HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSS-SECTIONAL STUDY IN CENTRAL SOUTH AFRICA

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Declaration of authorship:

I, Jacobus Charles Kotze, declare that the coursework master's degree mini-dissertation that I herewith submit in a publishable manuscript format for the master's degree qualification MMed Medical Virology at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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Abstract:

In South Africa, human immunodeficiency virus (HIV) infected individuals co-infected with hepatitis B virus (HBV) do not routinely undergo HBV viral load (VL) testing when on antiretroviral therapy in the public sector treatment programme. We set out to explore whether HIV VL can be used as a proxy for HBV treatment response, since HIV VL testing is routinely performed in HIV/HBV co-infected patients. The clinical utility of HIV VL testing in this context may be impacted by the slower rate of viral decay which has been described for HBV as compared to HIV. In total, 224 patient samples were tested for HBV VL to determine the relatedness between HIV VL and HBV VL results. Samples with detectable HBV VL were sequenced to identify HBV associated drug mutations, hepatitis B surface antigen (HBsAg) mutations and genotype. Chi-square test for independence (χ^2) was used to determine the relatedness between the viral loads, which indicated that the two viral loads are related *(p-value*<0.0001). However, in samples with an undetectable HIV VL, 29.27% (36/123) had a detectable HBV viral load, with 7.32% having an HBV VL >2000 IU/mL which has previously been linked to an increased risk of HBV related complications. Sequencing results showed that 10 samples had lamivudine resistance, however, no tenofovir resistance was detected. Three samples had immune escape mutations, two caused by the HBsAg mutations E164D and I195M and one by the immune-associated escape mutations N131T and D144A. The results from the study show that patients with HIV/HBV co-infection need to be monitored more closely in South Africa regarding HBV treatment response. The extensive use of lamivudine for HIV treatment in South Africa can be a driver of immune escape and further research needs to be done to determine the possible public health impact

Keywords:

HIV/HBV co-infection

HIV viral load

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Comparison

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HBV treatment

HIV/HBV treatment

Drug resistance

Lamivudine

Tenofovir

List of abbreviations:

ADV	Adefovir
ALT	Alanine transaminase
ART	Antiretroviral treatment
cccDNA	covalently closed circular DNA
CLIA	Chemiluminescence-based immunoassay
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DOH	Department of Health
DTG	Dolutegravir
EFV	Efavirenz
ETV	Entecavir
FTC	Emtricitabine
HAART	Highly active antiretroviral therapy
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
LdT	Telbivudine
NA	Nucleot(s)ide analogue
NHLS	National Health Services
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT	Reverse transcriptase

TAF	Tenofovir alafenamide
TDF	Tenofovir Disoproxil Fumarate
WHO	World Health Orginasation
VL	Viral load
YMDD	Tyrosine-methionine-aspartate-aspartate
3TC	Lamivudine

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Chapter 1: Literature Review

Introduction:

It is estimated that two billion people around the world have been infected with hepatitis B virus (HBV) and 360 million are chronic carriers.[1] The WHO has included viral hepatitis as one of its major public health priorities. HBV is one of the top leading causes of death worldwide with an estimated 786000 deaths per year attributable to hepatocellular carcinoma and liver cirrhosis.[2] Childhood vaccination and effective antivirals are available but the burden of disease still remains high especially in low-income countries where access to these remains low.[3–5]

Classification:

HBV is part of the *Hepadnaviridae* family and has a partially double-stranded circular DNA genome. HBV uses a transcriptional template called covalently closed circular DNA (cccDNA) that is found in the nucleus of hepatocytes.[6] This transcriptional template is essential for HBV maintenance and persistence within the hepatocyte. It forms a minichromosome that is difficult to eradicate and for this reason currently available treatments generally suppress rather than cure chronic HBV infection.[7]

A very distinctive feature of HBV, among DNA viruses, is that it uses reverse transcriptase during viral replication.[6] Reverse transcriptase has no proofreading mechanism and is error prone. Because of this HBV has a high mutation rate, which may be important in the potential for resistance to antiviral therapy.[6]

A diverse range of genotypes and subtypes are found across the world with genotypes A-J being mentioned in the literature. In Africa genotype A is the most prevalent in southern, central and eastern Africa with genotype A subtype 1 predominating.[8] Genotype D is more common in the northern countries and genotype E in western Africa.[8]

Epidemiology:

About a third of the world's population shows serological evidence of past or current infection with HBV.[9,10] During the 1990s there was a sharp increase in mortality from liver cancer (62%) and liver cirrhosis (29%) with HBV causing roughly 50% of the total mortality associated with liver cancer in 2010.[2]

Transmission occurs during close contact with infectious bodily fluids; mainly blood and semen.[7] Perinatal, percutaneous and sexual exposure are the main routes of infection but close person-to-person contact during early childhood is also thought to be a major route of infection.[11,12] Because of these different routes of infection, there are different modes of transmission depending on the level of prevalence of HBV. In areas where HBV prevalence is low (<2%), for example Europe or North America, the route of transmission is mainly sexual or parenteral and occurs in adolescents or young adults.[7] The perinatal route is the most common route of transmission in areas where the prevalence is high (≥8%), for example Southeast Asia.[13] In Africa where the prevalence is also high, most infections occur during early childhood.[13] The mode of transmission during early childhood is still not fully understood, but is thought to occur via close intimate non-sexual contact in overcrowded conditions, rituals involving scarification or tattooing and unsafe medical practices.[14,15] In areas where there is intermediate prevalence (2-7%), the mode of transmission is mixed.[16]

Infection that occurs during the perinatal period has a 90% chance of becoming a chronic infection, while infection in early childhood and adulthood has a 20-30% and 5% chance respectively.[17] This has a profound effect on disease progression with most of the morbidity and mortality associated with HBV occurring during chronic infection.

Pathogenesis:

HBV is not directly cytopathic and pathogenesis is mediated by the host immune response that is induced by active viral replication.[18] Infection in adults usually causes self-limited and transient hepatitis with a good cellular immune response.[19] The virus is then cleared with the establishment of protective antibodies.[20] This is different from chronic infection where the cellular immune response is blunted, with repetitive bouts of hepatitis causing necro-inflammation leading to cirrhosis.[20] The natural course of chronic HBV is typically divided into four phases depending on host virus-interactions. The immune tolerant phase is the first phase and is characterized by high HBV viral loads (>10^5 IU/mL), normal liver enzymes and positive HBeAg.[21] The second phase, known as the immune active phase, is where patients have a heightened immune response to the infection but do not clear it.[22] During this phase HBeAg positive patients seroconvert to HBeAb, there may be an increase in ALT and a decrease in HBV viral load.[23] If the patient has successful HBeAg seroconversion with suppression of HBV viral load, then the patient progresses to the inactive phase of infection.

This phase is characterized by the normalization of ALT levels, suppression of HBV viral load (<2000 IU/mL) and a fall in HBsAg levels.[24,25] A person can remain in this phase for the rest of their life or 20-30% of patients will experience HBV reactivation with raised viral loads and increased ALT levels.[7] Patients that have HBV reactivation are at increased risk of liver cirrhosis and hepatocellular carcinoma (HCC).[26] A small number of individuals with chronic HBV infection clear HBsAg, in most settings 0.5-1% per year, presumably as a result of successful immune responses that reduce or eliminate cccDNA.[7]

Africa and the HIV pandemic:

Measured by the prevalence of anti-HBc antibodies, Africa has a wide range of exposure to hepatitis B with older age and those with liver-related morbidities having the highest prevalence.[27,28] This also varies a lot between regions in Africa, with western Africa having an exposure prevalence of above 85% and eastern Africa between 65-85%.[29–31] Africa has a high prevalence of chronic HBV, above 8%, measured by the prevalence of HBsAg.[4] Due to the majority of infections occurring during early childhood, many people are either immune or chronically infected by early adulthood.[32] This is in contrast to human immunodeficiency virus (HIV), where most of the infections occur during adulthood.[33]

Because of these differences, the prevalence of chronic HBV in HIV-positive individuals is very similar to non-HIV infected individuals.[34–36] This is very different to developed countries where HBV and HIV share similar infection routes, especially parenteral routes, thus the prevalence of HBV is higher in HIV infected populations compared to the rest of the population.[13]

It is estimated that 5-20% of HIV infected individuals worldwide are co-infected with HBV.[37] HBV has become a major health concern in HIV infected patients and has become more evident after the introduction of highly active antiretroviral therapy (HAART) for HIV treatment. Since opportunistic infections have become less common in patients on HAART, complications from viral hepatitis have emerged as a leading cause of morbidity and mortality.[38–40]

HIV infection has a profound effect on the natural history of HBV infection. Co-infected patients have a higher chance of progressing to chronic HBV infection than the general adult population.[41] Co-infected patients also have a higher rate of HCC and liver cirrhosis than

HBV mono-infected patients, with more rapid progression, higher liver related mortality and decreased treatment response.[42–44] More challenges that have been encountered with co-infected patients include cross resistance between HIV and HBV drugs while on dual therapy for both conditions, increased liver injury due to direct hepatotoxicity or ART-related immune reconstitution hepatitis.[44]

HIV and HBV treatment:

While both HIV and HBV share use of a reverse transcriptase during viral replication, only two nucleot(s)ide analogues (NA), lamivudine (3TC) and tenofovir (TDF), show activity against both HIV and HBV. 3TC was the first to be used extensively to treat both infections, but it was soon realized that 3TC has a low barrier to resistance with high failure rates in the first year and 90% of lamivudine treated patients being resistant after 5 years in HBV infected individuals.[45] TDF has therefore become the treatment of choice in both infections due to its high barrier to resistance and superior efficacy compared to other NA's.[46] Thus TDF is the preferred choice for both HBV monoinfection, and for inclusion in ART regimens for HIV/HBV co-infected patients, with more than 95% of patients virologically suppressed after 5 years of treatment [with the majority of patients being virologically suppressed after prolonged periods of treatment].[47–49] Studies did show, however, that some HIV/HBV co-infected patients have prolonged low levels of HBV viraemia and even virologic breakthrough without resistance on effective tenofovir treatment.[49,50] Factors associated with detectable HBV viral load on tenofovir treatment include detectable HBeAg, CD4+ T cell count below 200 cells/mm³ and treatment compliance below 95%.[51] From these findings it is clear that co-infected patients must be started early on dual acting NA therapy and that compliance may still be an issue even when the HIV viral load is suppressed.[51] Co-infected patients that are receiving treatment for HIV and HBV should also not stop treatment for HBV due to the risk of severe HBV hepatitis flare and decompensation due to HBV reactivation.[48] Renal, bone density and liver derangements should be closely monitored because of possible drug toxicities when giving TDF.[48]

Other HBV treatment is available but has poor activity or is associated with HIV resistance mutations and is not recommended during HIV/HBV co-infection.[52] Adefovir (ADV) and telbivudine (LdT) are registered for HBV treatment and are only recommended during HBV mono-infection.[47,48] Entecavir (ETV) can also be given during HIV/HBV co-infection.[48] It is a

very potent anti-HBV drug with low HIV activity and can be used in certain circumstances for example in patients with renal failure. Care must be taken in regards to the drugs given to HIV because of the low activity of ETV against HIV and previous drug exposures can cause resistance to ETV.[53] The availability of ETV is limited in South Africa, thus the main treatment option is TDF.[54]

In South Africa, the first line treatment for HIV is a fixed daily combination of TDF, emtricitabine (FTC) and dolutegravir (DTG) or efavirenz (EFV), depending on when the patient started ART.[55] Patients who are co-infected with HBV must get a TDF-based regimen even when changing the patient to a 2nd line or 3rd line regimen for HIV. TDF is added to the standard second line regime if DTG is used for second line treatment or zidovudine is substituted with TDF if a protease inhibitor based second line regime is used.[55]

The importance of HBV viral load:

HBV viral load is used as a monitoring tool before and during HBV treatment and is an important factor in determining eligibility for treatment in mono-infected patients and predicting clinical outcome. Higher HBV viral loads have been linked to higher rates of liver cirrhosis and HCC, with viral loads of above 10^5 copies/mL having a worse outcome.[56] Many international guidelines suggest doing HBV viral loads periodically because any increase above 2000 IU/mL can have an impact on the course of disease.[47,48] International guidelines recommend that HBV viral loads are performed on a periodic basis to check patient compliance with treatment and also detect possible HBV drug resistance.[47,48] Because resistant strains of the virus will have higher replication during treatment, HBV viral load is a good marker to use for screening for possible viral resistance. In the South African hepatitis guidelines for HBV, HBV viral load is a very important factor in the monitoring of HBV infected patients but whether this practice is followed through is very difficult to say seeing that the monitoring of HBV is not specified in the HIV treatment guidelines.[54,55]

Drug resistance testing:

Drug resistance testing is usually recommended in patients that have past experience with nucleot(s)ide analogue treatment, failure to achieve viral suppression on current treatment and patients that experience virological breakthrough on current treatment.[47,48] Virological response is very important to monitor early during treatment to customize treatment for

the patient.[48,54] Primary non-response is defined by a less than one log_{10} decrease in viral load after three months of therapy.[48] In partial non-response, the viral load decreases by more than one log_{10} but still remains detectable after 12 months of treatment in compliant patients.[48] Virological breakthrough is defined as a one log_{10} increase in HBV viral load from baseline in a patient that had a virological response on treatment.[47,48]

