CHAPTER 1: INTRODUCTION

1.1 Background

DART was a randomized strategy trial that compared clinical monitoring only to laboratory and clinical monitoring to determine the impact of these interventions on the long term safety and efficacy of highly active antiretroviral therapy (HAART) in Zimbabwe and Uganda. The trial ran from the year 2003 up to 2009. The patients received lamivudine (3TC) and zidovudine (AZT) as Combivir (CBV) (a fixed dose combination of 3TC and zidovudine) together with either tenofovir (TDF) or nevirapine (NVP) or abacavir (ABC). There were 2468 patients enrolled to receive CBV/TDF, 547 patients received CBV/NVP and 300 patients received CBV/ABC. All patients were treatment naïve and had advanced acquired immuno-deficiency syndrome (AIDS) with a CD4 count of less than 200. Samples were taken at baseline and at 4, 12, 24 and 48 weeks and every 48 weeks thereafter until the last visit, up to 6 years.

As the DART study has closed, samples are now available for analysis in other sub-studies. The Hepatitis B Virus (HBV) sub-study has been initiated to study the efficacy of TDF/3TC combination therapy in suppressing HBV replication in treatment naïve patients co-infected with the human immunodeficiency virus (HIV). HBV is a major cause of viral hepatitis which is an inflammation of the liver caused by viral infection. TDF and 3TC are antiretroviral therapy (ART) drugs but are also active against HBV. Incidence of hepatitis flares and clinically significant liver disease events in patients treated for up to six years with TDF/3TC combination will be compared to those receiving 3TC. HBV TDF/3TC resistance development will be investigated by testing for viral rebound in follow up samples of baseline samples that will be found to have positive HBV e markers and detectable levels of
HBV DNA. HBV testing was not included in the main DART protocol and so it was not known which patients were HBV co-infected.

More than one third of the world’s population has been infected with HBV (Kane, 1996). According to the World Health Organisation (WHO) (2008) an estimated two billion people have been infected with HBV and more than 350 million have chronic HBV liver infections worldwide. It was previously reported that Zimbabwe has an overall HBV surface antigen (HBsAg) sero-prevalence rate of 15.4 % in the general population (Tswana et al., 1996) and is classified as a high HBV endemic area (See fig 1.1) but little is known of the relationship of HBV and HIV infection.

![Chronic HBV Prevalence Based on HBsAg Endemicity](image)

**Fig 1.1:** Chronic HBV prevalence based on HBsAg endemicity. (From World Health association [www.who.int](http://www.who.int))
1.2 Project Rationale:
Southern Africa, including Zimbabwe, is a high HBV endemic area (See fig 1.1) with an estimated 50 million chronic HBV infections and around 25 million HIV infected persons. Yet data from Africa on the prevalence and clinical implications of HIV/HBV co-infection are limited or unavailable. Although rates of hepatitis B have fallen because of HBV vaccination, trends among people with HIV have not been well studied. The prevalence of HIV infected persons carrying HBV serological markers in Zimbabwe is not known. This information will be important as the clinical impact of HBV infection in HIV positive patients has progressively grown since the introduction of HAART given the increase in survival rates experienced by these patients who now experience the sequelae of other chronic infections such as HBV infections. Liver disease due to chronic HBV infections has become an increasingly important source of morbidity and mortality in HIV infected patients in the HAART era.

In addition, the long term use of TDF/3TC combination therapy as a component of HAART in chronic HBV infections verses potential TDF/3TC resistance development in treatment naïve patients, not previously exposed to 3TC as part of earlier ART regimens, has not been studied in Africa. The ability of TDF/3TC to sustain the decrease in HBV viral load over time without resistance development is important in prevention of viral rebound given the inadequacies of 3TC in long term treatment of chronic HBV. There are currently no data for potential HBV resistance development or resistance mutations against TDF/3TC in treatment naïve HIV-1 positive individuals in Africa initiating TDF/3TC combination therapy. HBV e markers positive baseline samples identified in this project will be followed up to investigate TDF/3TC efficacy and resistance development in treatment of chronic HBV.
1.3 Aim of the study

To determine the HBV baseline serology profiles in the DART cohort and identify samples for follow up within the DART cohort to investigate HBV TDF/3TC resistance development in treatment naïve HIV/HBV co-infected patients.

1.4 Objectives

The objectives of the study were to:

1. Determine the prevalence of HBV co infection (HBsAg sero prevalence) in the DART cohort from Harare.
2. Investigate the level of exposure (HBV core antibody (anti-HBc) prevalence) to HBV in the same DART cohort.
3. Determine the different HBV serology marker combinations within the DART cohort.
4. Identify samples that will be followed up to investigate HBV TDF/3TC resistance, evidenced by viral rebound, in patients initiating TDF/3TC combination therapy as part of HAART.
CHAPTER 2: LITERATURE REVIEW

2. LITERATURE REVIEW

2.1 HBV structure and replication

The HBV virion or Dane particle is a double enveloped, 42nm diameter DNA virus with 3200 bases classified within the Family hepadnavirus (Hollinger and Liang, 2001; Murray et al., 2005). HBV is classified into 8 genotypes (A – H) based on 8% divergence in the entire genome sequence (Okamoto et al., 1988; Norder et al., 1994; Arauz-Ruiz et al., 2002).

Fig 2.1: Schematic diagram showing organisation of the HBV genome: HBV partially double stranded DNA virus has 4 partially overlapping open reading frames (ORFs) encoding the HBV polymerase (orange), the S proteins (pre-S1, pre-S2 and S), the HBV X protein (HBx) and HBV core proteins (precore and core) ((http://Abbott Molecular International Real Tiemm-em HBV.mht 2009).
As shown in fig 2.1, the circular partially double stranded HBV DNA has 4 open reading frames derived from the incomplete + strand coding for HBsAg, HBV core antigen (HBcAg) and viral polymerase gene (Carmona et al., 2006). The function of the fourth gene (X gene) is uncertain but is thought to be a transcriptional activator enhancing the expression of other proteins (CDC: http://virology-online.com/viruses/HepatitisB.htm). Another HBV marker, HBV e antigen (HBeAg), may be generated from the core protein by proteolytic cleavage (CDC: http://virology-online.com/viruses/HepatitisB.htm).

The virion envelope consists of an outer envelope containing host-derived lipids and all S gene polypeptides, the large (L), middle (M), and small (S) surface proteins, also known as pre-S1, pre-S2 and HBsAg (Mahoney and Kane, 1999; Robinson, 1995). The nucleocapsid contains core proteins HBcAg, viral DNA genome, an endogenous DNA polymerase (reverse transcriptase) enzyme, and protein kinase activity (Hollinger and Liang, 2001; O’Shea, 2009). HBsAg is released into the serum of infected people as spherical or filamentous particles whose structures are shown in the electron micrograph and illustration in Fig 2.2 (Carman and Thomas, 1992; Murray et al., 2005).
Fig 2.2: HBV particles. A. Schematic drawing of Dane particle and HBsAg particles in filamentous and spherical forms. B. As seen under electron microscopy X; 42 nm complete virions/Dane Particles. Y; spheres 22 nm in diameter. Z; filaments 22 nm in diameter, constitute the HBsAg. (Murray et al., 2005).
The HBV cycle, illustrated by Ganem et al. (2004), starts by virions binding to hepatocytes surface receptors (See Fig 2.3). The virions are then internalized and viral core particles migrate to the hepatocyte nucleus where their genomes are repaired to form a covalently closed circular DNA (cccDNA) that is the template for viral messenger RNA (mRNA) transcription. The viral mRNA that results is translated during protein synthesis in the cytoplasm to produce the viral surface, core, polymerase, and X proteins. There, progeny viral capsids assemble, incorporating genomic viral RNA (RNA packaging). This RNA is then reverse-transcribed into viral DNA by the polymerase.

