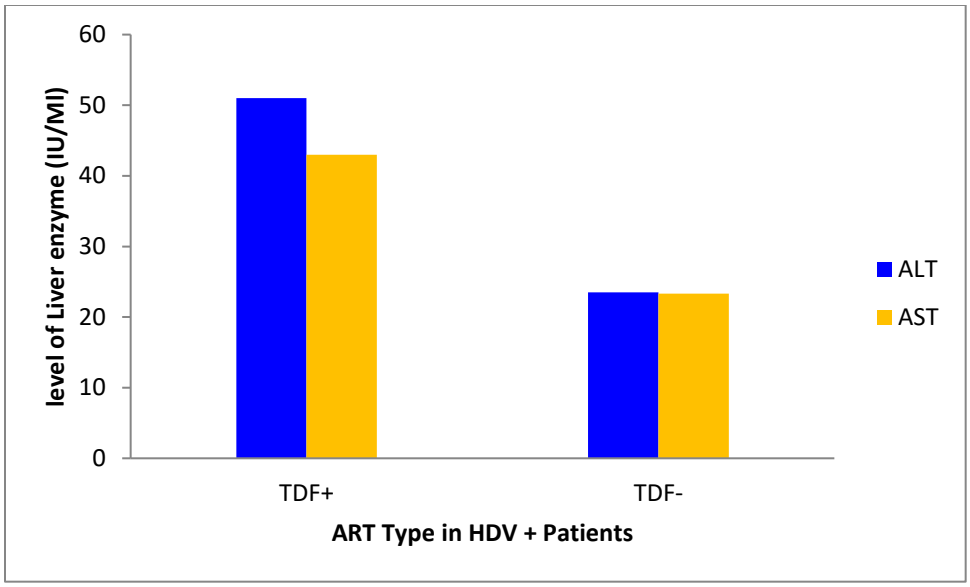

Figure 3.3: Effect of TDF on ALT and AST

Comparative presentation of level of liver enzymes in HDV positive patients receiving TDF against HDV positive patients who are on only lamivudine based ART regimen. No statistical analysis could be done as there is only one patient out of the 4 HDV+ patients who was on TDF.

3.6 HBV GENOTYPING

Of the 31 positives identified by real time PCR, amplification of the HBV S-gene was obtained for 17 (54.8%) specimen with successful sequences obtained for 15/17 (88.2%) samples. After aligning the generated sequences with reference strains from GenBank, all the 15 samples clustered with reference strains of genotype E (Fig 3.4). All the HBV 15 sequences obtained in this work have been submitted to the GenBank with the following accession numbers assigned to them:- MN996900, MN996901, MN996902, MN996903, MN996904, MN996905, MN996906, MN996907, MN996908, MN996909, MN996910, MN996911, MN996912, MN996913 and MN996914

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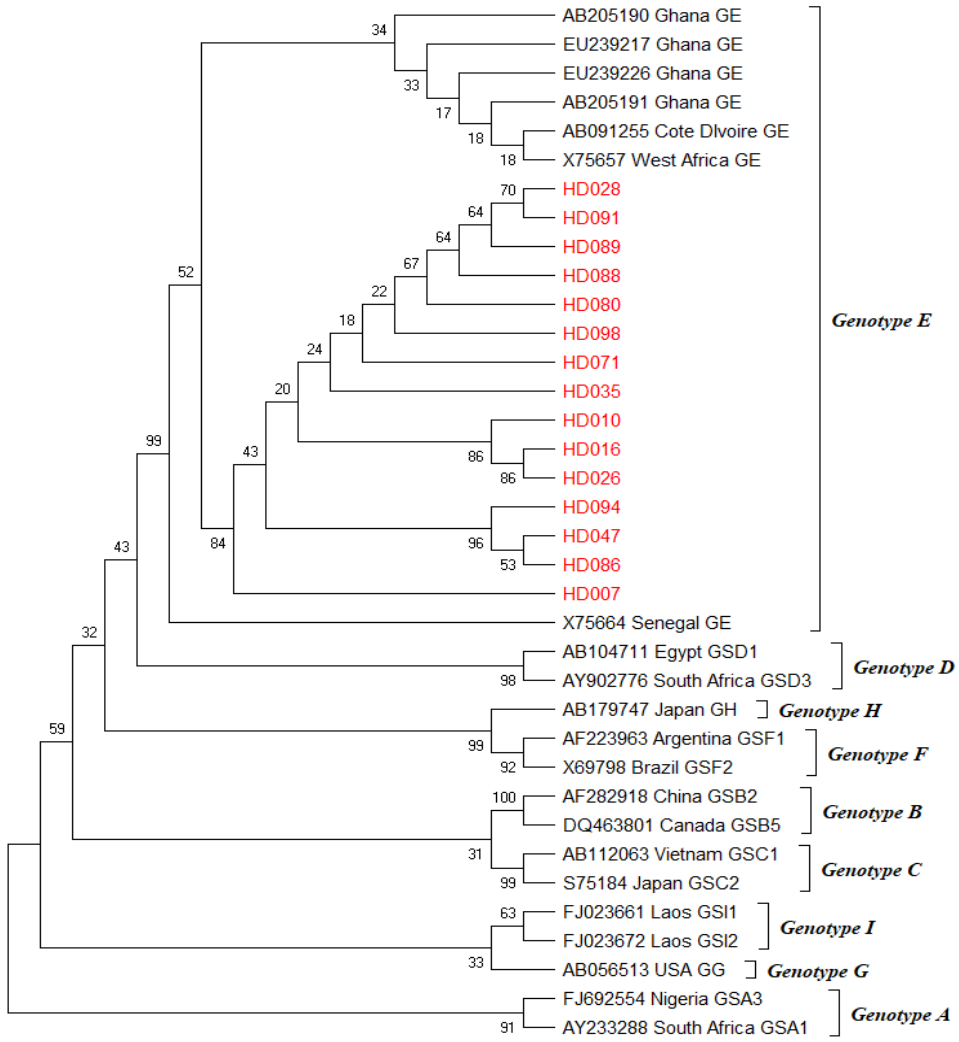

Figure 3.4: Phylogenetic tree showing the strain type of HBV

All samples clustered with genotype E reference strains from Ghana and Senegal. Sequences from this work are highlighted in red fonts.

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3.7 HDV GENOTYPING

Using primers targeting the R0 region of the HDV genome, 4 samples were amplified successfully and sequences obtained for 2/4 (50%) samples. The sequenced samples were aligned with reference strains from GenBank and clustered with Genotype 1 reference strains as shown in Fig 3.5.