As mentioned above, the antivirals used for HBV treatment are 3TC, LdT, ETV, ADV and TDF. Tenofovir alafenamide (TAF) has recently become an important drug in the treatment of HBV, not only in mono-infected patients but also in HIV/HBV co-infected patients.[48,57,58] TAF is a prodrug of tenofovir with lower doses needed, requiring only 10mg as compared to 300mg used with TDF.[59] TAF has a better side effect profile compared to TDF with regards to renal toxicity and bone mineral loss.[59]

Drug resistance to these nucleot(s)ide analogues involves changes to nucleotides at specific sites in the reverse transcriptase region of the pol gene.[60] This is caused by the absent proofreading capabilities of the HBV reverse transcriptase and high viral replication rate.[61] These two factors lead to the occurrence of random mutations in the viral genome, from which viral quasi-species arise.[60] Antiviral drugs cause selective pressure on the quasi-species present during natural infection with the selection of viral mutants that confer resistance to the particular antiviral agent present during failing treatment.[62] These major nucleotide changes usually come at a cost to the virus, leading to lowered viral replication.[63] This “fitness” cost can be overcome by acquiring accessory mutations or compensatory mutations at other sites on the viral genome leading to replicative capabilities similar to or even greater than the wild type virus.[63]

Major mutations involved with resistance to the L-nucleosides, 3TC and LdT, are found in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of the catalytic site of HBV pol.[63] L-Nucleosides are the enantiomers of the natural nucleosides and thus are the nonsuperimposable mirror image of d-nucleosides, in which all stereocenters have an inverted configuration.[64] Compared to natural nucleosides, the nucleobase of an l-nucleoside is designated to be β-oriented if it is cis to the 4 -hydroxymethyl group of the sugar moiety in its furanosyl configuration.[64] The palm region on the surface of the polymerase is the site of attachment for 3TC and is formed by rt204.[65] Mutations at this location reduces the surface area available between the polymerase and lamivudine

because the β-branched side chain of valine/isoleucine contains a methyl group that impinges on the sulphur atom in the L-oxathiolane ring of lamivudine.[65] The major mutations associated with lamivudine are rtM204I/V, with compensatory mutations at rtL180M and rtA181T.[65] These mutations cause lamivudine not to bind to the polymerase but cytosine, the natural nucleotide, can still bind in the presence of lamivudine.[65] The compensatory mutations, such as rtL180M and rtA181T, restore viral fitness to levels seen in the wild type virus.[65] Other compensatory mutations have been found like rtL80V/I, rtI169T, rtV173L, rtT184S/G, rtS202I, and rtQ215S.[66–70] Telbivudine shares lamivudine's resistance profile with a slightly higher barrier to resistance than lamivudine.[65] Telbivudine is rarely used in South Africa, if at all.

Resistance associated with the alkyl phosphonate ADV are found at sites rtA181T/V and rtN236T, with a mutation at rtI233V found recently.[71–74] TDF, also an alkyl phosphonate, has a very high barrier to resistance with a mutation at rtA194T causing decreased susceptibility during in vitro studies and rarely seen in patients failing TDF treatment.[75] RT194 is located in the DNA template binding region of the HBV reverse transcriptase.[65] The mutation rtA194T causes an alteration in the position of the DNA template in relation to the dNTP binding site affecting DNA polymerization.[65] A case study did report resistance to TDF during HIV/HBV coinfection with mutations at rtA194T and rtL180Mrt+M204V, but this could not be confirmed in a subsequent report.[76,77] ADV resistance does, however, reduce the efficacy of TDF and thus patients treated with ADV in the past should not receive TDF therapy.[78,79] Other cases of TDF resistance have been reported in the literature where multiple mutation combinations have caused resistance.[80,81] These combinations are numerous, and it is difficult to find the precise mechanism that leads to resistance, but they usually occur in patients with an extensive drug history.

The only cyclopentyl guanosine analogue that is active against HBV, ETV has a high barrier to resistance requiring multiple mutations to confer resistance. Entecavir shares the guanosine analogue group with abacavir and has, as mentioned previously, low activity against HIV. Cyclopentyl guanosine analogues are carbocyclic analogues of 2'-deoxyguanosine.[64] The oxygen in the furanose ring is replaced by a vinyl group in entecavir.[64] Multiple polymerase functions are affected by ETV such as priming, reverse transcription and DNA elongation.[65] The sites associated with resistance are rtL180M+rtM204V, with mutations at other sites

(rtT184G/S, rtS202I/G or rtM250V) also required.[68] There are two possible ways mutations can reduce the effectivity of ETV, by altering the binding of the primer strand and the template DNA or by altering the polymerase nucleotide binding pocket near the YMDD site.[65] What appears to be very important in the evolution of ETV resistance is previous exposure and resistance to 3TC.[65] The M204V mutation seen with 3TC usage increases the resistance to ETV significantly when associated with the M250V mutation.[65]

HBV viral load and resistance in South Africa:

Very little is known about the effectiveness of dual active therapy in HIV/HBV co-infected patients in South Africa. This is because HBV viral load testing is not done during the routine monitoring of patients on treatment in the public sector. HBV viral load is recommended though for HBV mono-infected patients on treatment.[82] HBV surface antigen is done once at the initiation of HIV treatment with alanine transaminase levels being done in the first few months after treatment to monitor for drug toxicity and immune reconstitution syndrome.[82]

An extensive search of published articles on ScienceDirect (https://www.sciencedirect.com/) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) could not find any articles pertaining to the efficacy of tenofovir-containing regimes on HBV viral load in South Africa. There have been HBV epidemiological and drug resistance studies done in HIV/HBV co-infected patients but they are few and small in patient numbers.[83,84]

Therefore, very little is known about HBV treatment response and drug resistance in HIV/HBV co-infected patients in South Africa where the prevalence of co-infection is high. In a recent systematic review, it was highlighted that very little is known about the prevalence of drug resistance mutations and surface antigen mutations stemming from drug resistance mutations and what impact this might have on the effective elimination of HBV in Africa.[85]

Aims and objectives:

This study sets out to determine whether adult patients have suppressed HBV viral loads on antiretroviral therapy for HIV recommended by the DOH and current WHO guidelines for HIV-HBV co-infections (TDF/3TC/ETV), and to investigate whether HBV drug resistance is a possible cause of an unsuppressed HBV viral load.

The objectives are to:

- Determine the prevalence of patients in the study group that have a detectable HBV viral load during standard antiviral therapy for HIV and HBV.
- Determine the correlation between HIV viral load and HBV viral load.
- Undertake HBV sequencing in patients with detectable HBV viral loads to determine whether there are any known or potential drug resistance mutations causing the increase in viral load.
- Determine any HBV surface antigen escape mutants that might be present in the overlapping region of the HBsAg and reverse transcriptase open reading frames.

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Chapter 2: Publishable Manuscript

HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSS-SECTIONAL STUDY IN CENTRAL SOUTH AFRICA

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Abstract:

In South Africa, human immunodeficiency virus (HIV) infected individuals co-infected with hepatitis B virus (HBV) do not routinely undergo HBV viral load (VL) testing when on antiretroviral therapy in the public sector treatment programme. We set out to explore whether HIV VL can be used as a proxy for HBV treatment response, since HIV VL testing is routinely performed in HIV/HBV co-infected patients. The clinical utility of HIV VL testing in this context may be impacted by the slower rate of viral decay which has been described for HBV as compared to HIV. In total, 224 patient samples were tested for HBV VL to determine the relatedness between HIV VL and HBV VL results. Samples with detectable HBV VL were sequenced to identify HBV associated drug mutations, hepatitis B surface antigen (HBsAg) mutations and genotype. Chi-square (χ^2) was used to determine the relatedness between the viral loads, which indicated that the two viral loads are related *(p-value*<0.0001). However, in samples with an undetectable HIV VL, 29.27% (36/123) had a detectable HBV viral load, with 7.32% having an HBV VL >2000 IU/mL which has previously been linked to an increased risk of HBV related complications. Sequencing results showed that 10 samples had lamivudine resistance, however, no tenofovir resistance was detected. Three samples had immune escape mutations, two caused by the HBsAg mutations E164D and I195M and one by the immune-associated escape mutations N131T and D144A. The results from the study show that patients with HIV/HBV co-infection need to be monitored more closely in South Africa regarding HBV treatment response. The extensive use of lamivudine for HIV treatment in South Africa can be a driver of immune escape and further research needs to be done to determine the possible public health impact

Introduction:

The WHO has included viral hepatitis as one of its major public health priorities. HBV infection is one of the leading causes of death worldwide with an estimated 786000 deaths per year attributable to hepatocellular carcinoma (HCC) and liver cirrhosis.[1] Vaccination and effective antivirals are available, but the burden of the disease remains high especially in low-income countries where access to these remains low.[2–4]

HIV infection has a profound effect on the natural history of HBV infection. Co-infected patients have a higher chance of progressing to chronic HBV infection than the general adult

population.[5] Co-infected patients also have a higher rate of HCC and liver cirrhosis than HBV mono-infected patients, with more rapid progression, higher liver-related mortality, and decreased treatment response.[6–8]

Very little is known about the effectiveness of dual active therapy in HIV/HBV co-infected patients in South Africa. This is because HBV viral load testing is not performed during the routine monitoring of patients on treatment in the public sector.[9] HBV VL testing is recommended for HBV mono-infected patients on treatment, but testing is not mentioned in the HIV treatment guidelines of South Africa, a population that has a high rate of HBV infection.[9,10] Co-infection with HBV among HIV infected patients is estimated to be between 5-15% in South Africa.[11] A recent systematic review highlighted the fact that there are also limited data about the prevalence of drug resistance mutations and surface antigen mutations stemming from drug resistance mutations, and what impact this might have on the effective elimination of HBV in Africa.[12]

In this cross-sectional study, we focused on HIV/HBV co-infected patients on antiretroviral therapy in central South Africa, namely the Free State and Northern Cape provinces. Because HIV treatment regimens are standardized in the public sector health care system in South Africa, this population group is broadly representative of the HIV/HBV population across the country. In South Africa, the first-line treatment for HIV is a fixed daily combination of tenofovir (TDF), emtricitabine (FTC)/lamivudine (3TC) and dolutegravir (DTG) or efavirenz (EFV), depending on when the patient started ART.[9] Patients that are co-infected with HBV must receive a TDF-based regimen even when changing to a second line or third line regimen for HIV, with TDF either substituted for zidovudine or as an additional drug in second line regimens. [9]

The main objective of the study was to determine the relatedness of HIV and HBV viral loads (HBV VL) in patients on dual therapy for HIV and HBV. The viral loads were used to assess the relationship between HIV and HBV viral loads to determine whether it is safe to assume that the HBV VL will be suppressed if the HIV viral load (HIV VL) is suppressed. Samples with a detectable HBV VL were sequenced to determine whether this was due to drug resistance. By sequencing the RT region of the POL gene, we were also able to infer hepatitis B surface antigen (HBsAg) mutations due to the overlapping open reading frames of the two genes.

Materials and methods:

Patient samples:

Study participants were any adult HIV/HBV coinfected patient on antiretroviral therapy in the Free State and Northern Cape provinces with a sample submitted to the Division of Virology at the National Health Laboratory Service (NHLS) Universitas Academic Laboratories for HIV viral load testing. Patient treatment history could not be reliably retrieved from the NHLS data and thus was a limiting factor, however, according to national Department of Health (DOH) HIV management guidelines, adult patients must be on treatment for HIV for at least 6 months before HIV VL testing is performed.[9] Patients were selected by matching weekly HIV VL samples to data retrieved from the NHLS to identify known HBsAg positive patients. Patients were therefore selected if they had a recent HIV VL done and a previous positive HBsAg. Patients were regarded as chronically infected with HBV if they had two positive HBsAg six months apart from the data retrieved from the NHLS databank. If the patient had incomplete testing done at the health care facility, the patient's HIV VL sample was then tested for HBsAg to confirm HBV chronicity. This was done to ensure that an undetectable HBV viral load was not due to an HBV negative sample.

Residual samples were retrieved following completion of HIV VL testing in the routine diagnostic laboratory The samples were deidentified and allocated a unique study number prior to further testing. After testing, samples with a detectable HBV VL and sufficient residual volume underwent HBV drug resistance testing using Sanger sequencing. The study the data set retrieved from the NHLS data bank included HBsAg results until 2018 and HIV VL collection started in February 2020 and ended in April 2021.

HBsAg and HBV viral load testing:

Samples with one previous positive HBsAg were retested on the automated serology analyser, LIAISON® XL from DiaSorin (DiaSorin S.p.A., Saluggia, Italy), to confirm chronic hepatitis B infection. The determination of HBsAg on the LIAISON-XL is a quantitative test and is a direct two-step chemiluminescence-based immunoassay (CLIA). The assay employs a set of monoclonal antibodies directed against highly conserved epitopes of the HBsAg internal region. The assay quantitation range is set from 0.03 to 150 IU/mL. Testing was performed according to the manufacturer's instructions.

All samples that were confirmed to have chronic HBV infection were tested using a fully automated quantitative real-time polymerase chain reaction system, the Roche COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 (RocheMolecular Diagnostics, Branchburg, NJ, USA), to determine the HBV VL. Patient HIV viral loads were categorized into three groups to compare HIV viral loads with HBV viral loads, namely <50 RNA copies/ml (virological suppression), 50-1000 RNA copies/ml, and >1000 RNA copies/ml (virological treatment failure), while HBV viral loads were divided into three groups of <20 IU/ml, 20-2000 IU/ml and >2000 IU/ml.