Fig 2.3: A schematic diagram of the Replication Cycle of HBV. (Ganem et al., 2004)
The polymerase, which also has ribonuclease H activity, initially synthesizes a negative strand of DNA using a protein primer and the RNA as a template followed by degradation of the RNA by the ribonuclease H activity as a positive strand DNA is formed (Yi-Brunozzi et al., 2008). The envelopment occurs before completion of the positive sense DNA resulting in genomes containing RNA-DNA circles (Mahoney and Kane, 1999; Ganem and Scheneider, 2001). When the RNA portions are removed by ribonuclease H activity the typical partially double stranded HBV DNA is formed. The virion is budded into the lumen of the endoplasmic reticulum, from which they are secreted via the Golgi apparatus out of the cell (Mahoney and Kane, 1999; Ganem and Scheneider, 2001). Sometimes the entire genome is integrated into the host cell DNA and such viral DNA has been found in hepatocellular carcinomas (Zuckerman, 1996).

2.2 HBV Pathogenesis and immune response

HBV mainly targets hepatocytes, but other cell types including biliary epithelial cells, pancreas, kidney, skin, spleen, bone marrow, peripheral blood mononuclear cells, can be infected (Zoulim, 2004). Replication in hepatocytes starts within 3 days of infection but symptoms may not appear for up to 45 days or more depending on viral dose, route of infection and age of the infected person (Gitlin, 1997). Symptoms are a result of cell mediated immunity and inflammation which also effect resolution of infection by destroying infected hepatocytes (WHO, 2002).

The HBV immune response system presents two stages, the immune tolerant stage and immune clearance stage. In the immune tolerant stage, common in people infected with HBV at birth (Lee, 1997), the immune system does not detect the virus and so it replicates unhindered. This is evidenced by normal alanine aminotransferase (ALT) and aspartate...
aminotransferase (AST) levels, high HBV DNA levels and patients are HBsAg positive, HBV surface antibody (anti-HBs) negative, HBeAg positive and HBV e antibody (anti-HBe) negative (Wright, 2006). In the immune clearance stage ALT/AST levels are high and HBV DNA levels may vary (Pan and Zhang, 2005). The immune system recognizes the virus and cytotoxic response is activated, resulting in cell death as it tries to kill infected hepatocytes and elevation of ALT/AST levels (Bozkaya, 2004). Anti-HBs and anti-HBe become positive.

An insufficient immune response will fail to clear the infection and results in the development of chronic hepatitis (Chisari and Ferrar, 1997). The HBsAg in serum binds to neutralizing antibody limiting its capacity to resolve the infection (Vanlandschoot et al., 2002). The HBsAg-anti-HBs complex elicits type III hypersensitivity reactions leading to conditions such as vasculitis, arthralgia, rash and renal damage (Venkateshan, 1990; Johnson and Couser, 1990).

The cellular immune response is illustrated by Ganem et al. (2004) in Fig 2.4. HBsAg particles and virions are taken up by antigen presenting cells, which degrade the viral proteins to peptides that are then presented on the cell surface bound to major histocompatibility complex (MHC) class I or II molecules. These peptide antigens can be recognized by CD8+ or CD4+ T cells, respectively, which are thereby sensitized. Antigen-presenting cells can also process and display viral antigens taken up by phagocytosis of killed infected hepatocytes. Virus-specific CD8+ cytotoxic T cells (with help from CD4+ T cells, as shown in Fig 2.4) can recognize viral antigens presented on MHC class I chains on infected hepatocytes. This recognition reaction can lead to either direct lysis of the infected hepatocyte or the release of interferon and tumor necrosis factor (TNF) α, which can down-regulate viral replication in surrounding hepatocytes without direct cell killing.
2.3 HBV clinical types of infection

Acute infection presents with a clinically apparent illness including symptoms such as fatigue, nausea, vomiting, arthralgias, fever, right upper quadrant pain, jaundice, dark urine, and clay coloured stools while some patients may have no symptoms (Gitlin, 1997; AIDS Education and Training Centers (AETC): [http://aidsetc.org/aidsetc.org/aidsetc?page=cm-511_hepb](http://aidsetc.org/aidsetc.org/aidsetc?page=cm-511_hepb)). About 25 % of HBV infected people develop acute infection (Murray et al., 2005). The four clinical phases of acute infection are the incubation period, the preicteric, icteric and convalescent periods (Murray et al., 2005). The associated symptoms are shown in Table 2.1. The incubation period is long (45 to 160 days) with an insidious onset of disease (CDC: [http://virology-online.com/viruses/HepatitisB.htm](http://virology-online.com/viruses/HepatitisB.htm)).
Acute liver failure or fulminant hepatitis presents with more severe symptoms and signs of severe liver damage and potentially causes death (Mahoney and Kane, 1999; Hollinger and Liang, 2001).

**Table 2.1:** Symptoms associated with acute infection phases (compiled from Murray *et al.*, 2005)

<table>
<thead>
<tr>
<th>Phase</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prodromal period</td>
<td>Fever, malaise, anorexia, nausea, vomiting, abdominal discomfort, chills</td>
</tr>
<tr>
<td>Icteric</td>
<td>Jaundice, dark urine, pale stools</td>
</tr>
<tr>
<td>Recovery</td>
<td>Decline in fever, renewed appetite</td>
</tr>
</tbody>
</table>

Chronic infection is defined as persistent HBsAg positivity for more than 6 months (Sterling, 2003). About 5 % to 10 % of HBV infections result in chronic disease (Murray *et al.*, 2005). Chronic hepatitis may be active with ongoing liver destruction leading to scarring of the liver, cirrhosis, liver failure or primary hepatocellular carcinoma (PHC) or it may be passive causing no problems (Ganem and Scheider, 2001; Murray *et al.*, 2005). Since chronic infection shows no signs and symptoms of infection it may go undetected and infected people become a major source of spread of the virus (Ganem and Scheider, 2001). Chronic infection may be detected by chance, by finding elevated liver enzyme levels on a routine blood chemistry profile (Hollinger and Liang, 2001). Fulminant hepatitis may occur if chronically infected people become co-infected with hepatitis D virus (HDV) (CDC, 1991; Mendez, 1991).
HBV infections may induce primary hepatocellular carcinoma (PHC) because of continued liver repair to counter the tissue damage (Robinson, 1995; Ganem and Schneider, 2001) or by integrating HBV DNA into the host chromosome and stimulating cell growth directly. Integration of viral DNA into the host chromosome could stimulate genetic rearrangement placing viral promoters adjacent to cellular growth control genes (Ganem and Schneider, 2001). It may take 30 to 50 years from initial HBV infection to PHC (WHO, 2002).

2.4 HBV and HIV co-infections

Co-infection with HBV and HIV is possible as the two have shared routes of infection primarily through blood exposure and sexual contact and from mother to child during childbirth. The association of HIV and HBV has become the focus of attention with cases, epidemiology, natural history and treatment being studied. (Piliero and Faragon, 2002; Thio, 2003). In chronic infections HBV genetic material remains in human cells and the virus may be reactivated as immune function deteriorates due to HIV infection.