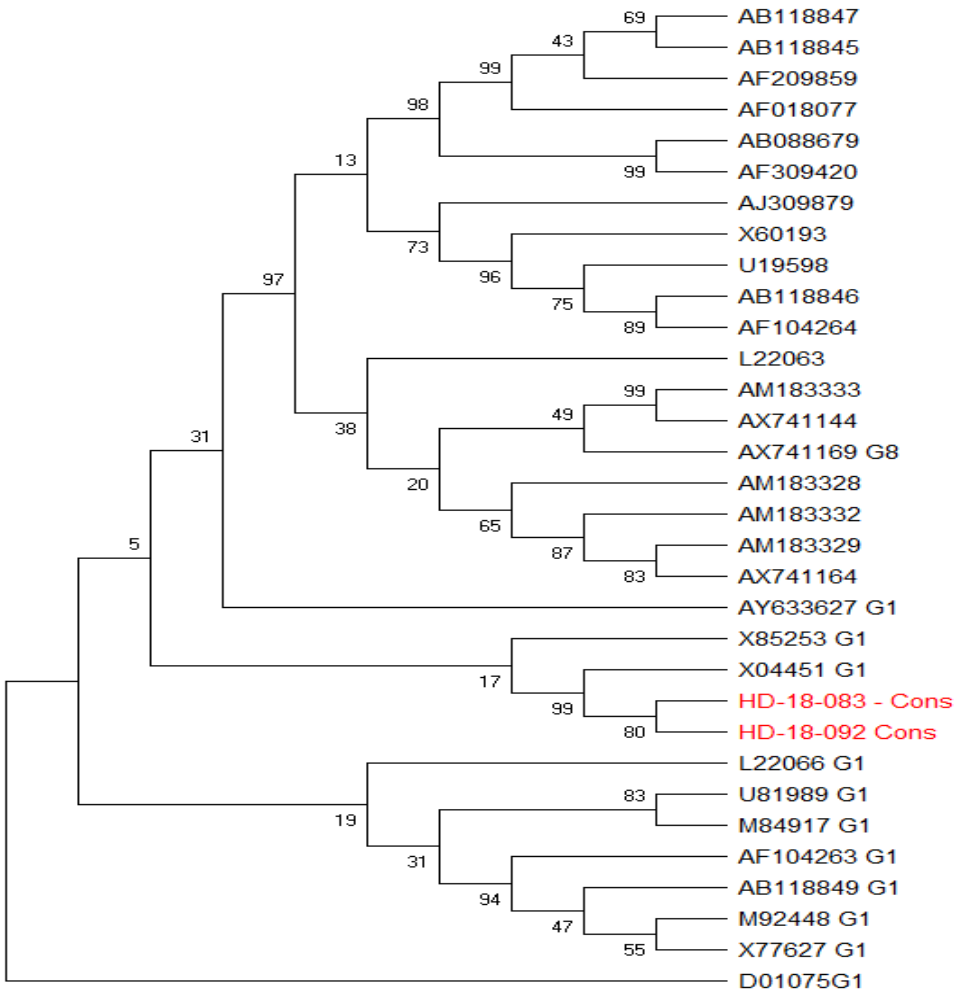

Figure 3.5: Phylogenetic tree showing the strains of HDV from this study as shown in red

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CHAPTER FOUR

4.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

4.1 DISCUSSION

Globally, an estimated 15 million HBV infected people are co-infected with HDV (Fernández-Montero et al., 2014) and although HDV causes the most severe form of viral hepatitis; it is often neglected during testing for viral hepatitis, thus, considered as a neglected disease (Soriano et al., 2013). Meanwhile, higher prevalence rates of HDV infection have been reported in places such as the Middle East, Mediterranean basin, Eastern Europe, Central Africa, Amazon Basin, and the Central Asia (Fernández-Montero et al., 2014). Since HIV, HBV, and HDV share similar transmission routes (Katwesigye et al., 2016), and HBV being the cause of a greater proportion of liver related deaths in HIV infected patients, infection with HDV has been shown to be relatively common among HIV/HBV co-infected patients (Fernández-Montero et al., 2014) leading to an aggressive form of liver disease such as cirrhosis, Liver cancer and death (Bekondi et al., 2010; Hughes et al., 2011). There is thus the need to test for markers of HDV in HBV chronic patients especially in HIV/HBV co-infected patients (Serrano et al., 2007). In Ghana, about 13.6% of HIV patients are also infected with HBV (Agyeman & Ofori-Asenso, 2016) with little information on HDV exposure or presence in these HIV/HBV co-infected patients as most studies focus on HDV in HBV infected individuals (Ampah et al., 2016).

This study was conducted to check for exposure, presence and genotypes of HDV in HIV/HBV co-infected individuals as well as look at the effect of infection with HIV, HBV, and or HDV on liver enzymes (ALT/AST) activity. Additionally, the genotypes of HBV circulating amongst these HIV/HBV co-infected patients and the effect of HBV DNA levels and other risk factors such as

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age, HIV RNA load, type of ART, duration of ART and gender on ALT and AST were evaluated in the study.

In this study, 2% anti-HDV IgG antibodies detected suggested serological evidence of exposure to HDV. All patient samples were tested for HDV RNA despite the serological testing as anti-HDV is often weak and might also be delayed by several months after onset of illness (Collier & Schillie, 2018). This resulted in the detection of HDV RNA in 2% of HIV/HBV co-infected patients thereby giving an overall prevalence of HDV observed to be 3.54% in this study. The 2% sero-prevalence observed in this study is similar to literature on the evidence of HDV in HIV/HBV co-infected patients in Ghana by Stockdale and colleagues in 2010 who observed a sero-prevalence of 2.2% in their study population. They also observed 40% of the 2.2% of their anti-HDV antibody positives having detectable RNA (Stockdale et al., 2017). The observed overall prevalence of 3.54% in this study when compared to the study by Stockdale and colleagues, in HIV/HBV co-infected patients in Ghana was higher as they reported a 2.2% prevalence of HDV in HIV/HBV co-infected patients in their study. In the African region, few studies have looked at the presence of HDV in HIV/HBV co-infected patients obtaining prevalence from as low as 1.5% to as high as 46% (Butler et al., 2018; Hønge et al., 2014; Katwesigye et al., 2016; Stockdale et al., 2018; Winter et al., 2016). Due the very few studies conducted to test for the presence and disease burden of HDV in HIV/HBV co-infected patients especially in Ghana, it is recommended that more studies be conducted across the country to provide data on the disease burden in this cohort of patients.

Some studies have reported HDV to have a negative effect on the replication of HBV without much or any effect on HBsAg production (Alfaiate et al., 2016; Pollicino et al., 2011) as HBsAg can be produced from either the HBV cccDNA or produced from HBV DNA integrated into the host genome (Kabaçam et al., 2012). In this study, all 4 HIV/HBV/HDV samples were reactive on

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the HIV confirmatory test and had detectable HDV RNA with out of 75% of them having undetectable HBV DNA but were HBsAg reactive. From the above results, we infer that HDV has an effect on the HBV, although the effect of the antiretroviral therapy (3TC, TDF) being administered cannot be overlooked since 70% of the HBsAg positive patients also had negative HBV DNA results. A research study reported no effect of 3TC on HDV (Niro et al., 2005) whilst other studies have reported a positive effect of TDF on HDV replication after a prolong treatment (Soriano et al., 2014). We therefore recommend further studies to be conducted in a larger population to clearly find the effect of HDV on HBV.

In this study, a total number of 12 HIV patients out of the 113 enrolled patients had HBsAg negative test results of which 4 (33.3%) had detectable HBV DNA in their serum indicating the presence of occult infection in our study patients. This condition called the occult hepatitis B virus infection is when an HBV positive patient has detectable HBV DNA in the absence of HBsAg in the serum (Hoffmann & Thio, 2007). Some research have shown that HIV infected individuals have increased prevalence of occult HBV whilst others have recorded a low prevalence of occult HBV in HIV infected patients (Mphahlele et al., 2002). Anti-HDV antibody and HDV RNA was detected in one of the 4 patients with occult HBV infection. Meanwhile, occult HBV according to researchers, may have an association with progression to liver cancer and cirrhosis in HIV infected patients (Bajjou et al., 2015; Hu, 2002; Mittal & Hu, 2017; Pogany et al., 2005). Further work is recommended to study the risk of occult HBV infection compared with chronic HBV infection in terms of liver disease infection and also the occurrence of HDV in occult HBV infected patients.