Sequencing:

Samples with a detectable HBV VL were sequenced with Sanger sequencing. Viral DNA was extracted from 300µl of patient samples using the NucliSENS® easyMag® from bioMérieux (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. A nested in-house PCR was used to amplify the region of interest. The target for amplification was a 645-bp fragment in the polymerase region of the HBV reverse transcriptase (RT) gene, covering amino acids 115-320, which includes the most important areas where drug-resistant mutations occur. The PCR primers used by the NHLS Laboratory at Charlotte Maxeke Johannesburg Academic Hospital for HBV drug resistance testing were used. For the first round PCR the following primers were used; forward primer 5' GTCTGCGGCGTTTTATCA 3' and reverse primer 5' GGAGTTCCGCACTATGGATCGG 3'. The PCR conditions for the first-round reaction was a denaturation step at 95 °C for 2 min followed by 35 PCR cycles at 95 °C (20 s), 54 °C (10 s) and 70 °C (15 s). The second-round PCR used the forward primer 5' GGTATGTTCCCGTTTGTCC 3' and reverse primer 5' GGCGAGAAAGTGAAAGCCT with the following PCR conditions; a denaturation step at 95 °C for 2 min followed by 30 PCR cycles at 95 °C (20 s), 57 °C (10 s) and 70 °C (9 s). KOD Hot Start DNA polymerase (Novagen, Nottingham, UK, cat. no. 71086) was used according to the manufacturer's instructions to perform the in-house PCR. The size of the PCR product was confirmed using gel electrophoresis. Applied Biosystems® (Applied Biosystems, Foster City, CA, USA) 3500xL series genetic analyser with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) was then used according to the manufacturer's specifications to perform Sanger sequencing using the nested primers mentioned previously. Unipro UGENE (http://ugene.net/) was used to analyse the chromatograms and generate consensus

sequences that was used with web-based software to call resistance-associated substitutions.

Resistance-associated substitution calling using web-based software:

The RT sequence of each HBV isolate was submitted to web-based software for phenotypic analysis of genotypic mutations in the reverse transcriptase region of the pol gene. Web-based software was used for sequence analysis, namely HBVseq from Stanford University (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) and Geno2pheno hbv from the Max Planck Institut Informatik (https://hbv.geno2pheno.org/). Predictions are categorized into susceptible, limited susceptibility, partly resistant, and resistant. The rules are based on literature reviews and articles that reported on HBV drug resistance mutations associated with patients failing therapy or *in vitro* studies. The amino acid sequence of HBsAg was compared against a list of escape mutations, which was also retrieved from literature reviews. The mutations in the HBsAg conferring immune escape that are caused by lamivudine are not listed on Geno2pheno hbv and were manually checked.

Statistical analysis:

Continuous variables were summarised by medians, minimum, maximum or percentiles. Categorical variables will be summarised by frequencies and percentages. Differences between groups was evaluated using Chi-Square for unpaired data. The analysis was done by the Department of Biostatistics, using Statistical Analyses Software (SAS 9.4).

Results:

Baseline characteristics and HBV viral loads of patients:

A total of 356 samples were collected, of which 224 patients were confirmed to have chronic HBV and were included for analysis. The gender distribution was almost equal with 110 (49.11%) female and 114 (50.89%) male study participants. The median age was 39 years with the youngest being 18 years and the oldest 64 years. Most of the samples came from the Free State province (204/224, 91.07%). The majority of patients had an undetectable HIV VL (123/224, 54.91%) with equal distribution in the groups with 50-1000 RNA copies/ml (51/224, 22.77%) and >1000 RNA copies/ml (50/224, 22.32%). In this cohort, 43.75% (98/224) of patients had a detectable HBV VL and 24.11% (54/224) had a VL above

2000 IU/mL indicating a high risk of HBV related complications. Table 1 shows the comparison of HIV viral load with HBV viral load.

Table 1. Multiway contingency table for HIV and HBV viral load comparison.

		HBV viral load IU/mL			
		<20	20-2000	>2000	Total
HIV viral load copies/mL	<50 (*N*=123) *n* (%)	87 (70.73)	27 (21.95)	9 (7.32)	123
	50-1000 (*N*=51) *n* (%)	27 (52.94)	6 (11.76)	18 (35.29)	51
	>1000 (*N*=50) *n* (%)	12 (24.00)	11 (22.00)	27 (54.00)	50
	Total	126	44	54	224

The majority of samples with an undetectable HIV viral load also had an undetectable HBV viral load (87/123, 70.73%), while 29.27% (36/123) had a detectable HBV viral load even though the HIV was suppressed. Nine samples in this group had an HBV viral load above 2000 IU/mL (7.32%) which is considered as poorly controlled HBV with a high probability of future complications due to HBV.[13,14]

The calculated χ^2 for the above contingency table was 51.5813 with a *p-value*<0.0001. This demonstrated that the two variables, HIV VL and HBV VL, are related.

In samples with an HIV viral load of between 50-1000 RNA copies/mL, which is considered undesirable for HIV control and treatment but does not indicate a regimen change, a higher percentage of patients had detectable HBV viral loads (24/51, 47.05%). A high proportion of

these patients, 18/24 (35.29%), had an HBV viral load above 2000 IU/mL showing poor HBV replication control. The samples that tested >1000 RNA copies/mL for HIV had a higher percentage of HBV viral load results above 2000 IU/mL compared to the other groups (27/50, 54%).

Sequencing results:

Of the 98 samples that had chronic HBV infection and a detectable HBV viral load, 47 had sufficient volume remaining and were therefore selected to undergo sequencing. The majority of the samples (46/47, 98%) were genotype A, while the remaining sample was genotype E. Ten samples had resistance to lamivudine, with 180M and 204V/I being the predominant mutations found. No tenofovir resistance was found in any of the samples. Eight out of the 10 patients had the I195M mutation in the HBsAg that is associated with lamivudine resistance. Two patients had mutations 173L, 180M and 204V in the RT region conferring nucleotide-induced immune-escape mutations at positions E164D and I195M in the HBsAg. One patient had a stop codon at position W196*W in the HBsAg with the associated RT mutation at M204IMV. Table 2 shows the details of the samples that had drug-resistance mutations.

Table 2. Details of samples with HBV drug resistance.

Patient ID	#9	#69	#107	#127	#164	#169	#187	#188	#197	#213
HBsAg	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
HIV RNA log_{10} (copies/mL)	3,18	LDL	1,88	4,74	2,62	1,38	LDL	LDL	2,48	1,74
HBV DNA log_{10} (IU/mL)	8,00	5,46	4,48	>8.23	8,08	>8.23	4,29	5,51	2,92	>8.23
HBV genotype	A1	A1	A1	A1	A1	A1	A2	A1	A1	A1
HBV RT mutations	L180LM, M204MV	L180M, M204V	V173LV, L180M, M204V	L180L M, M204I	M204IM	L180M, M204V	M204IMV	L180LM, M204IMV	V173L, L180M, M204V	L180M, M204V
SHB mutations	I195IM	I195M	E164DE , I195M	W196L	W196LW	I195M	I195IM, W196*W	I195IM, W196LW	E164D, I195M	I195M

Abbreviations: ID, Identification; LDL, Lower than Detectable Limit; RT, Reverse Transcriptase; SHB, Surface Hepatitis B.

One additional sample had immune-associated escape mutations N131NT and D144AD conferring vaccine and immune escape, as indicated in Table 3. The nucleotides at these positions had mixtures indicating that a subpopulation of HBV had the mutations.

Table 3. Details of the patient with immune-associated escape mutations.

Patient ID	HBsAg	HIV RNA log_{10} (copies/mL	HBV DNA log_{10} (IU/mL)	HBV genotype	HBV RT mutations	SHB mutations
#193	Positive	4,75	6,91	A1	None	N131NT, D144AD

Abbreviations: ID, Identification; RT, Reverse Transcriptase; SHB, Surface Hepatitis B.

Discussion:

Although HIV and HBV viral loads were shown to be related (χ^2 51.5813; *p-value*<0.0001), only approximately 71% of samples had a dually suppressed viral load, thus limiting the clinical utility of HIV VL testing as a proxy for HBV treatment response. In samples with an undetectable HIV viral load, 29.27% (36/123) had a detectable HBV viral load and 7.32% had an HBV viral load >2000 IU/mL, which raises concerns regarding HBV management. The percentage of samples with a detectable HBV viral load increased with higher levels of HIV viral load. In samples with HIV viral load of 50-1000 RNA copies/mL, 24/51 (47.05%) samples had detectable HBV viral loads and with HIV viral loads above 1000 RNA copies/mL, 27/50 (54%) samples had HBV viral loads above 2000 IU/mL. The sequencing results showed that 10 samples had lamivudine resistance and no samples had tenofovir resistance. From the 10 samples that had lamivudine resistance, two samples had immune escape mutations (E164D and I195M) caused by the lamivudine resistance mutations 173L, 180M and 204V.[15–17] These mutations in HBsAg are known to decrease antigen-antibody binding to levels seen with the immune-associated escape mutation G145R.[15,16] One sample had the immune-associated escape mutations N131NT and D144AD conferring vaccine and immune escape.[18,19]

Mutations due to lamivudine not only cause immune escape mutations but also mutations leading to stop codons in the HBsAg. The mutation W196*W in the HBsAg was found with the RT mutation M204I and in some studies, this combination has been associated with increased liver cirrhosis and hepatocellular carcinoma.[20,21] This variant also showed an increased cytopathic effect in *in vitro* cell culture.[21] Stop codons and immune-escape mutations also lead to failed HBsAg binding to antibodies in diagnostic assays potentially resulting in the misdiagnosis of patients.[16]

Limitations of the study were the inability to get reliable ARV treatment history for the patients and the absence of baseline HBV viral load data before initiation of treatment. Because of this, viral load dynamics and comparisons between length of treatment and viral load levels could not be studied. This could impact the HIV and HBV viral load comparison as HIV and HBV have different kinetics regarding time to suppression when on treatment, with HBV showing slower viral decay than HIV. [22–25] Therefore, patients with higher baseline HBV viral loads, positive HBeAg at base line or shorter duration of treatment, may be more likely to have detectable HBV viral loads even when the HIV viral load is suppressed. [22–25] Poor treatment adherence has also been cited as the main cause of delayed HBV VL suppression since higher optimal adherence level might be required for HBV suppression compared to HIV. [22,26,27] When the viral dynamics of HIV and HBV are compared, both have a rapid turnover and a massive production of plasma virus, but the most important difference is in the half-life of virus-producing cells, which is much shorter for HIV. [28] The total amount of plasma virus production is much more for HBV which is on average 10^{11} viral particles per day compared to HIV that is 10^{9} viral particles per day. [28] Further studies are warranted to determine whether the clinical utility of HIV viral load testing to monitor HBV treatment response can be improved by considering these factors. The South African DOH treatment guidelines are very clear on the treatment for HIV/HBV co-infected patients where an antiretroviral regimen containing TDF must be given to the patient. However, due to the unavailability of ARV treatment history in this study, it is possible that some of the patients may have been exposed to lamivudine as the only active HBV drug in regimens used prior to the introduction of TDF in South Africa in 2010 or that they were incorrectly placed onto regimens which do not include TDF. A study done in Ethiopia showed that giving therapy for HIV without HBV management leads to high rates of lamivudine resistance as seen in this study.[29] These mutations can occur in HIV/HBV co-infected patients within a short time period and prolonged exposure increases the chance of finding lamivudine mutations 173L, 180M and 204V.[30] It is worrying that immune escape variants were found from these randomly selected samples that could have major public health implications for example mother-to-child transmission, where immune-escape mutations can evade HBV vaccine induced antibodies and immunoglobulins given at birth.[16] Thus, monitoring drug response and making sure that patients are on the correct medication is very important not only for the patient but also regarding public health.

Eight of the ten samples that had resistance to lamivudine, had HIV viral loads below 1000 copies/mL. These patients do not require a regimen change of their antiretrovirals and thus most likely won't receive suppressive treatment for their HBV infection.[9] Many guidelines support the active monitoring of HBV to prevent future complications with the ideal HBV viral load being less than 2000 IU/mL.[13,14] A viral load above 2000 IU/mL is associated with increased rates of hepatocellular carcinoma and liver cirrhosis.[31,32] Our study highlighted the poor control and management of HBV in South Africa among HIV/HBV co-infected patients.

The development of 3TC resistance also has an impact on other treatment options for HBV as it causes cross-resistance to telbivudine and more importantly partial resistance to entecavir.[33] Entecavir, though not widely used in South Africa, is an important first line and rescue therapy option for patients with renal disease.[10,13,14]

No tenofovir resistance was found in our study but resistance could still become a problem if adequate monitoring of HBV treatment response is not implemented. Resistance to TDF has been described in the literature but is usually associated with extensive drug exposure and resistance.[34,35] Tenofovir is used as first line in HBV treatment and rescue therapy in patients with extensive drug resistance due to its high barrier to resistance, with good virological control after prolonged therapy even in HIV/HBV co-infected patients.[10,13,14,24,36,37]

A larger study and prolonged follow up is needed to see if there are any tenofovir drug mutations present among HIV/HBV co-infected patients in South Africa.

The extensive use of 3TC for HIV treatment can be a driver of immune escape and further research needs to be done to find the prevalence of these mutations. Drug level monitoring will also be a useful tool in addition to the investigations done in this study to see whether patients are receiving the correct treatment. A problem that was encountered while doing the study was the lack of repeat HBsAg testing for patients that tested positive at the health care facility. The majority of the samples required repeat HBsAg testing to confirm HBV chronicity. The results from the study show that patients with HIV/HBV co-infection need to be monitored more closely and HBV VL should be implemented as routine testing to monitor patients even if the HIV VL is suppressed.