There is a higher prevalence of HBV serological markers among HIV infected patients than in non HIV infected individuals (Hofer et al., 1998; Puoti et al., 2002). For example, 20 % of people acquiring HBV while already HIV positive develop chronic HBsAg antigenaemia, this rate is three times higher than that in HIV negative control subjects (Taylor et al., 1988; Thomson, 1996). HBsAg positivity prevalence of 9 % to 100 % in HIV-1 positive individuals has been reported in some parts of Africa including the Niger Delta in Nigeria, west Africa and Abidjan, Cote d’voire (Ejela et al., 2004; Mulders et al., 2004; Rouet et al., 2004).
2.5 HBV markers natural patterns and effect of HIV co-infection

The initial diagnosis of hepatitis B can be based on clinical symptoms and the presence of elevated ALT and AST in blood (Gitlin, 1997). The course and nature of the disease, *i.e.* acute or chronic, can be demonstrated by investigating the presence of HBsAg and HBeAg in serum and the pattern of antibodies to HBV antigens (Taswell *et al.*, 1985) as shown in Fig 2.5.

![Graph showing HBV markers in acute and chronic infection](image)

**Fig 2.5:** HBV markers in acute and chronic infection: Typical levels of ALT, HBV DNA, HBsAg, HBeAg, anti-HBc, anti-HBe, and anti-HBs antibodies are shown in acute self-limited HBV infection (Panel A) and in infections that become chronic (Panel B). (Prince, Lee and Brotman, 2001).
The graph shows that HBsAg is the first serological marker to appear followed by the appearance of HBeAg in serum. HBsAg persists throughout the course of the illness. The appearance of HBsAg occurs 2-8 weeks before biochemical evidence of liver dysfunction as shown by elevated ALT. Anti-HBc appears in the serum 2-8 weeks after the appearance of HBsAg and is associated with low infectivity of the serum. Anti-HBs is the last marker to appear late during convalescence. As shown in Fig 2.5B, HBV DNA and HBsAg persist in the serum at high levels years after exposure in chronic HBV infections. The other difference is that anti-HBe and anti-HBs appear early in acute infections but remain undetectable in chronic infections years after exposure.

HBV DNA assays are not routinely used for management and evaluation of hepatitis but are useful especially when serological patterns fall out of classical patterns (Sablon and Shapiro, 2005) as HBV DNA is a specific and active marker indicating HBV replication (Alberti et al., 1986; Jardi et al., 1996). For example, carriers on antiretroviral therapy and HBeAg negative individuals showing symptoms of infection.

Hepatitis B seroconversion starts with appearance of anti-HBe in the serum which, for those on treatment, marks the endpoint of treatment (Liaw, 2009; Liaw et al., 2010). At this point HBeAg is negative and HBe antibody (anti-HBe) is positive, however, HBsAg will remain positive and HBs antibody (anti-HBs) will be negative. Complete seroconversion occurs when there is negative HBsAg, positive anti-HBs and undetectable HBV. Table 2.2 shows the classification of HBV infection based on the presence of different HBV antibody and antigens.
Table 2.2: Classification of HBV infection

<table>
<thead>
<tr>
<th>HBV serological marker/s present</th>
<th>Classification of HBV</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM anti-HBc</td>
<td>Acute</td>
</tr>
<tr>
<td>HBsAg for more than six months</td>
<td>Chronic</td>
</tr>
<tr>
<td>Anti-HBs and anti-HBc</td>
<td>Past infection</td>
</tr>
<tr>
<td>Anti-HBs only</td>
<td>Vaccine recipient</td>
</tr>
</tbody>
</table>

Co-infection with HIV affects the classical HBV serology patterns since HIV modulates the natural history of HBV infection (Konopnicki et al., 2005). HIV infected chronic HBV positive individuals have been shown to be more likely to have evidence of HBV replication indicated by the presence of HBeAg, viral polymerase activity or HBV DNA in the serum (de Jongh et al., 1992) and lower incidences of spontaneous loss of HBeAg or HBsAg than HIV negative individuals (Calson et al., 2006). Accelerated loss of previously acquired HBV immunity with reappearance of HBsAg has been documented following HIV infection (Thomson, 1996). Occult HBV infection (HBsAg negativity associated with detection of HBV DNA in serum) has also been reported in HIV positive individuals (Calson et al., 2006).

2.6 HBV diagnosis

Biochemical, serological and molecular markers of HBV are used in the diagnosis of HBV infection. The tests and their interpretations are shown in Table 2.3.
Table 2.3: Hepatitis B tests and interpretation

<table>
<thead>
<tr>
<th>TEST</th>
<th>INTERPRETATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV surface antigen (HBsAg)</td>
<td>Presence denotes hepatitis B infection</td>
</tr>
<tr>
<td>Antibody to HBsAg (Anti-HBs)</td>
<td>Presence denotes immunity to antigen</td>
</tr>
<tr>
<td>HBV e antigen (HBeAg)</td>
<td>Early antigen that shows the virus is replicating in host liver cells</td>
</tr>
<tr>
<td>Antibody to HBeAg (Anti-HBe)</td>
<td>Anti HBe antibodies with undetectable DNA indicate that the virus is no longer replicating and the patient is much less infectious</td>
</tr>
<tr>
<td>Antibody to HBV core antigen (Anti-HBe)</td>
<td>Helps distinguish between acute and chronic HBV. Presence indicates:</td>
</tr>
<tr>
<td></td>
<td>1) Past infection (immunity) if anti-HBs antibody is also positive</td>
</tr>
<tr>
<td></td>
<td>2) Chronic infection if anti HBc-IgG and HBsAg present</td>
</tr>
<tr>
<td></td>
<td>3) Acute or recent infection if anti HBc-IgM present</td>
</tr>
<tr>
<td>HBV DNA</td>
<td>Viral load tests used as baseline indicator of infectivity, monitor treatment efficacy and detect drug resistance emergence.</td>
</tr>
<tr>
<td>* Alanine aminotransferase (ALT)</td>
<td>Presence indicates injured or dead liver cells. Elevation of ALT may indicate activation of immune response against the HBV virus.</td>
</tr>
<tr>
<td>* Aspartate aminotransferase (AST)</td>
<td>Enzyme is released into the blood as a response to cell injury or death indicating involvement of the immune system.</td>
</tr>
<tr>
<td>Alpha-Fetoprotein (AFP)</td>
<td>This tumor marker may indicate hepatocellular carcinoma when at elevated levels.</td>
</tr>
</tbody>
</table>

* Liver function tests (LFTs) that do not exclusively reflect the immune response to the HBV virus only (Table compiled from Murray et al., 2005)

The presence of these markers as illustrated in Fig 2.6 is used to confirm HBV infection and knowledge of the natural history of infection is used to determine the clinical stages and type of infection, i.e. acute or chronic.
2.6.1 Serological markers

Enzyme immunoassays (EIA) are used for the detection of HBV serological markers in human serum or plasma. As shown in Fig 2.6, HBsAg and HBeAg appear early in infection but HBsAg persists for a longer period. Anti-HBe appears towards resolution of infection. HBsAg is therefore the marker most commonly tested for to determine HBV infection. The EIAs for the detection of HBsAg employ capture antibodies often having specificity for epitopes present on the antigenic (a) determinant of the HBsAg. Loss of detection may occur due to mutations within and/or outside of the a determinant that affect conformational epitope recognition or HBsAg secretion or expression (Osiowy, 2006). Fig 2.6 also shows that reactivation of HBV is associated with elevated ALT and viral rebound although clinical symptoms will be minimal.