Within the HIV/HBV co-infected patients receiving HAART, a few important etiologies of liver enzyme elevations can be sectioned into three sets: - ART related, HBV chronicity and other risk factors such as age, presence of other hepatitis virus infection, HIV RNA load and even gender.

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Thus, determining the root cause of the elevation of the liver enzymes is very important as it informs the right therapy (Hoffmann & Thio, 2007). Elevated liver enzymes in HIV/HBV co-infections can be related to toxicity of the anti-retroviral as these drugs may cause injury to the liver through direct toxicity or may inhibit the activity of the DNA polymerase gamma in the mitochondria. Additionally, idiosyncratic reactions may occur with abacavir and nevirapine (Iorga et al., 2017; Kaplowitz, 2004). The presence of HBV in PLHIV is known to increase the risk of hepatotoxicity from ARVs three-fold to five-fold (Ngala et al., 2015; Puoti et al., 2003; Sulkowski et al., 2000) with studies in HIV/HBV co-infections in countries such as Taiwan and Thailand showing hepatoxicity to 15.3 to 16.0 episodes per hundred person years (Law et al., 2003; Sheng et al., 2004). The effect of ART on the liver enzyme in HIV/HBV co-infected patients in this study was analyzed using a univariate logistic regression which showed no association between ART and ALT/AST (p-value = 0.916), even though one HIV/HBV/HDV and nine HIV/HBV patients were observed to have high ALT levels. This observation is consistent with a study by Soriano et al in 2014 that checked for effect of tenofovir on HIV/HBV. Although they observed high ALT and AST in all but four of the study patients, a univariate logistic analysis they conducted did not associate the elevated or high liver enzyme ALT to the use of tenofovir drug.

Hepatitis B chronicity can affect liver enzyme levels as HBeAg seroconversion (undetectable HBV DNA or <2000 IU/mL) can be indicated by hepatic flares in ALT and AST levels. Some studies have observed high HBV DNA and lower liver enzyme elevations with more cirrhosis (Hoffmann & Thio, 2007) whilst another study correlated HBV DNA levels with serum ALT to be significant which led them to the conclusion that patients whose HBV DNA levels are elevated have an increase in liver inflammation (Milich, 1991). This study noted that patients with baseline HBV DNA greater than 2000 IU/mL had normal to low levels of ALT and AST. This reduced during

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follow-up testing and a logistic regression analysis of HBV DNA with ALT, AST levels revealed that suppression of HBV can be correlated with low levels of ALT, consistent with earlier submission although we didn't screen for cirrhosis or liver cancer. We thus recommend future studies to look at the presence or absence of cirrhosis in order to test the observation by Hoffman and his colleagues. Moreover, HBV positive patients are at risk of getting infected with hepatitis D virus which also affects ALT/AST levels (Hoffmann & Thio, 2007). However, in this study, infection type (i.e., whether a patient has HDV/HIV/HBV or HIV/HBV or HIV alone) did not have significant association to the ALT and AST levels when one-way ANOVA was tested (p-value > 0.05).

In our research work, all isolates obtained were determined to be genotype E after phylogenetic analysis and were observed to cluster around isolates from countries in the Western region of Africa. Hepatitis B is described as heterogeneous virus because the virus polymerase enzyme lacks proof reading ability leading to mutation rates estimated to be almost same as retroviruses and 10,000 times higher than other DNA genomes Currently, there are eight genotypes of HBV that have been described which are genotypes A – H. These HBV genotypes have distinct geographic distribution (Kramvis & Kew, 2007; Norder et al., 2004). Genotype A is restricted to Africa, Asia Europe, USA and Japan, with genotype B restricted to Asia, genotype C is restricted to Australia and Asia. Whilst genotype D causes a global infection, genotype E is restricted to West Africa but has been identified in the Central Africa. Genotype F is restricted to South America, genotype G is restricted to France and the USA and finally, genotype H restricted Central America. In Africa, genotype A is predominant in the southern, Eastern and central regions whilst genotype D is the predominant type in the Northern region with most of the isolates obtained in the West, being genotype E. Some studies in Ghana (Ampah et al., 2016; Bonney et al., 2013; Candotti et al., 2007)

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identified genotype E as the most predominant with low number of genotypes A and D also identified. Another study by Forbi and colleagues also observed mainly two genotypes which were HBV genotypes A and E with genotype E being predominant in the West (Ghana, Cote D'Ivoire) and Central (Cameroon) African countries whilst isolates from the Eastern part of Africa belonged to genotype A (Forbi et al., 2013). The observed genotype E in this study goes to confirm the earlier submissions that Western part of Africa predominantly has the HBV genotype E. The implication of this genotype on clinical outcomes of HIV/HBV infected patients, have not been well documented.

In this study, two out of four positive HDV patient samples were sequenced and genotypes determined to be that of genotype 1. Hepatitis Delta virus like all other viruses also have different variations in their genome which makes them to be grouped into 8 clades or genotypes currently. The various genotypes are mostly distinct to specific geographical setting with genotype 1 being the exception as it causes a global infection. Genotypes 2 and 4 are usually found in Asia, genotype 3 in the Americas whilst genotypes 5 to 8 are mostly restricted to Africa. Studies which have identified genotypes 5 to 8 outside of the African continent found the patients to be of African descent (Manns, 2017). In Ghana most of the studies carried out looking at the presence of HDV have been in HBV mono infected patients which have identified genotype 1 as the predominant HDV genotype (Cramer – unpublished) whilst isolates from the eastern part of Africa belong to genotype A. Limited studies have been done on the prevalence of HDV in HIV/HBV co-infected patients without genotyping results in Ghana. As such, to the best of our knowledge, this is the first or one of the few studies to identify HBV genotypes in HIV/HBV co-infected patients in Ghana. Due to loss to follow up of participants, clinical and virologic data of HDV on HBV replication for three of the four HDV positive patients samples could not be inferred. However,

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three out of the four samples had HBsAg detected whiles one sample had detectable HIV and HBV DNA but no HBsAg detected which is an indication of an occult infection of HBV in this HIV infected patient.

4.2 CONCLUSION

In this research study, the presence of occult hepatitis B infection, which is an essential factor in the development of cirrhosis and liver cancer, was identified in HBsAg negative patients. We observed that HBV DNA suppression, correlated with low levels of ALT whilst no association between HBV DNA suppression and AST was observed. Data obtained in this study established the occurrence of HDV in patients co-infected with HIV/HBV to be 3.54% in our study population. No correlation was observed between ALT/AST levels and other risk factors such as age, gender, type of ART, duration of ART, and HIV RNA load. Amongst the HIV/HBV co-infected patients recruited for this study, HBV genotype E and HDV genotype 1 were observed to be in circulation. The above findings support other research studies and therefore become imperative to look out for the presence of HDV in HIV/HBV co-infected patients as well as monitor HIV/HBV patients whose subsequent HBsAg test results turn out to be unreactive.