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Appendices:

Appendix A: Letter of approval from Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee

11-Oct-2019

Dear **Dr Jacobus Kotze**

Ethics Clearance: **HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSS-SECTIONAL STUDY IN CENTRAL SOUTH AFRICA.**

Principal Investigator: **Dr Jacobus Kotze**

Department: **School of Pathology Department (Bloemfontein Campus)**

APPLICATION APPROVED

Please ensure that you read the whole document

With reference to your application for ethical clearance with the Faculty of Health Sciences, I am pleased to inform you on behalf of the Health Sciences Research Ethics Committee that you have been granted ethical clearance for your project.

Your ethical clearance number, to be used in all correspondence is: **UFS-HSD2019/1744/0110**

The ethical clearance number is valid for research conducted for one year from issuance. Should you require more time to complete this research, please apply for an extension.

We request that any changes that may take place during the course of your research project be submitted to the HSREC for approval to ensure we are kept up to date with your progress and any ethical implications that may arise. This includes any serious adverse events and/or termination of the study.

A progress report should be submitted within one year of approval, and annually for long term studies. A final report should be submitted at the completion of the study.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National

Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP (2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this proposal for ethical clearance and we wish you every success with your research.

Yours Sincerely

Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00006240; REC 230408-011; IORG0005187; FWA00012784

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Appendix B: Letter of approval from Health Sciences Research Ethics Committee for amendment to study

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

Health Sciences Research Ethics Committee

11-Nov-2020

Dear **Dr Jacobus Kotze**

Ethics Number: UFS-HSD2019/1744/0110-0001

Ethics Clearance: **HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSSSECTIONAL STUDY IN CENTRAL SOUTH AFRICA.**

Principal Investigator: **Dr Jacobus Kotze**

Department: **School of Pathology Department (Bloemfontein Campus)**

SUBSEQUENT SUBMISSION APPROVED

With reference to your recent submission for ethical clearance from the Health Sciences Research Ethics Committee. I am pleased to inform you on behalf of the HSREC that you have been granted ethical clearance for your request as stipulated below:

1. We are requesting an amendment to the protocol to include getting access to the Department of Health's TIER.net database. It is an electronic patient management system that keeps track of patients receiving HIV treatment. On the database, the patient's treatment history and date of treatment initiation are recorded. We need this information to determine whether patients are on the recommended drug treatment for HIV/HBV co-infection. Getting this information from the NHLS LabTrack database has been difficult as the majority of patients' treatment details are lacking and insufficient. The amendment will not change the basic structure of the protocol and will only be an additional resource to gather information. The patients' identities will still be kept confidential and the details of doing so are outlined in the amendment.

2. We are also changing the inclusion criteria of the patients. Any patient on antiretroviral therapy for HIV that is HIV/HBV co-infected will be included in the study regardless of the current regime. This change in inclusion criteria will not change the outcomes of the study because HIV/HBV co-infected patients must be, according to guidelines, on treatment active for HBV regardless of the treatment regime for HIV. This change will ensure that an adequate sample size is collected and will allow the inclusion of patients for whom treatment regimen details are not available.

3. The testing and follow up of patients regarding HBV infection is poor in the public health sector. It has been difficult to determine whether a patient is chronically infected, and many patients only have one positive HBsAg test with no follow-up testing after 6 months. We are adding the serological test for HBsAg before doing the HBV viral load to ensure that the

patient is chronically infected, and an undetectable viral load is not due to an HBV negative sample but rather a suppression of HBV due to treatment. This extra testing will not impact the time until completion of the project or the budget.

The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services- (HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the HSREC of the Faculty of Health Sciences.

For any questions or concerns, please feel free to contact HSREC Administration: 051-4017794/5 or email EthicsFHS@ufs.ac.za.

Thank you for submitting this request for ethical clearance and we wish you continued success with your research. Yours Sincerely
Dr. SM Le Grange
Chair : Health Sciences Research Ethics Committee

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | E: ethicsfhs@ufs.ac.za
IRB 00011992; REC 230408-011; IORG 0010096; FWA 00027947

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Appendix C: Permission letter from NHLS

Office of the Business Manager

Practice No. 5200296

UNIVERSITAS ACADEMIC LABORATORIES

PO BOX 339 (G3)

C/O: CHEMICAL PATHOLOGY 1 st FLOOR BLOCK C FACULTY OF HEALTH SCIENCES UNIVERSITY OF FREE STATE

BLOEMFONTEIN

9301

REQUEST FOR APPROVAL OF LABORATORY RESOURCES FOR ACADEMIC PURPOSES

Date: 29 August 2019

Requestor: Dr Charles Kotze,

Project Name: "HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSS-SECTIONAL STUDY IN CENTRAL SOUTH AFRICA."

Dear Dr. Kotze,

Your request for use of laboratory facilities / data is hereby granted under following conditions:

1) That University Ethical Committee approval and approval from the Universitas Hospital management is obtained

2) All laboratory data remain confidential to the patient and doctor (anonymity is maintained)

3) This Office must be notified before any publication of any results / findings are made.

4) NHLS is recognised in all publications

5) That a successful K-Project application be made and relevant NHLS project cost centre be created to utilise testing at NHLS as per your protocol.

May your project be successful.

Regards,

Mr. Pakiso Letanta
Acting Business Manager

Physical Address: 1 Modderfontein Road, Sandringham, Johannesburg, South Africa
Chairperson: Prof Eric Buch Acting CEO: Dr Karmani Chetty
Postal Address: Private Bag X8, Sandringham, 2131, South Africa
Tel: +27 (0) 11 386 6000/ 0860 00 NHLS(6457) www.nhls.ac.za
Practice number: 5200296

Appendix D: Permission letter from Head of School of Pathology

29 Aug 2019

The Chair
Heath Sciences Research Ethics
Committee Faculty of Health
Sciences
University of the Free State.

Dear Dr Le Grange

Research Proposal: HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSS-SECTIONAL STUDY IN CENTRAL SOUTH AFRICA

MMed Registrar: Dr Charles Kotze
Supervisor: Prof. D Goedhals
Collaborators: Phillip Armand Bester, Sabeehah Vawda, Philippa Matthews, Cornel van Rooyen,

I grant approval for the above MMed study to be conducted within the School of Pathology (Division of Virology). Permission will also need to be obtained from the NHLS Business Manager to use data on the NHLS laboratory information system.

I wish Dr Kotze much success in his study.

Yours Sincerely

Jocelyn Naicker

Head: School of Pathology

Faculty of Health Sciences

Tel: 051 405 2914 | Cell: 0829071925

jocelyn.naicker@nhls.ac.za | www.nhls.ac.za

Office of the School of Pathology, Faculty of Health Sciences, Universitas Academic laboratories, University of the Free State

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Appendix E: Permission letter from supervisor

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Postgraduate Administration

Faculty of Health Sciences

University of the Free State

Bloemfontein

9 December 2021

To whom it may concern,

Re: Submission of MMed research report, Dr JC Kotze

This letter is to confirm that as supervisor, I approve the submission of Dr Kotze's research report. The Turnitin reports have been reviewed in detail and have been found to be acceptable. The similarities noted relate to scientific terminology, manufacturer and kit names. These cannot be changed as these are standard scientific terms which are used in the field. There are no sections of text which have been used verbatim from published sources.

Yours sincerely,

Dominque Goedhals

Affiliated Associate Professor: Division of Virology

Division of Virology 205 Nelson Mandela Drive/Rylaan

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Appendix F: Data forms

Assigned study #	Gender	Age	Lab	Location	1st line	HBsAg	HBsAgQ value	HBsAgQ result	Chronic HBV	HIVVL(copies/mL)	HIV VL (log)	HBVVL(IU/mL)	HBVVL(Log)
#1	M	48	JE	Free State	Y(?)	P	0.056	REACTIVE	Y	97	1.99	LDL	LDL
#2	F	44	JC	Free State	Y	P	#N/A	#N/A	Y	250	2.40	LDL	LDL
#3	F	57	JC	Free State	Y(?)	P	>150	REACTIVE	Y	69	1.84	LDL	LDL
#4	F	29	JG	Free State	Y(?)	P	8.2	REACTIVE	Y	LDL	LDL	LDL	LDL
#5	F	45	JA	Free State	Y(?)	P	4.5	REACTIVE	Y	<20	<1.30	LDL	LDL
#6	M	34	JC	Free State	?	P	>150	REACTIVE	Y	1280000	6.11	105	2.02
#8	F	23	JE	Free State	Y(?)	P	0.47	REACTIVE	Y	273000	5.44	LDL	LDL

#9	F	43	JB	Free State	?	P	>150	REACTIVE	Y	1520	3.18	100245916	8.00
#10	M	26	JE	Free State	Y(?)	P	0.087	REACTIVE	Y	LDL	LDL	LDL	LDL
#11	F	31	JC	Free State	Y(?)	P	>150	REACTIVE	Y	748	2.87	LDL	LDL
#12	F	38	JA	Free State	Y(?)	P	>150	REACTIVE	Y	<20	<1.30	LDL	LDL
#13	F	32	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	<20	<1.30
#14	F	33	KG	Northen Cape	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	61	1.79
#15	M	18	JE	Free State	?	P	#N/A	#N/A	Y	2830	3.45	323421	5.51
#16	F	30	JD	Free State	Y(?)	P	>150	REACTIVE	Y	567000	5.75	2378	3.38
#17	M	48	KH	Northen Cape	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	394	2.60
#18	M	40	JC	Free State	?	P	>150	REACTIVE	Y	321	2.51	18862	4.28
#19	M	54	HS	Free State	Y(?)	P	>150	REACTIVE	Y	71	1.85	2645273	6.42

#20	M	49	HU	Free State	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	LDL	LDL
#21	M	49	JE	Free State	Y	P	#N/A	#N/A	Y	<20	<1.30	LDL	LDL
#22	M	41	JG	Free State	Y(?)	P	>150	REACTIVE	Y	60	1.78	<20	<1.30
#23	F	59	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#24	F	27	JE	Free State	Y	P	>150	REACTIVE	Y	941	2.97	<20	<1.30
#25	F	28	JB	Free State	Y(?)	P	0.37	REACTIVE	Y	LDL	LDL	LDL	LDL
#26	F	51	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#27	M	36	JE	Free State	Y(?)	P	>150	REACTIVE	Y	77	1.89	LDL	LDL
#28	F	33	KH	Northen Cape	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	<20	<1.30
#29	M	46	JE	Free State	?	P	>150	REACTIVE	Y	<20	<1.30	LDL	LDL
#30	F	39	JG	Free State	Y(?)	P	>150	REACTIVE	Y	745	2.87	97536	4.99

#31	F	41	JA	Free State	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	22	1.34
#32	F	32	JD	Free State	Y(?)	P	#N/A	#N/A	Y	1340	3.13	>170000000	>8.23
#33	M	64	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#34	M	64	JA	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	6027	3.78
#35	M	37	JE	Free State	Y	P	>150	REACTIVE	Y	LDL	LDL	4010	3.60
#36	M	40	JE	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	<20	<1.30
#37	M	38	JE	Free State	?	P	#N/A	#N/A	Y	2900	3.46	267	2.43
#38	M	48	JG	Free State	Y(?)	P	#N/A	#N/A	Y	161	2.21	<20	<1.30
#39	F	33	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	99	2.00
#40	F	29	JG	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	19551	4.29
#41	M	34	JD	Free State	Y(?)	P	>150	REACTIVE	Y	36	1.56	235	2.37

#42	F	38	JB	Free State	?	P	#N/A	#N/A	Y	18600	4.27	973	2.99
#43	F	41	JG	Free State	?	P	0.15	REACTIVE	Y	<20	<1.30	LDL	LDL
#44	M	31	JC	Free State	Y(?)	P	>150	REACTIVE	Y	10200	4.01	>170000000	8.23
#45	F	58	JG	Free State	Y(?)	P	>150	REACTIVE	Y	42	1.62	LDL	LDL
#46	M	45	JD	Free State	Y	P	>150	REACTIVE	Y	227000	5.36	690886	5.84
#47	F	31	JC	Free State	Y(?)	P	0.13	REACTIVE	Y	287	2.46	LDL	LDL
#48	M	35	JA	Free State	Y(?)	P	#N/A	#N/A	Y	49	1.69	<20	<1.30
#49	F	25	JA	Free State	?	P	0.10	REACTIVE	Y	21300	4.33	LDL	LDL
#50	F	18	JG	Free State	?	P	>150	REACTIVE	Y	<20	<1.30	5116	3.71
#51	F	29	JD	Free State	Y(?)	P	0.54	REACTIVE	Y	LDL	LDL	LDL	LDL
#52	M	39	JG	Free State	?	P	>150	REACTIVE	Y	254000	5.40	2999775	6.48

#53	F	49	KD	Northen Cape	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	LDL	LDL
#54	F	37	JD	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	49	1.69
#55	M	31	JE	Free State	Y(?)	P	#N/A	#N/A	Y	177	2.25	34232	4.53
#56	M	35	JG	Free State	Y(?)	P	0.69	REACTIVE	Y	36	1.56	<20	<1.30
#57	F	45	JE	Free State	Y(?)	P	0.52	REACTIVE	Y	LDL	LDL	LDL	LDL
#58	F	36	JG	Free State	Y(?)	P	>150	REACTIVE	Y	407	2.61	335	2.53
#59	M	33	JE	Free State	?	P	#N/A	#N/A	Y	25900	4.41	67145	4.83
#60	M	44	JC	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	58	1.76
#61	M	26	JE	Free State	Y	P	110	REACTIVE	Y	268	2.43	LDL	LDL
#62	F	35	KD	Northen Cape	?	P	#N/A	#N/A	Y	13900	4.14	932	2.97
#63	M	34	JE	Free State	Y(?)	P	>150	REACTIVE	Y	<20	<1.30	LDL	LDL