![Fig 2.6: HBV testing: Natural history of HBV and the biochemical, serological and molecular markers used in its diagnosis (Thio, 2006)](image-url)
The serological markers of HBV infection vary depending on whether the infection is acute or chronic (Taswel et al., 1985). Table 2.4 shows profiles of HBV serology tests and their interpretations. Presence of both HBsAg and anti-HBc indicates a current infection, anti-HBs with anti-HBc reveals a past, resolved infection while anti-HBc alone may be interpreted as evidence of past infection with undetectable levels of anti-HBs or HBV chronic carrier with undetectable levels of HBsAg (Medrano et al., 1991).

**Table 2.4: HBV serology test profiles and interpretations (From European Guideline for the management of Hepatitis B and C virus infections 2010)**

<table>
<thead>
<tr>
<th>Stage of infection</th>
<th>HBsAg</th>
<th>HBeAg</th>
<th>IgM</th>
<th>Total anti-HBc</th>
<th>Anti-HBe</th>
<th>Anti-HBs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute (early)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++/++</td>
<td>-</td>
</tr>
<tr>
<td>Acute (resolving)</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Chronic (immune tolerant)</td>
<td>+</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Chronic (immune active)</td>
<td>+</td>
<td>+</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chronic (eAg Neg.)</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Chronic (inactive carrier)</td>
<td>+</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Resolved (immune)</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>+</td>
<td>_</td>
<td>+/-</td>
</tr>
<tr>
<td>Successful vaccination</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>_</td>
<td>+</td>
</tr>
</tbody>
</table>
2.6.2 Biochemical markers

The presence of elevated ALT and AST in blood may be used as initial diagnosis of HBV infection (Gitlin, 1997). These enzymes however do not exclusively reflect the immune response to the HBV virus only and so confirmation with tests for serological or molecular markers would be required.

2.6.3 HBV DNA detection

HBV DNA can be detected during the replicative phase of infection and becomes undetectable with resolution of infection. As shown in Fig 2.6, HBV DNA levels can rise again with reactivation of infection. This viral rebound may indicate treatment failure or HBV drug resistance for individuals on treatment. HBV DNA assays can be divided into signal amplification assays or DNA amplification tests based on the polymerase chain reaction (PCR) (Sablon and Shapiro, 2005). Signal amplification tests include liquid phase hybridization, antibody capture approach and branched DNA with sensitivities approaching 1 pg of DNA ($10^5$-$10^6$ genome copies) or even to $10^3$ genome copies (Bowden, 2002). HBV DNA detection based on nested PCR approach can detect as few as $10^2$-$10^3$ genome copies but such low titers result in problems with contamination and reproducibility leading to false positive results. This can be overcome by use of internal or external standards (Schutten and Niesters, 2001). Real-time PCR technology based on continuous quantitative monitoring during the exponential phase of the PCR reaction measures viral loads over a larger dynamic range compared to endpoint measurement PCR cycles (Schutten and Niesters, 2001; Bowden, 2002). In the HBV Abbott real time quantitative PCR (RT qPCR) the target region is a highly conserved region in the $N$-terminal third of the Surface gene allowing detection of genotypes A-H and ensuring that the assay is not affected by YMDD mutants, HBsAg escape mutants or
drug resistant mutants as this region only tolerates minor structural changes (http://Abbott Molecular International RealTiemm-eme HBV.mht, 2009).

2.7 HBV control and treatment in HIV co-infected individuals

An HBV vaccine composed of HBsAg has reduced the incidence of HBV infection in at risk groups (Murray et al., 2005). The vaccine induces protection in up to 95% of recipients (CDC: http://www.cdc.gov/ncidod/diseases/hepatitis/b/hebqafn.htm). The plasma derived vaccine consist of highly purified, formalin and/or heat inactivated hepatitis B subvirion particles of HBsAg with no detectable nucleic acid and so are non-infectious (Mahoney and Kane, 1999; Hollinger and Liang, 2001). It may also be genetically engineered as recombinant DNA yeast-derived or mammalian cell-derived were the S gene (pre-S1, pre-S2, S) is cloned and isolated (Mahoney and Kane, 1999; Hollinger and Liang, 2001). Zimbabwe has hepatitis B vaccination as part of its national immunization program as per the WHO extended program on immunization requirements. (http://www.who.int/csr/disease/hepatitis/whocdcsrlyo20022/en/index4.html).

There is no treatment available for acute infection and management is just supportive (Mahoney and Kane, 1999). However, Hepatitis B immune globulin is effective when administered within 48 hours of exposure and to infants born to mothers infected with HBV (Dienstag, 1989; Mahoney and Kane, 1999; Hollinger and Liang, 2001).

Treatment for chronic HBV is available, (see table 2.5) but when HBV and HIV co-exist, the treatment of chronic HBV poses specific problems and optimum treatment remains controversial (Nunez, 2003; AETC: http://aidsetc.org/aidsetc.org/aidsetc?page=cm-511_hepb). Chronic anti-HBV drugs show poorer performance, with lower response rates and
faster selection for HBV resistant strains in HBV/HIV co-infections while drugs active against both HBV and HIV if not properly used can lead to selection of resistance mutations in the HIV genome (Nunez et al., 2003).

Table 2.5: Hepatitis B treatment regimens in HIV co-infected patients

<table>
<thead>
<tr>
<th>Medication</th>
<th>Treatment Regimen</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon (INF)-alfa</td>
<td>5 million units (MU) daily or 10 MU 3 times weekly for 4-6 months*</td>
<td>Data on the effect against HBV/HIV co-infected patients is scarce. Interferon is contraindicated in patients with decompensated cirrhosis.</td>
</tr>
<tr>
<td>Pegylated interferon-alfa</td>
<td>180 micrograms per week for 4-6 months*</td>
<td></td>
</tr>
<tr>
<td>Lamivudine (3TC)#</td>
<td>150 mg twice daily or 300 mg daily (dosage as part of ART regimen) for 1 year or more*</td>
<td>Used only as part of an effective HIV ART regimen. High rate of HBV resistance occurs after 1-2 years of treatment. 3TC-resistant HBV is also resistant to emtricitabine. Most specialists recommend combination with a second agent (e.g., TDF or emtricitabine).</td>
</tr>
<tr>
<td>Tenofovir (TDF)#</td>
<td>300 mg daily: treatment duration unknown*</td>
<td>Used only as part of an effective HIV ART regimen. Active against lamivudine-resistant strains of HBV. Most specialists recommend combination with a second agent (e.g., lamivudine or emtricitabine).</td>
</tr>
<tr>
<td>Emtricitabine (FTC)# (NsRTI)</td>
<td>200 mg daily: treatment duration unknown*</td>
<td>Used only as part of an effective HIV ART regimen. FTC-resistant HBV is also resistant to 3TC. Most specialists recommend combination with a second agent (e.g., TDF or 3TC).</td>
</tr>
<tr>
<td>Adefovir (ADV), NtRTI</td>
<td>10 mg daily: treatment duration unknown*</td>
<td>Active against 3TC-resistant strains of HBV.</td>
</tr>
<tr>
<td>Entecavir (ETV) - Baraclude, NsRTI</td>
<td>0.5-1.0 mg daily: treatment duration unknown*</td>
<td>Active against 3TC-resistant strains of HBV. May have activity against HIV; pending further studies, should not be used in patients who are not on effective HIV ART regimen.</td>
</tr>
</tbody>
</table>

# Agents are active against both HIV and HBV
* The duration and expected efficacy of treatment vary according to the treatment strategy and the individual patient characteristics
(From AETC: http://aidsetc.org/aidsetc.org/aidsete?page=cm-511_hepb; Nunez et al., 2003)
The proper duration of therapy is not clear and the role of combination therapy has not been defined (AETC: http://aidsetc.org/aidsetc.org/aidsetc?page=cm-511_hepb).