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4.3 RECOMMENDATIONS

1. Future studies are recommended in a larger population to determine virologic and clinical implication of HDV in HIV/HBV co-infected patients.
2. Testing of HIV/HBV positive patients for the presence of HDV is recommended as HDV is known to increase liver damage progression to cirrhosis within a short period.
3. Molecular techniques including PCR should be applied to monitor HIV/HBV patients who are subsequently HBsAg unreactive in order to rule out occult cases.
4. Future studies should investigate HIV/HBV positive patients with low or normal levels of ALT and AST to be screened for the presence or absence of cirrhosis as studies suggest the presence of cirrhosis in these co-infected patients.
5. Whole genome sequencing of HBV genotype E is recommended to elucidate the virologic and clinical outcomes of patients who are infected with HBV genotype E. Added to this, studies looking at mutations in this genotype and their association with susceptibility to current ART as well as their importance in disease progression.

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APPENDICES

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH
Established 1979A Constituent of the College of Health Sciences
University of Ghana

INSTITUTIONAL REVIEW BOARD

Phone: +233-302-916438 (Direct)
+233-289-522574
Fax: +233-302-502182/513202
E-mail: nirb@noguchi.ug.edu.gh
Telex No: 2556 UGL GH

Post Office Box LG 581
Legon, Accra
Ghana

My Ref. No: DF.22
Your Ref. No:

2nd May, 2018

ETHICAL CLEARANCE

FEDERALWIDE ASSURANCE FWA 00001824 **IRB 00001276**

NMIMR-IRB CPN 090/17-18 **IORG 0000908**

On 2nd May, 2018, the Noguchi Memorial Institute for Medical Research (NMIMR) Institutional Review Board (IRB) at a full board meeting reviewed and approved your protocol titled:

TITLE OF PROTOCOL : **Hepatitis Delta Virus (HDV) in HIV/HBV (HEPATITIS B VIRUS) Co-Infected Patients in Ghana**

PRINCIPAL INVESTIGATOR : **Keren Okyerebea Attiku, MPhil Cand.**

Please note that a final review report must be submitted to the Board at the completion of the study. Your research records may be audited at any time during or after the implementation.

Any modification of this research project must be submitted to the IRB for review and approval prior to implementation.

Please report all serious adverse events related to this study to NMIMR-IRB within seven days verbally and fourteen days in writing.

This certificate is valid till 1st May, 2019. You are to submit annual reports for continuing review.

Signature of Chair: .....
Mrs. Chris Dadzie
(NMIMR – IRB, Chair)

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University of Ghana

Phone: +233-302-916438 (Direct)
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E-mail: nirb@noguchi.ug.edu.gh

My Reference: DF 22

NMIMR-IRB
P. O. Box LG 581
Legon, Accra
Ghana

July 26, 2019
Mphil.Cand. Keren Okyerebea Attiku
Department of Biochemistry, College of Basic and Applied Science, UG 1=Lego
P. O. Box LG 581
Legon
Accra

RE: Our Study # 090/17-18 **At:** NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH-IRB

Dear Keren Okyerebea Attiku:

Meeting Date: 5/8/2019 **At:** NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH-IRB
Protocol Title:
Hepatitis Delta Virus (HDV) in HIV/HBV (HEPATITIS B VIRUS) Co-Infected Patients in Ghana

This is to advise you that the above referenced Study has been presented to the Institutional Review Board, and the following action taken subject to the conditions and explanation provided below.

Internal #: 2348
Expiration Date: 5/7/2020
On Agenda For: Procedure
Reason 1: Amendment/Renewal **Reason 2:**
Description: The PI is requesting the following changes:
1. To increase the sample size from 120 to 138.This is because the previous sample size was based on a prevalence of HBV in HIV (7.3%).
2. Study objective 4 has been amended to read "To characterize HDV and HBV in HIV/HBV co-infected patients". This is to establish the circulating strains of HBV in the cohort of the study.
IRB ACTION: Renewed
Condition 1:
Action
Explanation: The amendment was approved.

Figure A. 1: Ethical Clearance from the Institutional Review Board of Noguchi Memorial Institute for the Medical Research.

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In case of reply the number
And the date of this
Letter should be quoted

My Ref. No. KBTH/MS/GS/19

Your Ref. No......

KORLE BU TEACHING HOSPITAL
P. O. BOX KB 77,
KORLE BU, ACCRA.

Tel: +233 302 667759/673034-6
Fax: +233 302 667759
Email: Info@kbth.gov.gh
pr@kbth.gov.gh
Website: www.kbth.gov.gh

10th January, 2019

KEREN OKYEREBEA ATTIKU
DEPT. OF BIOCHEMISTRY
COLLEGE OF BASIC AND APPLIED SCIENCE
UNIVERSITY OF GHANA
LEGON

INSTITUTIONAL APPROVAL: KORLE BU TEACHING HOSPITAL-SCIENTIFIC AND TECHNICAL COMMITTEE/INSTITUTIONAL REVIEW BOARD (KBTH-STC/IRB/000113/2018

Following approval of your study entitled "Hepatitis delta virus (HDV) in HIV/HBV (Hepatitis B virus) co-infected patients in Ghana" by the Korle Bu Teaching Hospital-Scientific and Technical Committee/Institutional Review Board.

I am pleased to inform you that institutional approval has been granted for the conduct of your study in Korle Bu Teaching Hospital.

Please contact the Head of Department to discuss the commencement date of the study.

Please note that, this institutional approval is rendered invalid if the terms of the Institutional Reviewed Board/Scientific and Technical Committee approval are violated.

Sincere regards,

Dr. Samuel Asiamah
Director of Medical Affairs
For: Chief Executive Officer

Cc: The Chief Executive
Korle Bu

Figure A. 2: Ethical Clearance from the Institutional Review Board of Korle-Bu Teaching Hospital.

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Consent Form

Research Topic: Hepatitis Delta Virus (HDV) in HIV/HBV Co-Infected Patients in Korle-Bu, Ghana

Principal Investigator: Keren Okyerebea Attiku

Address: NMIMR, P. O. Box LG 581 Legon Accra

Purpose of The Study/ General Information

The purpose of this study is to check for the presence of hepatitis D virus (HDV) disease in the blood of patients who are positive for HIV and hepatitis B virus (HBV) at the Korle-Bu Teaching Hospital in the Greater Accra Region of Ghana. Hepatitis D virus will only infect people who are positive for hepatitis B. We want to know whether the hepatitis D virus is causing more harm to the liver of patients who are HIV and HBV. We will also measure certain chemicals the body produce to show if the liver is working well or not. Information from this study will help doctors know the kind of treatment to give to HIV/HBV patients who are also positive for HDV. The information will also be used to develop future programs and interventions to address any effects the presence of HDV in HIV/HBV patients have on public health in Ghana.

We will appreciate that you take part in this research study which will carry out further laboratory investigations with your clinical specimen. It is intended to help you and other patients with similar diagnosis, manage the treatment regimens and prevent the progression into advance stages as well as the introduction of other forms of the virus.

Study Procedures

If you agree to be in this study, you will participate in a survey with a staff member where your age, sex, your background, health, and history of HIV/HBV treatment. Your name will not be taken or written instead, an Identification number (ID) will be given to your form to protect your privacy. Information about your diagnosis will be taken as well as the type of drugs you are taking. It will take up to 10 minutes to complete the survey form and any information you provide will be kept confidential. A trained laboratory person will take 5ml (1 tablespoon) of blood from you at the time of filling the survey form and 6 months after the first blood sample have been taken. The blood samples will be sent to Noguchi Memorial Institute for Medical Research, Legon, for analysis and storage.

Reason for the Research

Liver related deaths have been observed to be increasing in the HIV patients especially those who are also infected with HBV and HDV. Hepatitis D virus will only infect people who are positive for hepatitis B and causes the most severe form of liver disease and increases the time to develop liver cancer and death.