#64	M	45	JE	Free State	Y	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#65	M	44	JB	Free State	Y(?)	P	0.17	REACTIVE	Y	LDL	LDL	LDL	LDL
#66	F	30	JE	Free State	Y(?)	P	0.093	REACTIVE	Y	<20	<1.30	<20	<1.30
#67	F	32	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	51	1.71
#68	F	23	JD	Free State	?	P	#N/A	#N/A	Y	5490	3.74	LDL	LDL
#69	M	45	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	286595	5.46
#70	M	41	JG	Free State	Y(?)	P	#N/A	#N/A	Y	45	1.65	26	1.41
#71	M	39	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#72	M	43	JB	Free State	?	P	>150	REACTIVE	Y	30	1.48	147	2.17
#73	M	61	JB	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#74	M	44	JB	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	112	2.05

#75	M	39	JB	Free State	Y(?)	P	1.0	REACTIVE	Y	LDL	LDL	LDL	LDL
#76	F	35	HU	Free State	?	P	>150	REACTIVE	Y	47	1.67	<20	<1.30
#77	F	36	KH	Northen Cape	?	P	#N/A	#N/A	Y	1200000	6.08	LDL	LDL
#78	F	36	JG	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	30	1.48
#79	M	47	JA	Free State	Y(?)	P	0.19	REACTIVE	Y	177	2.25	LDL	LDL
#80	M	48	JC	Free State	Y(?)	P	110	REACTIVE	Y	76	1.88	6115	3.79
#81	F	29	JG	Free State	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	<20	<1.30
#82	M	46	JG	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	24	1.38
#83	M	36	JD	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	59	1.77
#84	M	40	JE	Free State	Y(?)	P	#N/A	#N/A	Y	6620	3.82	57243	4.76
#85	M	42	HT	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL

#86	M	54	JD	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	<20	<1.30
#87	M	32	JB	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	<20	<1.30
#88	M	52	JE	Free State	Y(?)	P	#N/A	#N/A	Y	100	2.00	1619	3.21
#89	F	36	JB	Free State	Y(?)	P	>150	REACTIVE	Y	155	2.19	10225841	7.01
#90	F	39	JA	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	LDL	LDL
#91	M	33	JD	Free State	Y(?)	P	0.22	REACTIVE	Y	LDL	LDL	LDL	LDL
#92	F	33	JE	Free State	Y(?)	P	>150	REACTIVE	Y	23	1.36	43964	4.64
#93	F	36	JE	Free State	Y(?)	P	5.5	REACTIVE	Y	4510	3.65	430	2.63
#94	F	45	JB	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	<20	<1.30
#95	F	46	JB	Free State	Y(?)	P	>150	REACTIVE	Y	<20	<1.30	LDL	LDL
#96	F	31	JD	Free State	Y(?)	P	>150	REACTIVE	Y	2560	3.41	<20	<1.30

#97	M	29	JG	Free State	Y(?)	P	#N/A	#N/A	Y	724	2.86	LDL	LDL
#98	M	41	KH	Northen Cape	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#99	M	52	KH	Northen Cape	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	<20	<1.30
#100	F	45	JE	Free State	Y(?)	P	0.076	REACTIVE	Y	<20	<1.30	LDL	LDL
#101	M	38	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	105	2.02
#102	M	40	JA	Free State	Y(?)	P	#N/A	#N/A	Y	3280	3.52	8714185	6.94
#103	M	55	KD	Northen Cape	Y(?)	P	#N/A	#N/A	Y	<50	<1.70	112	2.05
#104	F	26	JB	Free State	Y(?)	P	12	REACTIVE	Y	LDL	LDL	LDL	LDL
#105	F	62	JB	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#106	M	37	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	95	1.98
#107	M	52	JE	Free State	Y(?)	P	>150	REACTIVE	Y	75	1.88	30418	4.48

#108	F	55	JE	Free State	Y(?)	P	0.96	REACTIVE	Y	144	2.16	LDL	LDL
#109	M	34	JC	Free State	Y(?)	P	#N/A	#N/A	Y	47900	4.68	>170000000	>8.23
#110	F	56	JD	Free State	Y(?)	P	>150	REACTIVE	Y	25	1.40	865	2.94
#111	F	39	JE	Free State	Y(?)	P	>150	REACTIVE	Y	1400	3.15	320173	5.51
#112	F	39	HU	Free State	Y(?)	P	#N/A	#N/A	Y	8040	3.91	152	2.18
#113	M	37	JB	Free State	Y(?)	P	#N/A	#N/A	Y	77	1.89	671976	5.83
#114	M	58	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	93	1.97
#115	M	32	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	47	1.67
#116	M	47	JB	Free State	Y(?)	P	>150	REACTIVE	Y	656000	5.82	139	2.14
#117	F	29	JG	Free State	Y(?)	P	8.6	REACTIVE	Y	LDL	LDL	LDL	LDL
#118	M	56	JB	Free State	Y(?)	P	#N/A	#N/A	Y	60700	4.78	58	1.76

#119	F	37	HU	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	LDL	LDL
#121	F	22	JA	Free State	Y(?)	P	0.27	REACTIVE	Y	LDL	LDL	LDL	LDL
#122	F	42	KH	Northen Cape	Y(?)	P	0.18	REACTIVE	Y	437000	5.64	LDL	LDL
#123	M	28	JC	Free State	Y(?)	P	0.18	REACTIVE	Y	<20	<1.30	LDL	LDL
#124	F	30	JG	Free State	Y(?)	P	#N/A	#N/A	Y	3060	3.49	285811	5.46
#125	F	37	KH	Northen Cape	Y(?)	P	#N/A	#N/A	Y	73	1.86	LDL	LDL
#126	M	44	JE	Free State	Y(?)	P	#N/A	#N/A	Y	281	2.45	423836	5.63
#127	F	44	JE	Free State	Y(?)	P	>150	REACTIVE	Y	55200	4.74	>170000000	>8.23
#128	F	27	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	<20	<1.30
#129	M	45	JD	Free State	Y(?)	P	120	REACTIVE	Y	<20	<1.30	LDL	LDL
#130	M	41	JE	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	LDL	LDL

#131	M	36	JA	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	105	2.02
#132	M	42	JE	Free State	Y(?)	P	5.8	REACTIVE	Y	LDL	LDL	LDL	LDL
#133	M	48	JE	Free State	Y(?)	P	0.47	REACTIVE	Y	102	2.01	LDL	LDL
#134	M	57	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#135	M	38	JD	Free State	Y(?)	P	>150	REACTIVE	Y	3090000	6.49	16866	4.23
#136	F	48	JC	Free State	Y(?)	P	>150	REACTIVE	Y	323	2.51	458435	5.66
#137	F	37	KD	Northen Cape	Y(?)	P	#N/A	#N/A	Y	926	2.97	33497	4.53
#138	M	34	JE	Free State	Y(?)	P	>150	REACTIVE	Y	2110	3.32	57	1.76
#139	M	37	JA	Free State	Y(?)	P	0.80	REACTIVE	Y	<20	<1.70	LDL	LDL
#140	M	51	JB	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#141	F	33	JE	Free State	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	LDL	LDL

#142	F	49	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	<20	<1.30
#143	M	27	KD	Northen Cape	Y(?)	P	#N/A	#N/A	Y	968	2.99	12851	4.11
#144	F	46	JD	Free State	Y(?)	P	0.059	REACTIVE	Y	357000	5.55	LDL	LDL
#145	F	47	KD	Northen Cape	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	LDL	LDL
#146	M	35	JE	Free State	Y(?)	P	>150	REACTIVE	Y	37300	4.57	48	1.68
#147	F	57	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#148	F	34	JE	Free State	Y(?)	P	120	REACTIVE	Y	LDL	LDL	LDL	LDL
#149	M	55	JE	Free State	Y(?)	P	140	REACTIVE	Y	<20	<1.30	LDL	LDL
#150	F	63	KD	Northen Cape	Y(?)	P	0.12	REACTIVE	Y	<50	<1.70	LDL	LDL
#151	M	40	JC	Free State	Y(?)	P	>150	REACTIVE	Y	72200	4.86	74	1.87
#152	M	42	JC	Free State	Y(?)	P	#N/A	#N/A	Y	26200	4.42	76149	4.88

#153	M	47	JC	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#154	F	36	KA	Northen Cape	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#155	F	40	JC	Free State	Y(?)	P	>150	REACTIVE	Y	<20	<1.30	538	2.73
#156	F	36	JG	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	<20	<1.30
#157	F	36	JB	Free State	Y(?)	P	>150	REACTIVE	Y	1040000	6.02	85232	4.93
#158	M	56	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#159	F	34	JC	Free State	Y(?)	P	>150	REACTIVE	Y	2430	3.39	>170000000	>8.23
#160	F	38	JE	Free State	Y(?)	P	>150	REACTIVE	Y	82	1.91	76488	4.88
#161	M	49	JB	Free State	Y(?)	P	>150	REACTIVE	Y	<20	<1.30	LDL	LDL
#162	M	58	JE	Free State	Y(?)	P	>150	REACTIVE	Y	38	1.58	LDL	LDL
#163	F	39	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL

#164	M	48	JE	Free State	Y(?)	P	#N/A	#N/A	Y	419	2.62	119335761	8.08
#165	M	37	JB	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	LDL	LDL
#166	M	33	JA	Free State	Y(?)	P	#N/A	#N/A	Y	34	1.53	421	2.62
#167	M	38	JC	Free State	Y(?)	P	#N/A	#N/A	Y	85	1.93	LDL	LDL
#168	F	36	JE	Free State	Y(?)	P	>150	REACTIVE	Y	8560	3.93	<20	<1.30
#169	F	34	JE	Free State	Y(?)	P	>150	REACTIVE	Y	24	1.38	>170000000	>8.23
#170	F	38	JD	Free State	Y(?)	P	1	REACTIVE	Y	1200	3.08	LDL	LDL
#171	F	42	JC	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#172	F	42	JG	Free State	Y(?)	P	11	REACTIVE	Y	474	2.68	LDL	LDL
#173	M	36	JE	Free State	Y(?)	P	85	REACTIVE	Y	67	1.83	1074	3.03
#174	F	36	JG	Free State	Y(?)	P	100	REACTIVE	Y	231	2.36	LDL	LDL

#175	F	52	JC	Free State	Y(?)	P	>150	REACTIVE	Y	62	1.79	LDL	LDL
#176	M	34	JA	Free State	Y(?)	P	>150	REACTIVE	Y	251	2.40	720	2.86
#177	F	52	JD	Free State	Y(?)	P	>150	REACTIVE	Y	68	1.83	331286	5.52
#178	M	30	JC	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#179	F	36	JE	Free State	Y(?)	P	0.11	REACTIVE	Y	249	2.40	LDL	LDL
#180	M	45	JB	Free State	Y(?)	P	>150	REACTIVE	Y	22	1.34	558	2.75
#181	F	46	JA	Free State	Y(?)	P	>150	REACTIVE	Y	81200	4.91	>170000000	>8.23
#182	M	51	JE	Free State	Y(?)	P	>150	REACTIVE	Y	60	1.78	LDL	LDL
#183	F	29	JG	Free State	Y(?)	P	0.88	REACTIVE	Y	91	1.96	LDL	LDL
#184	M	39	JB	Free State	Y(?)	P	0.34	REACTIVE	Y	LDL	LDL	LDL	LDL
#185	M	50	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL

#186	M	54	JC	Free State	Y(?)	P	#N/A	#N/A	Y	51	1.71	452	2.66
#187	M	44	JA	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	19316	4.29
#188	M	37	JD	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	321322	5.51
#189	F	48	KD	Northen Cape	Y(?)	P	>150	REACTIVE	Y	17700	4.25	43668547	7.64
#190	F	55	JB	Free State	Y(?)	P	1.3	REACTIVE	Y	76	1.88	LDL	LDL
#192	F	46	JB	Free State	Y(?)	P	#N/A	#N/A	Y	45200	4.66	5818	3.76
#193	F	34	JA	Free State	Y(?)	P	>150	REACTIVE	Y	56300	4.75	8115871	6.91
#194	M	45	JD	Free State	Y(?)	P	0.069	REACTIVE	Y	LDL	LDL	LDL	LDL
#195	M	36	JE	Free State	Y(?)	P	>150	REACTIVE	Y	151	2.18	LDL	LDL
#196	M	42	JG	Free State	Y(?)	P	>150	REACTIVE	Y	165	2.22	LDL	LDL
#197	F	39	JB	Free State	Y(?)	P	#N/A	#N/A	Y	300	2.48	836	2.92