The optimal time for initiating anti-HBV therapy in HBV/HIV co-infected patients who do not yet meet the criteria for HAART has neither been established nor is it clear which drugs should be used. Nunez et al. (2003) however suggest that all HIV infected persons with active HBV replication (HBsAg positive and HBV DNA detectable) should be considered for HBV treatment. Interferon-alfa (INF-alfa) is an option but standard INF-alfa has a low response and there is lack of data on the effectiveness of pegylated INF-alfa (polyethylene glycol added to the interferon through a process known as pegylation) in subjects co-infected with HIV (AETC: http://aidsetc.org/aidsetc.org/aidsetc?page=cm-511_hepb; Nunez et al., 2003).

The European guidelines for the management of Hepatitis B and C virus infections (2010) recommend adefovir monotherapy as an alternative option for HIV patients who do not meet the criteria for HAART. However Nunez et al. (2003) note the potential risk for selection of resistance mutations in HIV with long-term use of adefovir monotherapy in as much as the use of 3TC, FTC or TDF monotherapy should be avoided as these drugs favour the selection of HIV resistance mutations. Patients who meet the criteria for HAART should be given regimens containing nucleoside/nucleotide combinations such as 3TC, FTC and/or TDF to treat HBV and prevent HBV resistance (AETC: http://aidsetc.org/aidsetc.org/aidsetc?page=cm-511_hepb; Nunez et al., 2003).

There are other NsRTI drugs still in development such as 2,3-Dideoxy-2,3-didehydro-L-fluorocyti-dine (L-fD4C), telbivudine and clevudine which have anti-HBV activity and
amdoxovir which has anti-HIV/HBV activity but data on these drugs is still scarce (Nunez et al., 2003).

2.8 HBV drug resistance

Some HAART drugs targeting reverse transcription have activity against both HIV and HBV because both viruses involve reverse transcription in their replicative processes. The nucleotide analogue 3TC (see Fig 2.7) is a nucleoside reverse transcriptase inhibitor (NsRTI) with both anti-HIV and anti-HBV properties widely used for chronic hepatitis B treatment (Dienstag et al., 1995; Benhamou et al., 1996; Lai and Yuen, 2000; Nunez et al., 2003; Leung, 2004). The modified sugar attached to 3TC inhibits the reverse transcriptase polymerase by preventing DNA chain elongation and viral replication (Gitlin, 1997; Zoulim, 1999). However, 3TC antiviral effects are usually reversed over time due to the occurrence of viral drug resistance caused by mutations in the HBV genome.

![Fig 2.7: Structure of 3TC and TDF](image)

Lamivudine [(-)-b-L-2’-3’-dideoxy-3’-thiacytidine]  
Tenofovir [(2R)-1-(6-aminopurin-9-yl)propan-2-yl]
The nucleotide reverse transcriptase inhibitor (NtRTI) TDF (see Fig 2.7) is also used as a component of HAART regimens and like 3TC has both anti-HIV and anti-HBV activity. TDF has been shown to be a potent inhibitor of HBV replication \textit{in vitro} even in the presence of 3TC resistance mutations (Nunez \textit{et al.}, 2003; Lada \textit{et al.}, 2004).

Drug resistance is conferred by the appearance of one or several mutations within the HBV polymerase gene which confer to the mutant viral population a phenotypic advantage over the wild-type pre-therapeutic viral quasi-species, as they induce a reduction of drug susceptibility of mutant strains in vivo (Durantel \textit{et al.}, 2005). As shown in Fig 2.8, resistance develops as a function of factors related to the patient, the virus and the drug.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{d028.png}
\caption{Factors affecting the intrinsic potency/durability of HAART regimens (Vezinet, 2004)}
\end{figure}

Some factors that influence the speed of emergence for drug resistance on therapy are: level of residual viral replication, genetic barrier for a given drug in the regimen, degree of selective pressure exerted by a given drug and magnitude of resistance conferred by a given
mutation or mutations *i.e.* degree of growth advantage over wild-type under drug pressure (Ruiz, 2004). The genetic barrier (number of mutations required for resistance to occur) is important in maintaining the potency of a drug (Vezinet, 2004). Other factors contributing to emergence of drug resistance in HAART regimens are: viral population size, level of viral replication (low or high generation time) and an error-prone reverse transcriptase with high mutation rates (Ruiz, 2004).

HBV has a high generation time with as many as $1 \times 10^{12}$ to $1 \times 10^{13}$ virions or complete HBV viruses produced per day in infected individuals (Nowak and Bonhoeffer, 1996). In addition HBV replicates via reverse transcription and the polymerase protein lacks a “proof reading” function (Bartholomeusz and Locarnini, 2006). Antiviral drug resistant mutants on the other hand emerge as a function of the following factors: viral mutation frequency, the intrinsic mutability of the antiviral target site, the selective pressure exerted by the drug, the magnitude and rate of virus replication, the overall replication fitness of the mutant and the availability of replication space (Bartholomeusz and Locarnini, 2006). Viral persistence is due to the long half-life of hepatocytes because of a defective immune response against infected cells, and the persistence of viral cccDNA in infected cells (Zoulim, 2004).

**2.9 Drug resistance mechanisms**

The mechanisms of nucleoside/nucleotide analogue resistance include steric hindrance, sub-optimal nucleophilic attack geometry and pyrophosphorolysis.

Due to the overlap of the reading frames of the HBV polymerase (Pol) with the frame-shifted HBsAg (Poch *et al.*, 2004) drug resistant mutations in the HBV Pol can directly impact on the nature of HBsAg and its function, including properties of viral neutralization. Mutations
affecting the YMDD locus typically alter the ability of the dNTP binding pocket to accommodate the nucleoside/nucleotide analogue thus the primary mechanism of resistance is by steric hindrance within the dNTP binding pocket (Sarafianos et al., 1999; Gao et al., 2000), illustrated by Clavel and Hance (2004) in Fig 2.9.

**Fig 2.9:** Resistance caused by mutation in the HBV genome as a result of interference with the incorporation of a nucleoside analogue (From Clavel and Hance, 2004)

In sub-optimal nucleophilic attack geometry the catalytic efficiency of the enzyme is adversely affected and since the catalysis of the incoming dNTP to the elongating DNA strand is dependent on a precise spatial arrangement of the 5'-phosphate and the 3'-hydroxyl group, alterations in the geometry of the reaction constituents can greatly diminish the efficiency of catalysis of the nucleoside/nucleotide analogue (Doo and Liang, 2001).
The other resistance mechanism is due to ATP mediated excision of the nucleoside analogue, *i.e.* pyrophosphorolysis or pyrophosphate exchange (Illustrated by Clavel and Hance in Fig 2.10) (Arion *et al.*, 1998; Meyer *et al.*, 1999; Urban *et al.*, 2001).

**Fig 2.10** Resistance by ATP-mediated excision of the nucleoside analogue (From Clavel and Hance, 2004)
2.10 HBV polymerase resistance mutations

Detectable HBV viremia in patients who have already received 3TC as part of HAART usually indicates resistance of HBV to the drug (Nunez et al., 2003). Drug resistance to 3TC is associated with mutations in the conserved catalytic polymerase/reverse transcriptase domain of the gene (Zoulin, 2002), located at the YMDD motif (Doo and Liang, 2001). Genotypic resistance to 3TC occurs in approximately one quarter of patients after one year of treatment (Lai et al., 1998). The rate increases to 40% after two years and to as much as 50% and 70% after 3 and 4 years respectively (Lai et al., 1998). The drug resistance is associated with mutations in the tyrosine-methionine-aspartate-aspartate (YMDD) motif largely due to the pressure exerted by long term therapy in chronic hepatitis patients (Zoulim, 2001; Doo and Liang, 2001).