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Currently, most of the drugs that are used in the management of HIV and HBV, does not have an effect on HDV. There is only one type of drug that really seem to work in HDV management. You are therefore being asked to take part in this research to find out whether you are positive for HDV or not. The results will help the doctors in Ghana be aware of the importance to check for the hepatitis D in people who are also positive for HBV and give better care to such people. This will also help the people in charge of the hospitals in Ghana be made aware that HDV should be tested for all HBV positive people and to also bring in the right medicine for treatment.

Why have I been asked to participate? You have been chosen because we are looking for hepatitis D virus in HIV/HBV positive patients. We will like to 5ml (1 tablespoon) of blood from you at the time of filling the survey form and 6 months after the first blood sample have been taken because you have tested positive for HIV and HBV and you are non-alcoholic.

Do I have to take part? You are free to choose to take part of this study or not. If you decide not to take part in this study, it will not affect the care you will receive in anyway. If you agree to participate you are free to end your participation at any time you want to.

Will I be paid any money? There will be no money paid to you if you take part in this study.

What will happen to me if I take part? If you take part, a trained staff member will collect information from your medical records. Your name will not be written down and you will be asked a few questions. You will be asked to provide 5ml (1 tablespoon) of blood at the time of filling the survey form and 6 months after the first blood sample have been taken for research purposes. The blood for this study will not have your name on it so there will be no way to provide the test results back to you.
I would also like to store some of your blood to be used for later testing after obtaining permission from an ethics board. Any later test results cannot be linked back to you because I will not be writing your name on any of the blood test tubes. You may take part in the study without having your blood stored for later testing. We will store your samples for 5 years

Will my taking part in this research study be kept private? All information and blood samples will be kept private. Names will not be written on the forms or the blood tubes. All information will be kept well and taken to Noguchi Memorial Institute for Medical Research where they will be stored in a locked cabinet that can only be opened by an assigned research staff and the people in charge of this study. When

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the study is over and the results are submitted to the Biochemistry department of the University of Ghana and also published, your name will not appear in any of such documents.

What will I get by participating in this research study?

By participating in the study, you are helping me to identify hepatitis D virus in HIV/HBV patients at the Korle-Bu Teaching Hospital, Ghana. The information will help generate future studies and improve HIV and hepatitis programs in Ghana.

What is the risk of being in this research study?

Should you take part in this study, the risk associated is small. All the things that will be used to take the 5ml (1 tablespoon) of blood at the time of filling the form and 6 months later, are clean and safe. They have never been used before and will be thrown away after each use. Some people may feel dizzy when blood is drawn and there may be some pain or discomfort from the needle stick. You may develop a bruise or swelling where the needle goes into your arm. If you have any pain, bleeding, or swelling from taking blood, please let us know so we can take care of it.

What will happen to the results of this research study?

The results will be written into my thesis which will not have any individual names. This thesis will be used by the Ministry of Health/ Ghana Health Service and other donors to improve the management of liver diseases caused by hepatitis D virus in Ghana.

Do I receive the results? No. The results will not be given to you or your doctor. It will be used to develop future programs and interventions to address the testing of any HIV/HBV patient for the presence of HDV and inform on the drug regimen of choice as well as inform on the effect of hepatitis D virus on the public health in Ghana.

Confidentiality

Your personal information and laboratory results will be held in the strictest confidence, and no identifying information of any kind will be released to any other person or agency without your specific permission in writing. Your samples collected for research purposes will be labelled with a code number and will be taken to Noguchi Memorial Institute for Medical Research for processing. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

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Volunteer Agreement

The document detailing the risks, discomfort, benefits and procedures involved in the research work entitled *Hepatitis delta virus (HDV) in HIV/HBV co-infected patients in Ghana* has been read and adequately explained to me. I have been given full opportunity to have any questions I may have answered to my satisfaction. Therefore, I agree to participate as a volunteer.

Do you agree to be in the study? _____ Yes___ No☐ ☐

Do you agree to have your blood stored for future studies? _____Yes___☐No___ ☐

_____ _____

Signature (or thumb print) of consenting individual Date

If a volunteer cannot read the document, then a witness is needed; I was present during the reading and explanation of the consent document to the volunteer; all questions from the volunteer were duly answered and the volunteer agreed to participate in this study.

Signature of witness..... Date.....

I certify that the purpose and the nature of the research, the potential benefit and possible discomfort associated with this research has been explained to the volunteer who has agreed to voluntarily participate

Signature of person who obtained the consent..... Date.....

If You Have a Problem or Have Other Questions

Please call Keren Okerebea Attiku (0242 329146) if you have further questions about the research.

4

Figure B. 1: Consent form endorsed by the Institutional Review Board of Noguchi Memorial Institute for the Medical Research.

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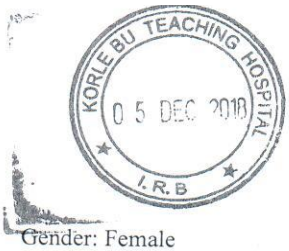

CONSENT FORM

Personal Details

Title and Name: Keren Okyerebea Attiku

Place of Birth: Korle-Bu, Ghana

Gender: Female

Date of Birth: 9th March, 1986

Phone Number: +233242329146

Home Address: No. 4 Naa Amui Street, Ashaiman.

Postal Address: P. O. Box, LG 581, Legon

Email:kaaoma@gmail.com; koattiku@st.ug.edu.gh

Nationality: Ghanaian

Occupation: Biomedical Research Scientist

Person to notify in case of an emergency: Bryan Edwin Attiku, 0243072523

Applicant Information

Title: **Hepatitis Delta Virus (HDV) in HIV/HBV Co-Infected Patients in Ghana**

Principal Investigator: Keren Okyerebea Attiku

Address: Department of Biochemistry, Cell and Molecular Biology, University of Ghana, P.O. Box LG 54, Volta Road, Legon.

General information about Research: The purpose of this study is to check for the presence of hepatitis D virus (HDV) disease in the blood of patients who are positive for HIV and hepatitis B virus (HBV) at the Korle-Bu Teaching Hospital in the Greater Accra Region of Ghana. Hepatitis D virus will only infect people who are positive for hepatitis B. We want to know whether the hepatitis D virus is causing more harm to the liver of patients who are HIV and HBV. We will also measure certain chemicals the body produce to show if the liver is working well or not. Information from this study will help doctors know the kind of treatment to give to HIV/HBV patients who are also positive for HDV. The information will also be used to develop future programs and interventions to address any effects the presence of HDV in HIV/HBV patients have on public health in Ghana. We will appreciate that you take part in this research study which will carry out further laboratory investigations with your clinical specimen. It is intended to help

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you and other patients with similar diagnosis, manage the treatment regimens and prevent the progression into advance stages as well as the introduction of other forms of the virus.

If you agree to be in this study, you will participate in a survey with a staff member where your age, sex, your background, health, and history of HIV/HBV treatment. Your name will not be taken or written instead, an Identification number (ID) will be given to your form to protect your privacy. Information about your diagnosis will be taken as well as the type of drugs you are taking. It will take up to 10 minutes to complete the survey form and any information you provide will be kept confidential. A trained laboratory person will take 5 mL (1 teaspoon) of blood from you at the time of filling the survey form and 6 months after the first blood sample have been taken. The blood samples will be sent to Noguchi Memorial Institute for Medical Research, Legon, for analysis and storage.