#198	M	35	JG	Free State	Y(?)	P	>150	REACTIVE	Y	91300	4.96	35918374	7.56
#199	F	24	JB	Free State	Y(?)	P	0.86	REACTIVE	Y	221	2.34	LDL	LDL
#200	F	33	JA	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#201	M	51	JC	Free State	Y(?)	P	>150	REACTIVE	Y	68600	4.84	>170000000	>8.23
#202	M	54	KD	Northen Cape	Y(?)	P	#N/A	#N/A	Y	<20	<1.30	LDL	LDL
#203	F	37	JB	Free State	Y(?)	P	0.18	REACTIVE	Y	<20	<1.30	LDL	LDL
#204	F	34	JC	Free State	Y(?)	P	>150	REACTIVE	Y	<50	<1.70	580	2.76
#205	M	47	JC	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#206	M	34	JB	Free State	Y(?)	P	0.1	REACTIVE	Y	LDL	LDL	LDL	LDL
#207	F	40	JE	Free State	Y(?)	P	#N/A	#N/A	Y	14400	4.16	>170000000	>8.23
#208	F	32	JE	Free State	Y(?)	P	>150	REACTIVE	Y	321	2.51	7721268	6.89

#209	F	38	JB	Free State	Y(?)	P	1.1	REACTIVE	Y	LDL	LDL	LDL	LDL
#210	M	42	JC	Free State	Y(?)	P	>150	REACTIVE	Y	2930	3.47	9680863	6.99
#211	F	54	JE	Free State	Y(?)	P	0.81	REACTIVE	Y	LDL	LDL	LDL	LDL
#212	F	30	JB	Free State	Y(?)	P	0.1	REACTIVE	Y	2790	3.45	<20	<1.30
#213	F	41	JE	Free State	Y(?)	P	>150	REACTIVE	Y	55	1.74	>170000000	>8.23
#214	M	36	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#215	F	20	JA	Free State	Y(?)	P	0.11	REACTIVE	Y	LDL	LDL	LDL	LDL
#216	F	42	JA	Free State	Y(?)	P	>150	REACTIVE	Y	122	2.09	201398	5.30
#217	M	29	KG	Northen Cape	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	LDL	LDL
#218	F	55	JG	Free State	Y(?)	P	>150	REACTIVE	Y	28	1.45	LDL	LDL
#219	M	52	JE	Free State	Y(?)	P	>150	REACTIVE	Y	420	2.62	LDL	LDL

#220	M	52	JG	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	327	2.51
#221	F	31	JG	Free State	Y(?)	P	0.19	REACTIVE	Y	LDL	LDL	LDL	LDL
#222	M	36	JA	Free State	Y(?)	P	>150	REACTIVE	Y	107000	5.03	2797150	6.45
#223	F	42	KD	Northen Cape	Y(?)	P	0.23	REACTIVE	Y	58000	4.76	LDL	LDL
#224	M	62	HS	Free State	Y(?)	P	14	REACTIVE	Y	LDL	LDL	LDL	LDL
#225	M	38	JE	Free State	Y(?)	P	>150	REACTIVE	Y	LDL	LDL	<20	<1.30
#227	F	32	JA	Free State	Y(?)	P	#N/A	#N/A	Y	LDL	LDL	67	1.83
#228	M	62	JC	Free State	Y(?)	P	#N/A	#N/A	Y	9690	3.99	LDL	LDL

Sample ID	Genotype	Escape mutants	SHB mutations	RT Mutations	3TC	3TC muts	ADV	ADV muts	ETV	ETV muts	TDF	TDF muts	LdT	LdT muts
9	A1		I195IM, S207N, C221CG	M129L, V163I, L180LM, M204MV, L229LW, I253V, S256G, H271C	resistant	180M,204V	susceptible	none	partly resistant	204V,180M	susceptible	none	resistant	204V
15	A1		K122R, A194V, S207N	N122H, Y126H, M129L, N131D, V163I, I253V, D263E, I282V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
16	A1		S207N	N122H, M129L, V163I, I253V, D263E, V266I	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
30	A1		A194V, S207N	N122H, Q125E,	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

				M129L, V163I, I253V										
32	A1		S207N	N122H, M129L, V163I, I253V, V266I	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
34	A1		S207N	N122H, M129L, W153R, V163I, I253V, D263E, V266MT	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
40	A1		A194V, S207N	N122Y, Q125E, M129L, V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
42	E			M267L	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
46	A1		I110IL, A194V, P203PR, S207N, L209LV, Y225FY	S117PS, N118NT, N122H, Q125E, M129L, V163I, L217LR, N238NT, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

50	A2		F179FI, L209V	I187IN, L217R, N248IN	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
52	A1		A194V, S207N	N122H, M129L, V163I, I253V, V266K	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
55	A1		A194V, S207N	N122H, M129L, V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
62	A1		K122R, A194V, M197T, S207N	Y126H, M129L, N131D, V163I, I253V, V266I	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
69	A1		A194V, I195M, S207N	N122H, M129L, V163I, L180M, M204V, I253V, V266I	resistant	180M,204V	susceptible	none	partly resistant	204V,180M	susceptible	none	resistant	204V
84	A1		A194V, S207N	V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
89	A1		A194V, S207N	N122H, M129L,	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

				V163I, I253V, V266I										
107	A1		E164DE, I195M, S207N	N122H, M129L, V163I, V173LV, L180M, M204V, I253V, V266I	resistant	173L,180M,204V	susceptible	none	partly resistant	204V,180M	susceptible	none	resistant	204V
109	A2		L209V	L217R	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
111	A1		S171FS, S207N	N122H, M129L, V163I, I253V, V266I	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
124	A1		L175LS, A194V, S207N	N122H, M129L, V163I, I253V, K270T	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
126	A1	129	Q129R, V168A, T189I, A194V, S207N	N122H, Q125E, M129L, V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
127	A1		L192FL, A194V,	N122H, Y126H, M129L,	resistant	180M,204I	susceptible	none	partly resistant	204I,180M	susceptible	none	resistant	204I

			W196L, S207N	M145L, V163I, L180LM, A200AV, M204I, I253V										
135	A1		S207N	V163I, I253V, H271C	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
136	A1		K122R, A194V, S207N	N122H, Y126H, M129L, Q130P, N131D, V163I, I253V, I282V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
137	A1		A159V, A194V, Y200F, S207N	M129L, V163I, L164M, I253V, H271C	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
152	A1		A194V, S207N	M129L, V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
157	A1		A194V, S207N	N122H, M129L, V163I, I253V,	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

				V266I, M309L										
159	A1		A194V, S207N	N122H, Q125E, M129L, V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
160	A1		A194V, S207N	N122H, Q125E, M129L, V163I, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
164	A1		S167L, W196LW, S207N, I213IT, F220FL	N122H, M129L, V163I, M204IM, L229LV, I253V, V278I	resistant	204I	susceptible	none	partly resistant	204I	susceptible	none	resistant	204I
166	A2		L209V	L217R	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
169	A1		A194V, I195M, S207N, F220FL	N122H, Q125E, M129L, V163I, L180M, M204V, L229LV, I253V, L276I	resistant	180M,204V	susceptible	none	partly resistant	204V,180M	susceptible	none	resistant	204V
173	A1		S207N	N122H, M129L,	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

				W153R, V163I, I253V, V266LV										
177	A1		A194V, S207N	N122H, Q125E, M129L, V163I, I253V, L276I	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
181	A1		A194V, S207N	M129L, V163I, I253V, V266K	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
186	A1		A194V, S207N	N122H, M129L, N131D, V163I, I253V, I282V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
187	A2		I195IM, W196*W, L209V	M204IMV, L217R	resistant	204I,204V	susceptible	none	partly resistant	204V,204I	susceptible	none	resistant	204I,204V
188	A1		I195IM, W196LW, S207N	V163I, L180LM, M204IMV, I253V, V266I, H271C	resistant	180M,204I,204V	susceptible	none	partly resistant	204V,204I,180M	susceptible	none	resistant	204I,204V

189	A1		E164EV, S207N	M129L, V163I, I253V, V266I, H271C, C314S	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
192	A1		S207N	M129L, V163I, I253V, S256G, H271C	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
193	A1	131N, 144A	N131NT, D144AD, A194V, S204RS, S207N	N122H, M129L, Q139HQ, V163I, S213ST, I253V	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
197	A1		E164D, V184A, I195M, S207N	N122H, M129L, V163I, V173L, L180M, M204V, I253V, V266I	resistant	173L,180M,204V	susceptible	none	partly resistant	204V,180M	susceptible	none	resistant	204V
201	A1		K122R, A194V, S207N	N122H, N124H, Y126H,	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

				M129L, N131D, V163I, I253V, T259S										
207	A1		S207N	N122H, M129L, W153R, V163I, I253V, D263E, V266M	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
208	A1		A194V, S207N	N122H, M129L, V163I, I253V, V266I	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none
213	A1		I195M, S207N	N122H, M129L, W153R, V163I, L180M, M204V, I253V, D263E, V266M	resistant	180M,204V	susceptible	none	partly resistant	204V,180M	susceptible	none	resistant	204V
216	A1		T143M, A194V, S207N	N122H, Q125E,	susceptible	none	susceptible	none	susceptible	none	susceptible	none	susceptible	none

				M129L, V163I, I253V										

Appendix G: Lab techniques

Samples with one previous positive HBsAg were retested on the automated serology analyser, LIAISON® XL from DiaSorin (DiaSorin S.p.A., Saluggia, Italy), to confirm chronic hepatitis B infection. The determination of HBsAg on the LIAISON-XL is a quantitative test and is a direct two-step chemiluminescence-based immunoassay (CLIA). The assay employs a set of monoclonal antibodies directed against highly conserved epitopes of the HBsAg internal region. The assay quantitation range is set from 0.03 to 150 IU/mL. Testing was performed according to the manufacturer's instructions.

All samples that were confirmed to have chronic HBV infection were tested using a fully automated quantitative real-time polymerase chain reaction system, the Roche COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0 (RocheMolecular Diagnostics, Branchburg, NJ, USA), to determine the HBV VL.

Samples with a detectable HBV VL were sequenced with Sanger sequencing. Viral DNA was extracted from 300µl of patient samples using the NucliSENS® easyMag® from bioMérieux (bioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. A nested in-house PCR was used to amplify the region of interest. The target for amplification was a 645-bp fragment in the polymerase region of the HBV reverse transcriptase (RT) gene, covering amino acids 115-320, which includes the most important areas where drug-resistant mutations occur. The PCR primers used by the NHLS Laboratory at Charlotte Maxeke Johannesburg Academic Hospital for HBV drug resistance testing were used.[NO_PRINTED_FORM] For the first round PCR the following primers were used; forward primer 5' GTCTGCGGCGTTTTATCA 3' and reverse primer 5' GGAGTTCCGCACTATGGATCGG 3'. The PCR conditions for the first round reaction was a denaturation step at 95 °C for 2 min followed by 35 PCR cycles at 95 °C (20 s), 54 °C (10 s) and 70 °C (15 s). The second-round PCR used the forward primer 5' GGTATGTTCCCGTTTGTCC 3' and reverse primer 5' GGCGAGAAAGTGAAAGCCT with the following PCR conditions; a denaturation step at 95 °C for 2 min followed by 30 PCR cycles at 95 °C (20 s), 57 °C (10 s) and 70 °C (9 s). KOD Hot Start DNA polymerase (Novagen, Nottingham, UK, cat. no. 71086) was used according to the manufacturer's instructions to perform the in-house PCR. The size of the PCR product was confirmed using gel electrophoresis. Applied Biosystems® (Applied Biosystems, Foster City, CA, USA) 3500xL series genetic analyser with BigDye® Terminator v3.1 Cycle Sequencing Kit

(Applied Biosystems) was then used according to the manufacturer's specifications to perform Sanger sequencing using the nested primers mentioned previously.

Appendix H: Copy of the research protocol approved by the HSREC

HBV VIRAL LOAD AND DRUG RESISTANCE AMONG HIV-HBV CO-INFECTED PATIENTS: A CROSS-SECTIONAL STUDY IN CENTRAL SOUTH AFRICA

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1. Introduction

It is estimated that 2 billion people around the world have been infected with hepatitis B virus (HBV) and 360 million are chronic carriers [1]. The WHO has included viral hepatitis as one of its major public health priorities. HBV is one of the top leading causes of death worldwide with an estimated 786000 deaths per year attributable to hepatocellular carcinoma and liver cirrhosis [2]. Childhood vaccination and effective antivirals are available but the burden of disease still remains high especially in low-income countries where access to these remains low [3,4,5].

Epidemiology:

About a third of the world's population shows serological evidence of past or current infection with HBV [6,7]. During the 1990s there was a sharp increase in mortality from liver cancer (62%) and liver cirrhosis (29%) with HBV causing roughly 50% of the total mortality associated with liver cancer in 2010 [2].

Transmission occurs during close contact with infectious bodily fluids; mainly blood, semen and saliva [8]. Perinatal, percutaneous and sexual exposure are the main routes of infection but close person-to-person contact during early childhood is also thought to be a major route of infection [9,10]. Because of these different routes of infection, there are different modes of transmission depending on the level of prevalence of HBV. In areas where HBV prevalence is low (<2%), for example Europe or North America, the route of transmission is mainly sexual or parenteral and occurs in adolescents or young adults [8]. The perinatal route is the most common route of transmission in areas where the prevalence is high (≥8%), for example Southeast Asia [11]. In Africa where the prevalence is also high, most infections occur during early childhood [11]. The mode of transmission during early childhood is still not fully understood, but is thought to occur via close intimate non-sexual contact in overcrowded conditions, rituals involving scarification or tattooing and unsafe medical practices [12,13]. In areas where there is intermediate prevalence (2-7%), the mode of transmission is mixed [14].

Infection that occurs during the perinatal period has a 90% chance of becoming a chronic infection, while infection in early childhood and adulthood has a 20-30% and 5% chance respectively [15]. This has a profound effect on disease progression with most of the morbidity and mortality associated with HBV occurring during chronic infection.