Figure 2.11 shows some HBV polymerase resistance mutations. The reverse transcription region (RT), responsible for RNA- and DNA dependent DNA synthesis contains seven motifs that are labeled A to G in Fig 2.11. Mutations to 3TC are shown to occur at B domain position rtL180M (Substitution of leucine for methionone at position 180 in the reverse transcriptase polypeptide chain) and at C domain position rtM204V/I while mutation to TDF is shown in the D domain at position rtN236T (Sablon and Sapiro, 2005). The rtL180M, rtM204V/I and rtN236T mutations at the YMDD motif have also been associated with patients co-infected with HBV and HIV (Shaw et al., 2006). Amini-Bavil-Olyaee et al. (2009) report an rtA194T polymerase mutation in HBV/HIV coinfected patients during TDF treatment that may be associated with TDF resistance. Further, rtM550V and the rtL526M variant mutations have been cited in HIV-HBV co-infected patients (Dore et al., 1999; Thibault et al., 1999; Lai and Yuen, 2000; Leung, 2004). The double resistant mutation rtM550V and rtL526M wasdisplayed in more than 90% of HIV-HBV co-infected patients
under 3TC therapy and is associated with prolonged 3TC treatment (Thibault et al., 1999; Yeh et al., 2000). ADV, ETV and ADV/3TC mutation positions are also shown in Fig 2.11.

![Fig 2.11: Key HBV polymerase resistance mutations (Lorcanini, 2008)](image)

**2.11 Monitoring HBV drug resistance**

Genotypic methods of monitoring HBV drug resistance include direct DNA Sequencing (Aberle et al., 2001; Clarke and Bloor, 2002), Clonal Analysis (Stuyver et al., 2000; Zoulim, 2002), Restriction Fragment Length Polymorphism - RFLP (Chayama et al., 1998; Allen et al., 1999; Jardi et al., 1999; Niesters et al., 2002), DNA hybridization (Cane et al., 1999; Aberle et al, 2001; Stuyver et al., 2001; Whalley et al., 2001; Punia et al., 2004) and the indirect method of viral load assays. The various genotypic methods of monitoring HBV drug resistance have their limitations, for example, direct DNA sequencing may fail to detect viral resistance in populations with the mutated virus making a large fraction of up to 30% of the entire HBV population (Aberle et al, 2001).
CHAPTER 3: MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Study design

The project was part of the larger retrospective HBV sub study investigating the effect of TDF/3TC combination therapy on chronic HBV infection in HIV-1 co-infected patients enrolled in the DART study. This project investigated baseline HBV serology patterns and the prevalence of HBV co-infection in this cohort. This information was used to identify samples that will be followed up in the HBV sub study to investigate the efficacy of TDF/3TC in suppressing HBV replication in HIV-1 positive individuals initiating TDF/3TC combination therapy.

**Fig 3.1:** Relationship of project to the HBV sub study and the DART cohort.
3.2 Samples
Clinical samples were obtained from archived (at -80 °C) baseline samples from the DART study of HIV-1 positive patients initiating TDF/3TC combination therapy as part of HAART. Demographic data on sex and age at baseline of trial participants was obtained from the DART study data base. The samples had been twice screened for HBsAg. A total of 957 baseline samples were investigated. All patients were treatment naïve and had advanced acquired immuno deficiency syndrome (AIDS) with a CD4 count of less than 200.

3.3. Ethics and consent
The main HBV sub study protocol was submitted for ethical review and has Medical Research Council of Zimbabwe (MRCZ) and DART virology sub-committee approval.

3.4 Serology assays
Serology tests were done using the Abbott Murex kits (Murex Biotech Limited, UK) and the DiaSorin kits (DiaSorin S.p.A., Italy) per the kit manufacturer’s instructions using the test plan below (Fig. 3.2). The kits used were Abbott Murex HBsAg, Abbott Murex HBsAg confirmatory, Abbott Murex anti-HBc total, Abbott Murex anti-HBs tests and DiaSorin ETI – EBK PLUS and ETI – AB – EBK PLUS for HBeAg and anti-HBe tests respectively. The tests are enzyme immunoassays for the detection of HBV markers in human serum or plasma. Assay reagents, stored at 2 - 8 °C, and samples were brought to room temperature before use.
3.4.1 Confirmation of HBsAg positivity

Samples that were positive in both HBsAg screening tests with the Murex HBsAg version 3 assay were confirmed using the Murex HBsAg confirmatory version 3 assay (n = 225). A 96 well microplate coated with mouse monoclonal antibody to HBsAg was used. Two assay wells were assigned for each test sample, a control well and a specific well. The plate layout was as shown in fig 3.3.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>PC</td>
<td>PC</td>
<td>S07</td>
<td>S07</td>
<td>S15</td>
<td>S15</td>
<td>S23</td>
<td>S23</td>
<td>S31</td>
<td>S31</td>
<td>S39</td>
<td>S39</td>
</tr>
<tr>
<td>B</td>
<td>NC</td>
<td>NC</td>
<td>S08</td>
<td>S08</td>
<td>S16</td>
<td>S16</td>
<td>S24</td>
<td>S24</td>
<td>S32</td>
<td>S32</td>
<td>S40</td>
<td>S40</td>
</tr>
<tr>
<td>C</td>
<td>S01</td>
<td>S01</td>
<td>S09</td>
<td>S09</td>
<td>S17</td>
<td>S17</td>
<td>S25</td>
<td>S25</td>
<td>S33</td>
<td>S33</td>
<td>S41</td>
<td>S41</td>
</tr>
<tr>
<td>D</td>
<td>S02</td>
<td>S02</td>
<td>S10</td>
<td>S10</td>
<td>S18</td>
<td>S18</td>
<td>S26</td>
<td>S26</td>
<td>S34</td>
<td>S34</td>
<td>S42</td>
<td>S42</td>
</tr>
<tr>
<td>E</td>
<td>S03</td>
<td>S03</td>
<td>S11</td>
<td>S11</td>
<td>S19</td>
<td>S19</td>
<td>S27</td>
<td>S27</td>
<td>S35</td>
<td>S35</td>
<td>S43</td>
<td>S43</td>
</tr>
<tr>
<td>F</td>
<td>S04</td>
<td>S04</td>
<td>S12</td>
<td>S12</td>
<td>S20</td>
<td>S20</td>
<td>S28</td>
<td>S28</td>
<td>S36</td>
<td>S36</td>
<td>S44</td>
<td>S44</td>
</tr>
<tr>
<td>G</td>
<td>S05</td>
<td>S05</td>
<td>S13</td>
<td>S13</td>
<td>S21</td>
<td>S21</td>
<td>S29</td>
<td>S29</td>
<td>S37</td>
<td>S37</td>
<td>S45</td>
<td>S45</td>
</tr>
<tr>
<td>H</td>
<td>S06</td>
<td>S06</td>
<td>S14</td>
<td>S14</td>
<td>S22</td>
<td>S22</td>
<td>S30</td>
<td>S30</td>
<td>S38</td>
<td>S38</td>
<td>S46</td>
<td>S46</td>
</tr>
</tbody>
</table>