Possible risks and discomfort: Should you take part in this study, the risk associated is small. All the things that will be used to take the 5 mL (1 teaspoon) of blood at the time of filling the form and 6 months later, are clean and safe. They have never been used before and will be thrown away after each use. Some people may feel dizzy when blood is drawn and there may be some pain or discomfort from the needle stick. You may develop a bruise or swelling where the needle goes into your arm. If you have any pain, bleeding, or swelling from taking blood, please let us know so we can take care of it.

Possible benefits:

There will be no direct benefits to participants.

Voluntary participation: You are free to choose to participate or not to participate. If you decide not to participate this is will not affect the care you will receive in anyway. Any earlier data taken from you will be destroyed. If you agree to participate you are free to end your participation at any time.

Confidentiality: If you take part, a trained staff member will collect information from your medical records. Your name will not be written down and you will be asked a few questions. You will be asked to provide 5ml (1 teaspoon) of blood at the time of filling the survey form and 6 months after the first blood sample have been taken for research purposes. The blood for this study will not have your name on it so there will be no way to provide the test results back to you.

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Your personal information and laboratory results will be held in the strictest confidence, and no identifying information of any kind will be released to any other person or agency without your specific permission in writing. Your samples collected for research purposes will be labelled with a code number and will be taken to Noguchi Memorial Institute for Medical Research for processing. In the event of any publication or presentation resulting from the research, no personally identifiable information will be shared.

Sample storage

I will also like to store some of your blood to be used for later testing to answer research questions generated at the end of this study. Any later test results cannot be linked back to you because I will not be writing your name on any of the blood test tubes. You may take part in the study without having his/her blood stored for later testing. We will store your samples for 5 years.

Compensation: There is no compensation available.

Voluntary participation and right to leave the study: You are free to choose to participate or not to participate. If you decide not to participate this is will not affect the care you will receive in anyway. Any earlier data taken from you will be destroyed. If you agree to participate you are free to end your participation at any time.

Contacts for additional information: Please call Keren Okyerebea Attiku (0242329146) if you have further questions about the research.

Your rights as a participant

This research has been reviewed and approved by the Institutional Review Board of Korle Bu Teaching Hospital for Medical Research (KBTH-IRB). If you have any questions about your rights as a research participant you can contact the IRB Office between the hours of 8am-5pm through the landline

0302666766 or email addresses: rdo@kbth.gov.gh

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Volunteer Agreement

The above document describing the benefits, risks and procedures for the research title (name of research) has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

....

Name and signature or mark of parent or guardian **Date**

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

....

Name and signature of witness: **Date:**

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

....

Name Signature of Person Who Obtained Consent: **Date:**

4

Figure B. 2: Consent form endorsed by the Institutional Review Board of Korle-Bu Teaching Hospital.

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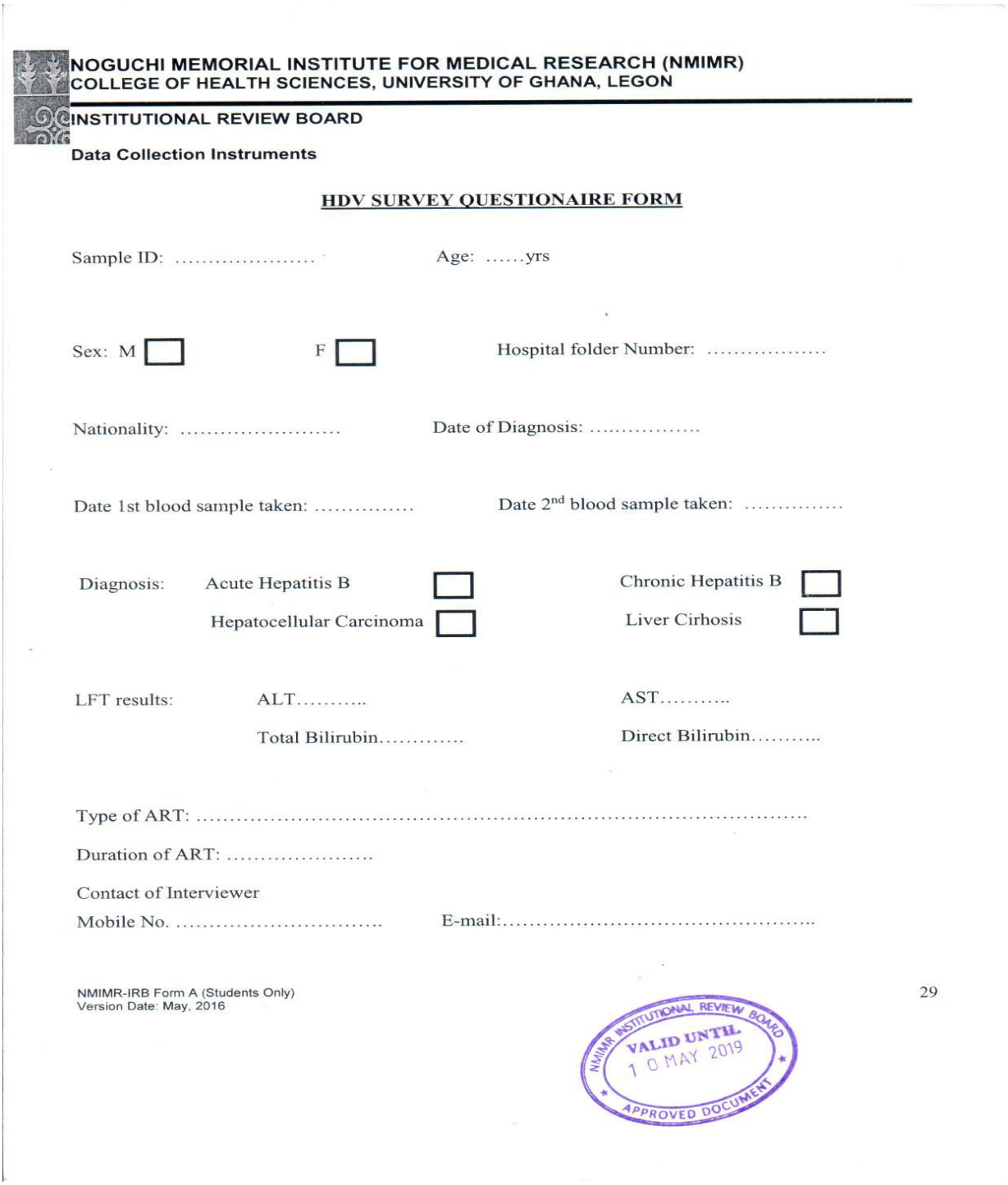

NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH (NMIMR)
COLLEGE OF HEALTH SCIENCES, UNIVERSITY OF GHANA, LEGON

INSTITUTIONAL REVIEW BOARD

Data Collection Instruments

HDV SURVEY QUESTIONAIRE FORM

Sample ID: ..... Age: .....yrs

Sex: M ☐ F ☐ Hospital folder Number: .....

Nationality: ..... Date of Diagnosis: .....

Date 1st blood sample taken: ..... Date 2nd blood sample taken: .....

Diagnosis: Acute Hepatitis B ☐ Chronic Hepatitis B ☐
Hepatocellular Carcinoma ☐ Liver Cirhosis ☐

LFT results: ALT..... AST.....
Total Bilirubin..... Direct Bilirubin.....

Type of ART: .....

Duration of ART: .....

Contact of Interviewer
Mobile No. ..... E-mail:.....