The virion:

HBV is part of the Hepadnaviridae family and has a partially double-stranded circular DNA genome. HBV uses a transcriptional template called covalently closed circular DNA (cccDNA) that is found in the nucleus of hepatocytes [16]. This transcriptional template is essential for HBV maintenance and persistence within the hepatocyte. It forms a minichromosome that is difficult to eradicate and for this reason currently available treatments generally suppress rather than cure chronic HBV infection [8].

A very distinctive feature of HBV, among DNA viruses, is that it uses reverse transcriptase during viral replication. Reverse transcriptase has no proofreading mechanism and is error-prone. Because of this HBV has a high mutation rate, which may be important in the potential for resistance to antiviral therapy.

Pathogenesis:

HBV is not directly cytopathic and pathogenesis is mediated by the host immune response that is induced by active viral replication [17]. Infection in adults usually causes self-limited and transient hepatitis with a good cellular immune response [18]. The virus is then cleared with the establishment of protective antibodies [19]. This is different from chronic infection where the cellular immune response is blunted with repetitive bouts of hepatitis causing necro-inflammation leading to cirrhosis [19]. The natural course of chronic HBV is typically divided into four phases depending on host virus-interactions. The immune tolerant phase is the first phase and is characterized by high HBV viral loads ($>10^5$ IU/mL), normal liver enzymes and positive HBeAg [20]. The second phase, known as the immune clearance phase, is where patients have a heightened immune response to the infection but do not clear it [21]. During this phase HBeAg positive patients seroconvert to HBeAb, there may be an increase in ALT and a decrease in HBV viral load [22]. If the patient has successful HBeAg seroconversion with suppression of HBV viral load, then the patient progresses to the inactive phase of infection. This phase is characterized by the normalization of ALT levels, suppression of HBV viral load

(<2000 IU/mL) and a fall in HBsAg levels [23,24]. A person can remain in this phase for the rest of their life or 20-30% of patients will experience HBV reactivation with raised viral loads and increased ALT levels [8]. Patients that have HBV reactivation are at increased risk of liver cirrhosis and hepatocellular carcinoma (HCC) [25]. A small number of individuals with chronic HBV infection clear HBsAg, in most settings 0.5-1% per year, presumably as a result of successful immune responses that reduce or eliminate cccDNA [8].

Africa and the HIV pandemic:

Measured by the prevalence of anti-HBc antibodies, Africa has a wide range of exposure to hepatitis B with older age and liver-related morbidities having the highest prevalence [26,27]. This also varies a lot between regions in Africa, with western Africa having an exposure prevalence of above 85% and eastern Africa between 65-85% [28,29,30]. Africa has a high prevalence of chronic HBV, above 8%, measured by the prevalence of HBsAg [4]. Due to the majority of infections occurring during early childhood, many people are either immune or chronically infected by early adulthood [31]. This is in contrast to human immunodeficiency virus (HIV), where most of the infections occur during adulthood [32].

Because of these differences, the prevalence of chronic HBV in HIV-positive individuals is very similar to non-HIV infected individuals [33,34,35]. This is very different to developed countries where HBV and HIV share similar infection routes, especially parenteral routes, thus the prevalence of HBV is higher in HIV infected populations compared to the rest of the general population [11].

It is estimated that 5-20% of HIV infected individuals worldwide are co-infected with HBV [36]. HBV has become a major health concern in HIV infected patients and has become more evident after the introduction of HAART for HIV treatment. Since opportunistic infections have become less common in patients on HAART, complications from viral hepatitis has emerged as a leading cause of morbidity and mortality [37,38,39].

HIV infection has a profound effect on the natural history of HBV infection. Co-infected patients have a higher chance of progressing to chronic HBV infection than the general adult population [40]. Co-infected patients also have a higher rate of HCC and liver cirrhosis than HBV mono-infected patients [41,42].

HIV and HBV treatment:

Both HIV and HBV share use of a reverse transcriptase during viral replication. Thus theoretically all the nucleot(s)ide analogues (NA) that are active against HIV should also work against HBV. But this has not been the case, with only the two NA's lamivudine and tenofovir showing activity against both HIV and HBV.

Lamivudine was the first to be used extensively to treat both infections, but it was soon realized that lamivudine has a low barrier to resistance with failure rates of 90% after 5 years in HBV infected individuals [43]. Tenofovir has therefore become the treatment of choice in both infections due to its high barrier to resistance and superior efficacy compared to other NA's [44]. Thus tenofovir is the preferred choice for both HBV monoinfection, and for inclusion in ART regimens for HIV-HBV co-infected patients, with more than 95% of patients virologically suppressed after 5 years of treatment [with the majority of patients being virologically suppressed after prolonged periods of treatment] [45,46,47]. Studies did show, however, that some HIV-HBV co-infected patients have prolonged low levels of HBV viraemia and even virologic breakthrough without resistance on effective tenofovir treatment [47,48]. Factors associated with detectable HBV viral load on tenofovir treatment include detectable HBeAg, CD4+ T cell count below 200 cells/mm^3 and treatment compliance below 95% [49]. From these findings it is clear that co-infected patients must be started early on dual acting therapy and that compliance may still be an issue even when the HIV viral load is suppressed [49].

Other HBV treatment is available but has poor activity or is associated with HIV resistance mutations and is not recommended during co-infection [50]. Adefovir, telbivudine and entecavir are registered for HBV treatment and are only recommended during HBV mono-infection [45,46].

In South Africa, the first line treatment for HIV is a fixed daily combination of tenofovir, emtricitabine and efavirenz. Patients that are co-infected with HBV must get a tenofovir-based regimen even when changing the patient to a 2nd line or 3rd line regimen for HIV.

The importance of HBV viral load:

HBV viral load is used as a monitoring tool before and during HBV treatment, and is an important factor in determining eligibility for treatment and predicting clinical outcome. Higher HBV viral loads have been linked to higher rates of liver cirrhosis and HCC, with viral loads of above 10^5 copies/mL having a worse outcome [51]. Many international guidelines suggest doing HBV viral loads periodically because any increase above the lower limit of detection can have an impact on the course of disease [45,46]. International guidelines recommend that HBV viral loads are performed on a periodic basis to check patient compliance with treatment and also detect possible HBV drug resistance [45,46]. Because resistant strains of the virus will have higher replication during treatment, HBV viral load is a good marker to use for screening for possible viral resistance.

Drug resistance testing:

Drug resistance testing is usually recommended in patients that have past experience with nucleot(s)ide analogue treatment, failure to achieve viral suppression on current treatment and patients that experience virological breakthrough on current treatment [45,46]. Virological breakthrough is defined as a 1 $\log_{10}$ increase in HBV viral load from nadir in a patient that had a virological response on treatment [45,46].

As mentioned above, the antivirals used for HBV treatment are lamivudine (3TC), telbivudine (LdT), entecavir (ETV), adefovir dipivoxil (ADV) and tenofovir disoproxil fumarate (TDF). Drug resistance to these nucleot(s)ide analogues involves changes to nucleotides at specific sites in the reverse transcriptase region of the pol gene [52]. This is caused by the absent proofreading capabilities of the HBV reverse transcriptase and high viral replication rate [53]. These two factors lead to random mutations being inserted into the viral genome leading to the potential generation of a wide variety of quasi-species [52]. Antiviral drugs cause selective pressure on the quasi-species present during natural infection with the selection of viral mutants that confer resistance to the particular antiviral agent present during treatment [54]. These major nucleotide changes usually come at a cost to the virus, leading to lowered viral replication [55]. This “fitness” cost can be overcome by acquiring accessory mutations at other sites on the viral genome leading to replicative capabilities similar to or even greater than the wild type virus [55].

Major mutations involved with resistance to the L-nucleosides, 3TC and LdT, are found in the tyrosine-methionine-aspartate-aspartate (YMDD) locus of the catalytic site of HBV pol [55]. These mutations are rtM204I/V, rtL180M and rtA181T/V57 with compensatory mutations such as rtL80V/I, rtI169T, rtV173L, rtT184S/G, rtS202I, and rtQ215S found at other sites [57,58,59,60,61].

Resistance associated with the alkyl phosphonate ADV are found at sites rtA181T/V and rtN236T, with a mutation at rtI233V found recently [62,63,64,65]. TDF, also an alkyl phosphonate, has a very high barrier to resistance with a mutation at rtA194T causing decreased susceptibility during in vitro studies [66]. A study did report resistance to TDF during HIV-HBV coinfection with mutations at rtA194T and rtL180Mrt+M204V, but this could not be confirmed in a subsequent report [67,68]. ADV resistance does, however, reduce the efficacy of TDF and thus patients treated with ADV in the past should not receive TDF therapy [69,70].

The only D-Cyclopentane ETV has a high barrier to resistance requiring multiple mutations to confer resistance. The sites associated with resistance are rtL180M+rtM204V, with mutations at other sites (rtT184G/S, rtS202I/G or rtM250V) also required [71].

HBV viral load and resistance in South Africa:

Very little is known about the effectiveness of dual active therapy in HIV-HBV co-infected patients in South Africa. This is because HBV viral load testing is not done during the routine monitoring of patients on treatment in the public sector. HBV viral load is recommended though for HBV mono-infected patients on treatment [72]. HBV surface antigen is done once at the initiation of HIV treatment with alanine transaminase levels being done in the first few months after treatment to monitor for drug toxicity and immune reconstitution syndrome [72].

An extensive search of published articles on ScienceDirect (https://www.sciencedirect.com/) and PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) could not find any articles pertaining to the efficacy of tenofovir-containing regimes on HBV viral load in South Africa. There have been HBV epidemiological and drug resistance studies done in HIV-HBV co-infected patients but they are few and small in patient numbers [73,74].

Therefore very little is known about HBV treatment response and drug resistance in HIV-HBV co-infected patients in South Africa where the prevalence of co-infection is high.

2. Aims and objectives

This study sets out to determine whether adult patients have suppressed HBV viral loads on antiretroviral therapy for HIV recommended by the DOH and current WHO guidelines for HIV-HBV co-infections (TDF/3TC/ETV), and to investigate whether HBV drug resistance is a possible cause of an unsuppressed HBV viral load.

The objectives are to:

- Determine the prevalence of patients in the study group that have a detectable HBV viral load during standard antiviral therapy for HIV and HBV.
- Determine the correlation between HIV viral load and HBV viral load.
- Undertake HBV sequencing in patients with detectable HBV viral loads to determine whether there are any known or potential drug resistance mutations causing the increase in viral load.
- Determine any HBV surface antigen escape mutants that might be present in the overlapping region of the HBsAg and reverse transcriptase open reading frames.

3. Methodology

3.1 Study design

The study will focus on HIV-HBV co-infected patients on antiretroviral therapy in central South Africa, namely the Free State and Northern Cape provinces. Because HIV treatment is standardized across South Africa, this population group is broadly representative of the HIV-HBV population across South Africa. We will use a cross-sectional study approach.

3.2 Sample/ study participants

Residual samples will be selected and retrieved from the 7000-8000 HIV viral loads that are received by the Division of Virology at the NHLS Universitas Academic Laboratories on a weekly basis. Patient samples will be selected by matching known HBsAg positive patient data from NHLS LabTrak to weekly HIV viral load samples. HBV data for the Free State and Northern Cape provinces were retrieved from the NHLS for another study (HSREC number UFS-HSD2018/0115/2703) and additional data will be retrieved from LabTrak by the researchers.

Data on the following parameters will be retrieved from the NHLS LabTrak database and used as inclusion criteria:

1. Patient's HBsAg serostatus and HIV viral load.
2. Area from which the sample originates by using the letter prefix of the episode number on patient sample. The letter prefix indicates the laboratory where the sample was sent from.
3. Patient demographics for example age and sex.
4. ARV treatment history if available.
5. Hepatitis B serological markers including HBeAg, HBeAb, HBc IgM if available.

1. Study participants will be any adult patient on first line antiretroviral therapy that is HIV-HBV co-infected in the Free State and Northern Cape provinces, with a sample submitted to the Division of Virology at the NHLS Universitas Academic Laboratories for HIV viral load testing. According to national guidelines and recommendations from the DOH, patients must be on treatment for HIV for at least 6 months before doing an HIV viral load [72]. First line

treatment for HIV in South Africa contains tenofovir that covers HBV infection. Since all co-infected patients must be on a tenofovir containing regimen is it safe to assume that patients are on treatment for HBV. The second line regimen for co-infected patients includes continuation of TDF with standard second line ARVs 3TC/FTC + zidovudine + lopinavir/ritonavir. Patient information pertaining to the current treatment regimen will be retrieved from the NHLS LabTrak database since all treatment history must be recorded by the registering laboratories when indicated on the request form.

The Department of Health also stores data about patient drug history and date of initiation on a centralized data bank called TIER.net. This is an electronic patient management system with modules to capture patient data on HIV testing and treatment. A request will be made at the Department of Health of the Free State, Bophelo House, to retrieve this patient data on treatment history (current and previous regimens) and treatment initiation dates. Only information for patients that have been identified from the NHLS data base as having HIV/HBV co-infection will be requested. The patient information will kept confidential and no personal information or patient identifiers will be entered on the data sheet, only the drug history. The data retrieved from the TIER.net data bank will be stored on the study supervisor's computer that is password protected and stored in a secure location.

2. Any plasma sample from a HIV/HBV co-infected patient will be retrieved and stored in -80°C freezers according to laboratory guidelines. Identification pertaining to the patient will be removed and the sample will be given a unique study number.