Fig 3.3: Plate layout for the HBsAg confirmatory test.
Twenty five microlitres (25μl) of control reagent (buffer solution) or specific reagent (buffer containing horse anti-HBs) were added to the required respective wells followed by addition of 75μl of Murex HBsAg negative control (NC) and positive control (PC) and test samples (S). The plate was gently mixed by tapping the side, covered with a lid and incubated for 30 minutes at 37°C, following which 50μl of conjugate (horseradish-peroxidase labelled goat antibody to HBsAg) was added to each well. The plate was gently mixed by tapping the side, covered with a lid and incubated at 37 °C for another 30 minutes after which the plate was washed 5 times with an automated microplate Biotek ELx50 stripwasher using working strength (1:20 dilution) glycine/borate wash solution. The plate was then inverted and tapped onto absorbent paper before addition of 100μl substrate solution (3,3’,5,5’-tetramethylbenzidine - TMB) to each well, following which the plate was covered with a lid and incubated at 37 °C for 30 minutes. After the incubation, 50μl of stop solution (0.5M – 2M sulphuric acid) was added to each well and absorbance for each well read at 450nm within 15 minutes using a Biotek ELx808 microplate reader.

The cut off value was calculated as the mean absorbance of negative controls + 0.05

The inhibition of reactive samples with specific reagent was calculated using the following formula:

\[
\frac{[(SC – NC) – (SS – NS)] \times 100}{(SC – NC)}
\]

Where:

SC = absorbance of sample with control reagent
NC = absorbance of negative control with control reagent
SS = absorbance of sample with specific reagent
NS = absorbance of negative control with specific reagent
A sample was confirmed reactive if the absorbance with the control reagent was equal to or greater than the cut-off value and the inhibition by specific reagent was equal to or greater than 50%. If absorbance with the control reagent was less than the cut off value for a sample tested undiluted, the result was considered to be indeterminate. Providing the absorbance in the control well is less than 2.0, samples giving less than 50% inhibition by the specific reagent are considered negative and therefore false reactive in Murex HBsAg Version 3. For the assay to be valid it had to meet the manufacturer’s quality control requirements in terms of negative and positive controls range values (Appendix 1).

### 3.4.2 Anti-HBc (total) detection

All samples (n = 957) were tested for presence of antibodies to HBc antigen. To each required well of a 96 well microplate coated with recombinant HBcAg was added 50μl of sample diluent (buffer solution) followed by 50μl of sample or control as illustrated in fig 3.4

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NC</td>
<td>S05</td>
<td>S13</td>
<td>S21</td>
<td>S29</td>
<td>S37</td>
<td>S45</td>
<td>S53</td>
<td>S61</td>
<td>S69</td>
<td>S77</td>
<td>S85</td>
</tr>
<tr>
<td>B</td>
<td>NC</td>
<td>S06</td>
<td>S14</td>
<td>S22</td>
<td>S30</td>
<td>S38</td>
<td>S46</td>
<td>S54</td>
<td>S62</td>
<td>S70</td>
<td>S78</td>
<td>S86</td>
</tr>
<tr>
<td>C</td>
<td>PC</td>
<td>S07</td>
<td>S15</td>
<td>S23</td>
<td>S31</td>
<td>S39</td>
<td>S47</td>
<td>S55</td>
<td>S63</td>
<td>S71</td>
<td>S79</td>
<td>S87</td>
</tr>
<tr>
<td>D</td>
<td>PC</td>
<td>S08</td>
<td>S16</td>
<td>S24</td>
<td>S32</td>
<td>S40</td>
<td>S48</td>
<td>S56</td>
<td>S64</td>
<td>S72</td>
<td>S80</td>
<td>S88</td>
</tr>
<tr>
<td>E</td>
<td>S01</td>
<td>S09</td>
<td>S17</td>
<td>S25</td>
<td>S33</td>
<td>S41</td>
<td>S49</td>
<td>S57</td>
<td>S65</td>
<td>S73</td>
<td>S81</td>
<td>S89</td>
</tr>
<tr>
<td>F</td>
<td>S02</td>
<td>S10</td>
<td>S18</td>
<td>S26</td>
<td>S34</td>
<td>S42</td>
<td>S50</td>
<td>S58</td>
<td>S66</td>
<td>S74</td>
<td>S82</td>
<td>S90</td>
</tr>
<tr>
<td>G</td>
<td>S03</td>
<td>S11</td>
<td>S19</td>
<td>S27</td>
<td>S35</td>
<td>S43</td>
<td>S51</td>
<td>S59</td>
<td>S67</td>
<td>S75</td>
<td>S83</td>
<td>S91</td>
</tr>
<tr>
<td>H</td>
<td>S04</td>
<td>S12</td>
<td>S20</td>
<td>S28</td>
<td>S36</td>
<td>S44</td>
<td>S52</td>
<td>S60</td>
<td>S68</td>
<td>S76</td>
<td>S84</td>
<td>S92</td>
</tr>
</tbody>
</table>

**Fig 3.4:** Plate layout for the anti-HBc test

The wells were covered with a lid and then incubated for 30 minutes at 37 °C, following which the plate was washed 5 times with an automated microplate Biotek ELx50 stripwasher using working strength glycine/borate wash solution. The plate was then inverted and tapped onto absorbent paper before addition of 50μl of conjugate (monoclonal anti-HBc conjugated to horseradish peroxidase) and incubated for 30 minutes at 37 °C. After the incubation, the
plate was washed as before and 100μl of substrate solution (TMB) was added to each well. The plate was then covered with a lid and incubated for 30 minutes at 37 °C away from direct sunlight, following which 50μl of stop solution (0.5M – 2M sulphuric acid) was added to each well and absorbance for each well read at 450nm within 15 minutes using a Biotek ELx808 microplate reader.

The cut off value was calculated by adding the mean absorbance of the positive control to the mean absorbance of the negative control and then dividing the result by 2. Samples giving an absorbance greater than the cut-off value were considered non-reactive in the assay. Samples giving absorbance less than the cut-off value were considered reactive in Murex anti-HBc (total). For the assay to be valid it had to meet the kit manufacturer’s quality control requirements in terms of negative and positive controls range values.

### 3.4.3 Anti-HBs detection

Samples found to be HBsAg negative but anti-HBc (total) reactive (n = 407) were tested for the presence of anti-HBs. To each required well of a 96 well microplate coated with inactivated human HBsAg, ad and ay subtypes, was added 25μl of assay diluent (buffer solution) followed by 75μl of negative control, 10mlU/ml calibrator – C1 (10mlU anti-HBs/ml) and 100mlU/ml calibrator – C2 (100mlU anti-HBs/ml) and samples as illustrated in fig 3.5. The wells were covered with a lid and incubated for 60 minutes at 37 °C under humid conditions following which the plate was washed 5 times with an automated microplate Biotek ELx50 stripwasher using working strength glycine/borate wash solution. The plate was then inverted and tapped onto absorbent paper before addition of 50μl of conjugate (inactivated human HBsAg, ad and ay subtypes, conjugated to horseradish peroxidase) to each well.
The plate was covered with a lid and incubated for another 60 minutes at 37 °C under humid conditions and then washed as before. After washing, 100μl of substrate solution (TMB) was added to each well. The wells were covered with a lid and incubated for 30 minutes at 37 °C under humid conditions away from direct sunlight, following which 50μl of stop solution (0.5M – 2M sulphuric acid) was added to each well and absorbance for each well read at 450nm within 15 minutes using a Biotek ELx808 microplate reader.