NMIMR-IRB Form A (Students Only)
Version Date: May, 2016

29

NMIMR INSTITUTIONAL REVIEW BOARD
VALID UNTIL
10 MAY 2019
APPROVED DOCUMENT

Figure C: Approved questionnaire used during the patient interview process.

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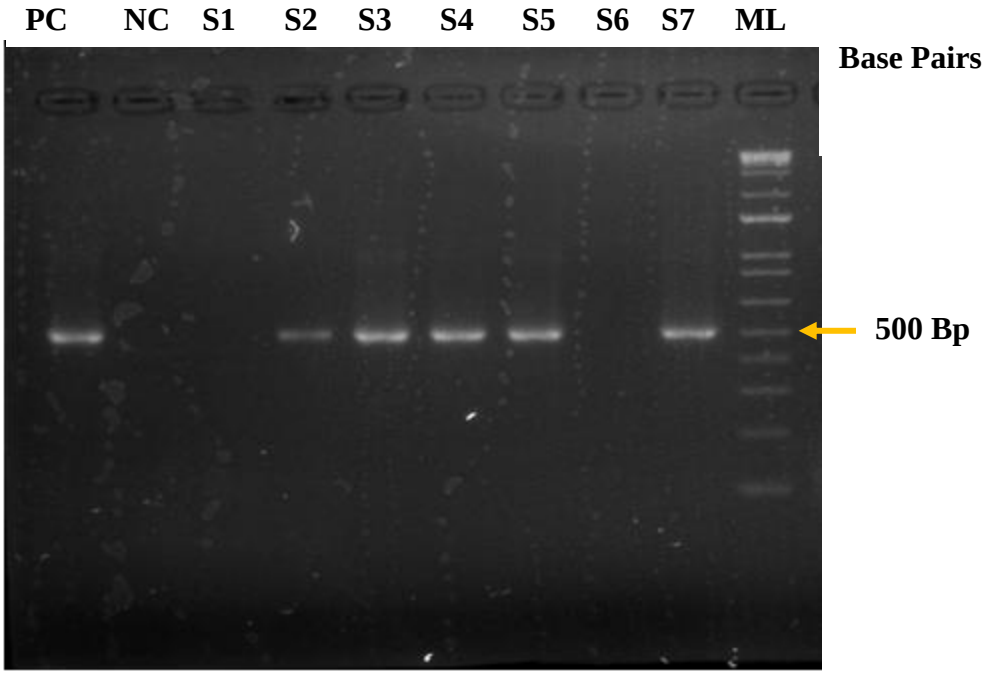

Figure D. 1: A representative result of hepatitis B virus S-gene amplification.

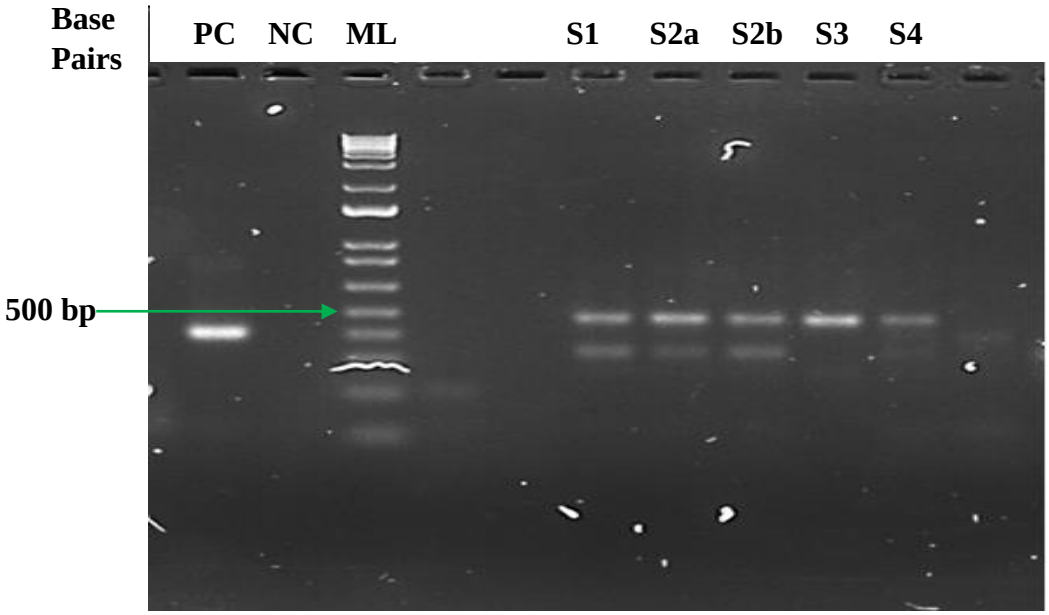

Figure D. 2: Amplification of the R0 region of hepatitis D virus.

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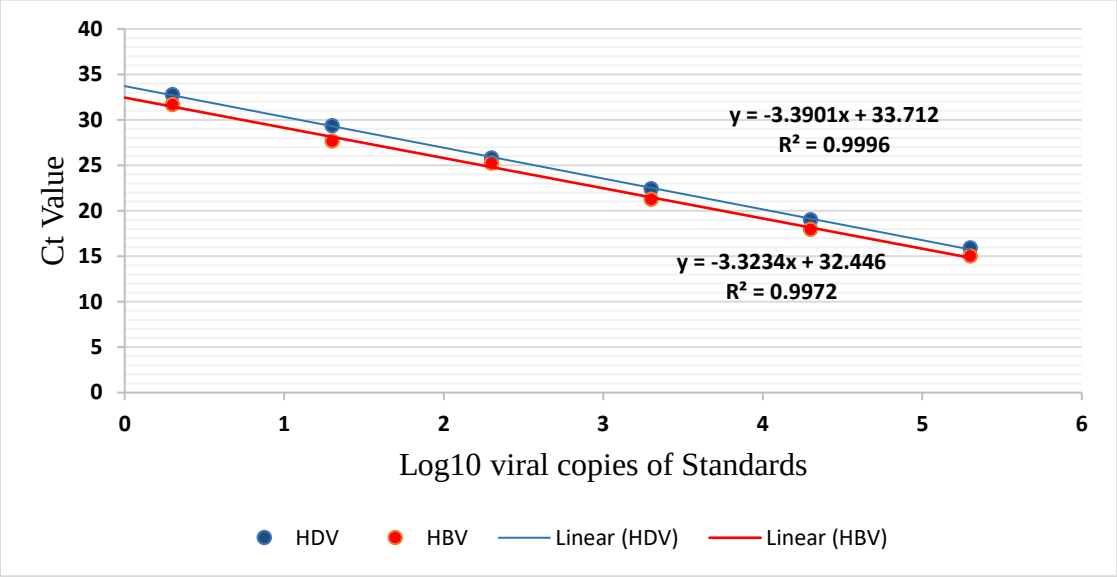

Figure D. 3: Determination of cut-off threshold for detection and quantitation of HBV and HDV.