3. We will stratify all samples according to HIV viral load, in order to assess the distribution of HIV viral loads in this population subset. HIV viral loads from patient samples will be grouped according to the log value of the viral load.

Grouping will be done as following:

Log(HIV VL) = χ value

The χ value is grouped according to the following ranges:

0; 1 to <2; 2 to <3; 3 to <4; 4 to <5; 5 to <6; and ≥6

Samples with a value below the limit of quantification of the assay and samples with an undetectable viral load will be grouped together and regarded as supressed HIV viral load (i.e. χ value = 0).

4. HBsAg serological testing will be done to confirm HBV chronicity before doing HBV viral load testing. This is done to ensure that an undetectable HBV viral load is not due to an HBV negative sample and that the patient is truly chronically infected with HBV.

5. Approximately 300 samples with sufficient residual plasma (minimum 650 µL) will be selected for further analysis of HBV on the basis of HIV viral load, in order to ensure our sample selection is representative of the complete distribution of HIV viral loads in the population subset. This will allow us to determine the range of HBV viral loads across a diverse population group according to compliance. Higher HIV VL values are usually associated with poor compliance while on treatment and not mutations causing drug resistance but this is not definitive and drug resistance testing will still be done on these samples.

6. Samples with detectable HBV viral loads will be used for Sanger sequencing of the HBV RT gene. Depending on the number of samples with detectable HBV viral loads, a subset of approximately 50-60 samples may be selected across the HIV viral load range.

7. Sequence data will also be used to detect possible HBV surface antigen escape mutants. This can be done due to the overlapping reading frames of the HBV surface antigen and RT genes. When the sequence data is analysed these “escape mutants” can be inferred using data from the RT region.

3.3 Measurement

The quantitative data of the HBV and HIV viral loads will be stored in Excel and used to determine the proportion of patients with undetectable HBV viral loads and to identify if a correlation exists between HBV and HIV viral load levels.

Patient samples will be removed after completion of HIV viral load testing and after routine diagnostic results have been released. HBsAg testing will first be done on samples where the diagnosis of chronic HBV is in doubt using a commercial instrument such as the

LIAISON® XL from DiaSorin then HBV viral load testing will be done using a commercial instrument such as the Roche Cobas® 8800 both available at the Division of Virology. Viral DNA will then be extracted from patient samples that have a detectable HBV viral load, using the NucliSENS® easyMag® from bioMérieux or similar. PCR-based direct sequencing will be used to generate HBV sequence data. An in-house designed assay will be used for this purpose.

The PCR-based direct sequencing will utilize a nested PCR with primers covering the region of interest, namely the reverse transcriptase region of the pol gene. The amplification product (±1200bp) will then be identified using gel electrophoresis.

Applied Biosystems® 3500xL series genetic analyser with BigDye® Terminator v3.1 Cycle Sequencing Kit will be used according to the manufacturer's specifications to perform Sanger sequencing. The sequencing data will then be retrieved from the genetic analyser and stored in FASTA format following analysis on ChromasPro or similar software.

The RT sequence of each HBV isolate will then be submitted to a web-based software for phenotypic analysis of genotypic mutations in the reverse transcriptase region of the pol gene.

We will use web-based software for sequence analysis, such as HBVseq from Stanford University (https://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html) and Geno2pheno hbv from the Max Planck Institut Informatik (https://hbv.geno2pheno.org/). The web-based software will compare the consensus sequence of our patient sample to known wild type sequences of HBV to determine genotypic mutations and the genotype of the HBV.

For geno2pheno each input sequence is aligned against an HBV consensus sequence of the RT domain, the HBV genotype and HBV subgenotype are inferred using reference sequences from GenBank. The aligned nucleotide sequence is translated to the amino acid level with respect to both the RT ORF and the overlapping HBsAg ORF. All polymorphisms with respect to both reading frames are extracted. Prediction results are staged in four categories, namely susceptible, limited susceptibility, partly resistant, and resistant. The rules are based on literature research and incorporate recent research results. Similarly, the amino acid

sequence of HBsAg is compared against a comprehensive list of escape mutations, which was crafted from the literature which is listed in the tool.

In HBVseq each submitted nucleotide sequence is aligned to the consensus genotype A HBV RT amino acid sequence and if successful, the sequence is then compared to a list of genotype-specific reference sequences. The submitted sequence is assigned a genotype closest to the reference sequence. The nucleotide sequence generated by local alignment is gap-stripped and translated in the correct reading frame using the standard genetic code. The resulting amino acid is considered a mutation if it differs from the consensus genotype reference sequence. Each of the mutations identified in a sequence is used to interrogate HBVrt DB to ascertain the mutation's prevalence according to genotype and treatment. HBVrt DB is a database comprised of reference sequences from GenBank that is kept up to date periodically.

HBVseq reports on the genotype and major drug mutations of the sample while Geno2pheno hbv reports on the genotype, subgenotype and major drug mutations. Geno2pheno hbv is able to summarize the results into pdf format that can be stored easily. HBVseq also gives information about the quality of the sample in regards to stop codons or frameshifts. Both software can be used simultaneously as a quality control check.

If we have sufficient residual sample volume, we will consider further sequencing of a small subset of samples using an Illumina platform in order to collect data about drug resistance polymorphisms present as minor quasispecies. The selection of these samples will be determined by volume, HBV viral load, and detection of HBV drug resistance using Sanger sequencing.

4. Analysis of data

1. We will present an analysis of the distribution of HIV viral loads in the whole sample set collected, representing the whole HIV/HBV co-infected population. Included in the analysis will be patient demographics like age, sex and area where sample was collected.

2. In the subgroup of 300 samples from HIV/HBV co-infected samples selected for further analysis, we will report the distribution of HIV viral loads and HBV viral loads.

3. We will determine the relationship between HIV viral load and HBV viral load, to determine the extent to which:

(i) these two parameters correlate;

(ii) complete suppression of HIV viral load is predictive of complete suppression of HBV viral load;

(iii) HBV viral load is suppressed in different groups stratified according to HIV viral load and other patient parameters (e.g. age, sex)

4. We will evaluate our RT sequences for HBV genotype (A-I) and percentage similarity to the identified genotype using the above web based software.

5. We will determine the frequency of drug resistance mutations in the RT region of all the samples that are sequenced. We will seek any association between the presence/absence of such mutations and the HBV and HIV viral loads.

6. In sequences derived from individuals with suppressed HIV viral load but detectable HBV viraemia (suggestive of possible drug resistance in HBV) we will also review the entire RT sequence for other polymorphisms that could represent novel or putative drug resistance mutations.

7. Sequence data will be used to detect any HBsAg escape mutants and whether these mutations are associated with drug resistance mutations or random mutations.

8. If we are able to undertake Illumina sequencing, we will quantify the prevalence of drug resistance mutations within hosts (% of minor variants expressing drug resistance mutation).

This will provide useful insights into the extent to which consensus sequencing is representative of the whole within-host population.

5. Implementation of data

The data obtained from this study will allow us to better understand the dynamics of HIV-HBV co-infection in South Africa. Co-infected patients in South Africa are being treated for HBV but not monitored for treatment response or possible drug resistance. Therefore the data obtained from this study can be used to inform policies and further help prevent patients that are HIV-HBV co-infected from progressing to liver-related diseases.

6. Time schedule

The time schedule of the project is to collect and run samples from approximately October 2019 to July 2020, followed by data analysis from August-September 2020. The MMed report will be drafted from October-December 2020. The MMed project will be finished at the end of 2020.

STAGE	DURATION	DATE
Literature review	2 months	1 December 2018 - 31 January 2019
Writing protocol	2 months	1 February - 31 March 2019
Review of protocol by study leader	12 days	1 - 12 April 2019
Adjustments to protocol	5 days	1 - 5 June 2019
Submission to Department of Biostatistics	14 days	July 2019
TURNITIN plagiarism check	2 days	July 2019
Adjustments to protocol if necessary	5 days	August 2019
Submission to HSREC supervisor	1 day	August 2019
Feedback and adjustments to protocol if necessary	6 days	August 2019
HSREC submission	2 days	September 2019
HSREC approval	2 months	September - October 2019
Perform study	10 months	October - July 2020
Transferring data to spreadsheet	1 week	August 2020

Analysis of data	2 months	August – September 2020
Writing final research report	3 months	October - December 2020
Submission of final report	1 day	January 2021

7. Budget

Funding will be sought from the NHLS Research Trust. Additional funding for sequencing is available from the Wellcome Trust Clinical Fellowship of Prof Philippa Matthews.

ITEM	**Cost per unit**	**Number of units**	**COST**
HBV viral load	R 250	300	R 75 000
HBV resistance testing	R 600	50	R 30 000
HBsAg serological testing	R 33	300	R 10 000
TOTAL			R 115 000

8. Ethical aspects

Only residual samples that are left over from HIV viral load testing will be used during the study and no additional samples will be collected from patients. Thus no patient contact will occur during the study period. Information pertaining to drug history will only be retrieved from data stored on NHLS LabTrak and no patient information will be retrieved from patient files at the local health facilities.

Patient information will be kept confidential by assigning each patient sample with a study number. The patient episode numbers and assigned study numbers will be logged in excel or a paper document. The original file or document will be kept confidential and stored with the study supervisor. Permission to perform the study will be obtained from the Business Manager at NHLS Universitas Academic Laboratories and from the Head of the School of Pathology.

9. Outcomes

The project will be completed in fulfilment of the research requirements for an MMed degree. The results will be submitted for publication in a peer reviewed journal and presented at a local and/or international congress.

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10. Appendices

Data forms:

	Demographics					Treatment		Serostatus [Positive(P),Negative(N)]						Viral load (VL)			
Assigned study #	Gender	Age	Lab	Location		1st line		HBsAg	HBeAg	HBeAb	HBc IgM	HBcTA		HIV VL	HIV VL (log)	HBV VL	Genotype
eg. 01	Male	40	JC	Kroonstad		Yes		P	P	N	N	P		20000	4.30	40000	A

Mutations								
	Mutations associated with drug resistance							
		Confered resistance analysis [Susceptible(S), Partly resistant(P), Resistant(R)]					HBV surface protein mutations	
All mutations found	Mutations associated with drug resistance	Lamivudine	Adevofir	Entecavir	Tenofovir DF	Telbivudine	All mutations	Mutations associated with escape mutants
53,112,173,180,204	173L,180M,204V	R	S	P	S	R	None	None

Appendix I: Instructions to authors of the named peer reviewed journal

Journal of Viral Hepatitis

PREPARING THE SUBMISSION

Cover Letters

Cover letters are not mandatory; however, they may be supplied at the author's discretion.

Parts of the Manuscript

The manuscript should be submitted in separate files: main text file; figures.

Main Text File

The text file should be presented in the following order:

i. A short informative title that contains the major key words. The title should not contain abbreviations (see Wiley's best practice SEO tips);

ii. A short running title of less than 40 characters;

iii. The full names of the authors;

iv. The author's institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;

v. Acknowledgments;

vi. Abstract and keywords;

vii. Main text;

viii. References;

ix. Tables (each table complete with title and footnotes);

x. Figure legends;

xi. Appendices (if relevant).

Figures and supporting information should be supplied as separate files.

Authorship

Please refer to the journal's authorship policy the Editorial Policies and Ethical Considerations section for details on eligibility for author listing.

Acknowledgments

Contributions from anyone who does not meet the criteria for authorship should be listed, with permission from the contributor, in an Acknowledgments section. Financial and material support should also be mentioned. Thanks to anonymous reviewers are not appropriate.

Conflict of Interest Statement

Authors will be asked to provide a conflict of interest statement during the submission process. For details on what to include in this section, see the section 'Conflict of Interest' in the Editorial Policies and Ethical Considerations section below. Submitting authors should ensure they liaise with all co-authors to confirm agreement with the final statement.

Abstract

Please provide an abstract of no more than 250 words containing the major keywords. It should summarize the aim of the study and describe the work undertaken, results and conclusions.

Keywords

Please provide up to five keywords. Keywords should be taken from those recommended by the US National Library of Medicine's Medical Subject Headings (MeSH) browser list at www.nlm.nih.gov/mesh.

Main Text

- Introduction: This section should not exceed more than 500 words and should not have a separate heading. The Introduction should briefly discuss the objectives of the study and provide the background information to explain why the study was undertaken, and what hypotheses were tested.
- Materials and methods: Animal preparation and experimentation should cite the approving governing body. Equipment and apparatus should cite the make and model number and the company name and address (town, county, country) at first mention.
- Results: Tables and text should not duplicate each other.
- Discussion: This section should not exceed more than 1,500 words and should be concise. The Discussion should include a brief statement of the principal findings, a discussion of the validity of the observations, a discussion of the findings in light of other published work dealing with the same or closely related subjects, and a statement of the possible significance of the work. Authors are encouraged to conclude with a brief paragraph that highlights the main findings of the study.

References

All references should be numbered consecutively in order of appearance and should be as complete as possible. In text citations should cite references in consecutive order using Arabic superscript numerals. For more information about AMA reference style please consult the AMA Manual of Style

Sample references follow:

Journal article

1. King VM, Armstrong DM, Apps R, Trott JR. Numerical aspects of pontine, lateral reticular, and inferior olivary projections to two paravermal cortical zones of the cat cerebellum. J Comp Neurol 1998;390:537-551.

Book

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3. American Cancer Society. Cancer Facts & Figures 2003. http://www.cancer.org/downloads/STT/CAFF2003PWSecured.pdf Accessed March 3, 2003

Tables

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