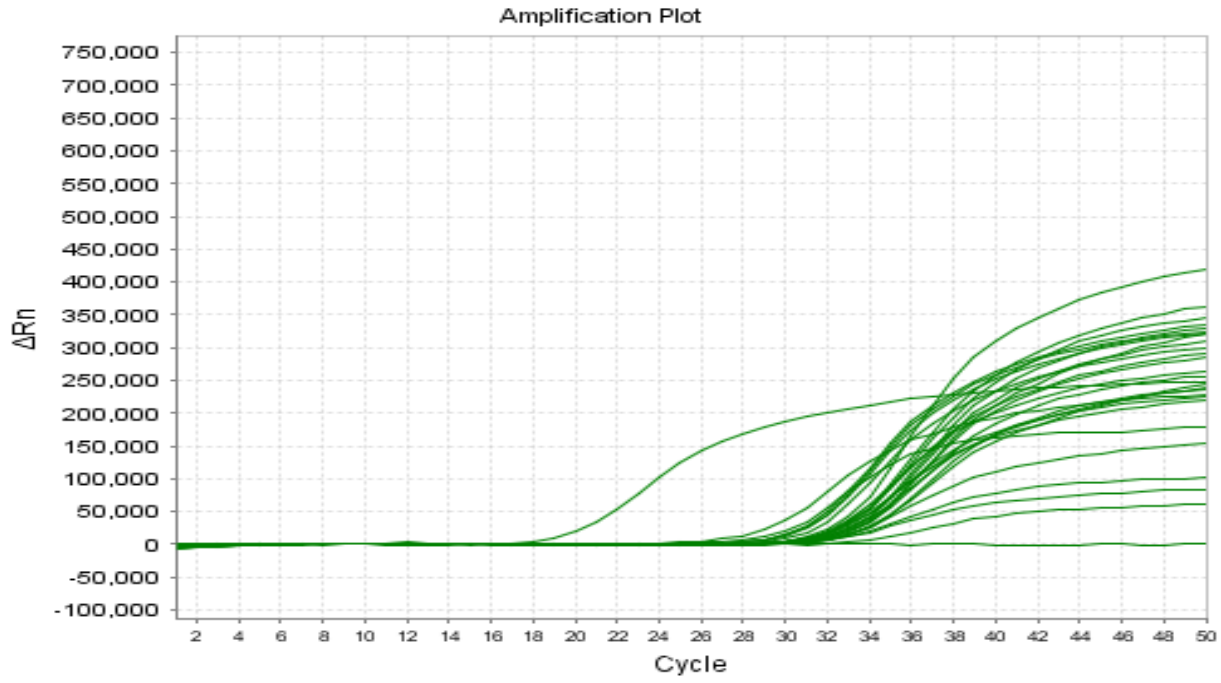

Figure D. 4: Sample amplification plot indicating a successful DNA extraction.

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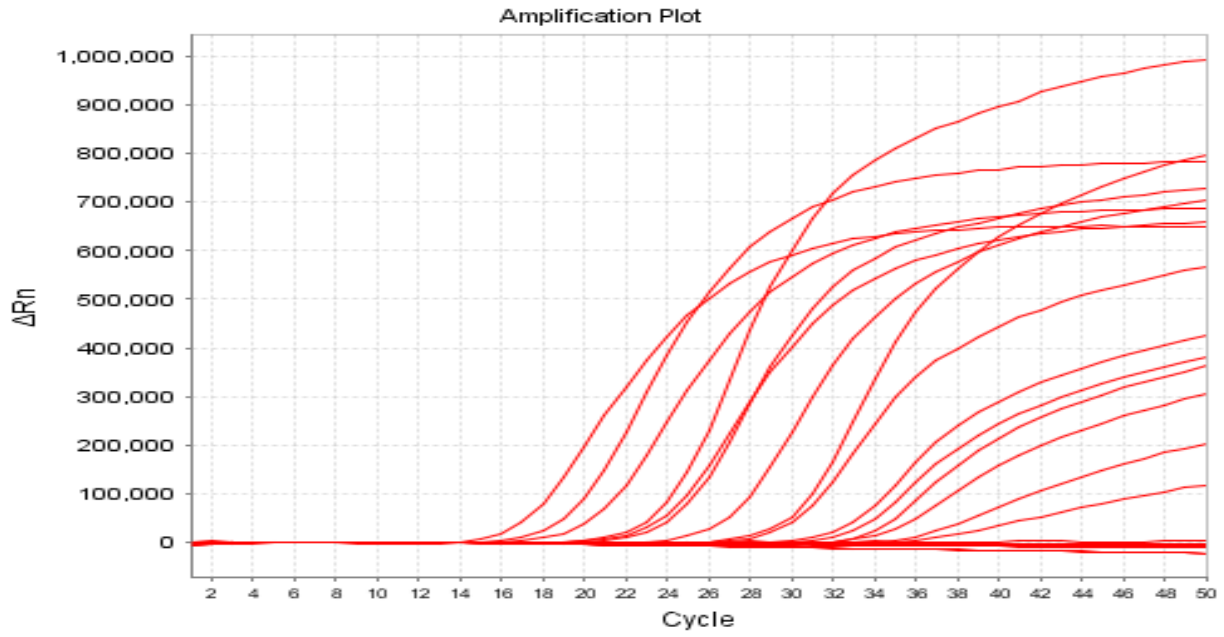

Figure D. 5: Sample Amplification plot showing amplification of hepatitis viruses.

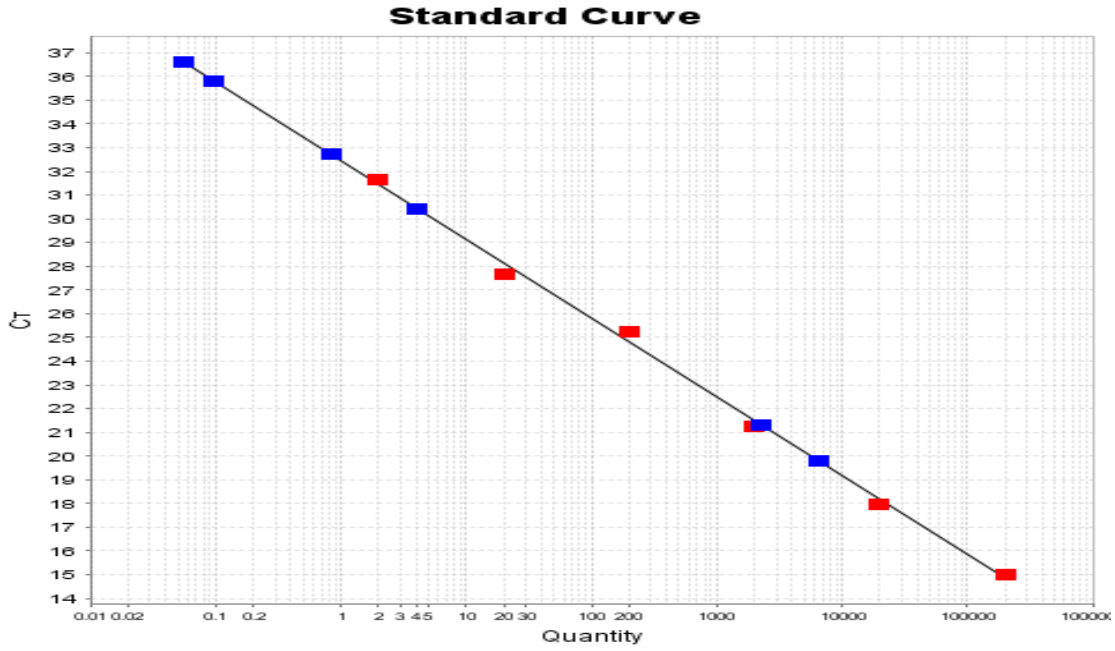

Figure D. 6: Representative Graph showing the distribution of HBV amplified samples by real time PCR (blue) amongst the Standards (red).

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