Samples giving an absorbance less than the mean absorbance of the 10mIU/ml calibrator were considered non reactive in Murex anti-HBs. Samples giving an absorbance equal to or greater than the mean absorbance of the 10mIU/ml calibrator were considered reactive. For the assay to be valid it had to meet the kit manufacturer’s quality control requirements in terms of negative control and calibrator range values.

### 3.4.4 HBeAg and anti-HBe detection

Samples found to be HBsAg positive or HBsAg indeterminate and HBc (total) reactive (n = 133) were tested for the presence of HBeAg and anti-HBe.
3.4.4.1 HBeAg detection

To each required well except the blank (BL) well of a 96 well microplate coated with mouse monoclonal antibody to HBeAg was added 50μl incubation buffer followed by dispensing of 100μl calibrator (C), negative and positive controls and samples into their respective wells as shown in fig 3.6.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>BL</td>
<td>S03</td>
<td>S11</td>
<td>S19</td>
<td>S27</td>
<td>S35</td>
<td>S43</td>
<td>S51</td>
<td>S59</td>
<td>S67</td>
<td>S75</td>
<td>S83</td>
</tr>
<tr>
<td>B</td>
<td>C</td>
<td>S04</td>
<td>S12</td>
<td>S20</td>
<td>S28</td>
<td>S36</td>
<td>S44</td>
<td>S52</td>
<td>S60</td>
<td>S68</td>
<td>S76</td>
<td>S84</td>
</tr>
<tr>
<td>C</td>
<td>C</td>
<td>S05</td>
<td>S13</td>
<td>S21</td>
<td>S29</td>
<td>S37</td>
<td>S45</td>
<td>S53</td>
<td>S61</td>
<td>S69</td>
<td>S77</td>
<td>S85</td>
</tr>
<tr>
<td>D</td>
<td>C</td>
<td>S06</td>
<td>S14</td>
<td>S22</td>
<td>S30</td>
<td>S38</td>
<td>S46</td>
<td>S54</td>
<td>S62</td>
<td>S70</td>
<td>S78</td>
<td>S86</td>
</tr>
<tr>
<td>E</td>
<td>NC</td>
<td>S07</td>
<td>S15</td>
<td>S23</td>
<td>S31</td>
<td>S39</td>
<td>S47</td>
<td>S55</td>
<td>S63</td>
<td>S71</td>
<td>S79</td>
<td>S87</td>
</tr>
<tr>
<td>F</td>
<td>PC</td>
<td>S08</td>
<td>S16</td>
<td>S24</td>
<td>S32</td>
<td>S40</td>
<td>S48</td>
<td>S56</td>
<td>S64</td>
<td>S72</td>
<td>S80</td>
<td>S88</td>
</tr>
<tr>
<td>G</td>
<td>S01</td>
<td>S09</td>
<td>S17</td>
<td>S25</td>
<td>S33</td>
<td>S41</td>
<td>S49</td>
<td>S57</td>
<td>S65</td>
<td>S73</td>
<td>S81</td>
<td>S89</td>
</tr>
<tr>
<td>H</td>
<td>S02</td>
<td>S10</td>
<td>S18</td>
<td>S26</td>
<td>S34</td>
<td>S42</td>
<td>S50</td>
<td>S58</td>
<td>S66</td>
<td>S74</td>
<td>S82</td>
<td>S90</td>
</tr>
</tbody>
</table>

Fig 3.6: Plate layout for the HBeAg/anti-HBe tests

A cardboard sealer was applied before the plate was gently tapped and then incubated for 2 hours at 37 °C under humid conditions. After incubation, the plate was washed 5 times with an automated microplate Biotek ELx50 stripwasher using working strength glycine/borate wash solution. The plate was then inverted and tapped onto absorbent paper before addition of 100μl enzyme tracer (mouse monoclonal antibody to HBeAg conjugated to horseradish peroxidase) diluted 1:50 with tracer diluent (buffer solution) to each well except for the blank well. Another 1 hour incubation at 37 °C under humid conditions followed by a wash step as before was done after which 100μl chromogen/substrate solution (TMB/Hydrogen peroxide system) was added into all wells. The plate was then incubated for 30 minutes at room temperature away from direct light, following which 100μl stop solution (0.4N sulphuric acid) was added into all wells and absorbance for each well read at 450nm within 1 hour using a Biotek ELx808 microplate reader.
The cut-off value was determined by adding 0.060 to the mean absorbance of the calibrator values after subtraction of the substrate blank. Samples with absorbance values above the cut-off value were considered reactive for HBeAg and samples with absorbance values below the cut-off value were considered non reactive for HBeAg. For the assay to be valid it had to meet the kit manufacturer’s quality control requirements in terms of the blank, negative and positive controls and calibrator range values.

3.4.4.2 Anti-HBe detection

To each required well except the blank (BL) well of a 96 well microplate coated with mouse monoclonal antibody to HBeAg was added 50μl incubation buffer followed by dispensing of 50μl calibrator (C), negative and positive controls and samples into their respective wells as shown in fig 3.5. Fifty microlitres (50μl) of neutralizing solution (containing recombinant HBeAg) was then added into all wells except for the blank well. A cardboard sealer was applied before the plate was gently tapped and then incubated for 2 hours at 37 °C under humid conditions. After incubation the plate was washed 5 times with an automated microplate Biotek ELx50 stripwasher using working strength glycine/borate wash solution. The plate was then inverted and tapped onto absorbent paper before addition of 100μl enzyme tracer (mouse monoclonal antibody to HBeAg conjugated to horseradish peroxidase) diluted 1:50 with tracer diluent (buffer solution) to each well except for the blank well. Another 1 hour incubation at 37 °C under humid conditions followed by a wash step as before was done after which 100μl chromogen/substrate solution (TMB/Hydrogen peroxide system) was added into all wells. The plate was then incubated for 30 minutes at room temperature away from direct light, following which 100μl stop solution (0.4N sulphuric acid) was added into all wells and absorbance for each well read at 450nm within 1 hour using a Biotek ELx808 microplate reader.
The cut-off value was determined by multiplying the mean absorbance of the calibrator values by 0.500 after subtraction of the substrate blank. Samples with absorbance values above the cut-off value were considered non reactive for anti-HBe and samples with absorbance values below the cut-off value were considered reactive for anti-HBe. For the assay to be valid it had to meet the kit manufacturer’s quality control requirements in terms of the blank, negative and positive controls and calibrator range values.

3.6 Statistical analysis

Demographic descriptive statistics was used to analyse the results. The Z-test for difference in proportions was used to compare prevalence data between gender and between age groups.
CHAPTER 4: RESULTS

4. RESULTS

4.1 Demographic data

Demographic data on sex and age at baseline of participants is shown in Fig 4.1.

A total of 957 participants were tested and all participants were HIV positive. There were more females (57.7 %) than males (42.3 %). The majority of participants were in the 30-34 (23.6 %) and 35-39 (26 %) age group.

Fig 4.1: Gender ratios and age groups at Baseline
4.2 HBV markers investigated

Figure 4.2 shows the serology markers tested for and the number of positives for each marker.

![Bar chart showing HBV serology markers](image)

**Fig 4.2:** Comparison of numbers obtained for HBV serology markers tested.

4.2 HBsAg sero prevalence

One hundred and sixty four (164) subjects out of 957 were confirmed HBsAg positive giving a 17.1 % HBsAg sero prevalence in the cohort (Males 18.3 %, Females 15.8 %). The prevalence in different age groups at baseline and distribution by gender is shown in Fig 4.3. The HBsAg confirmatory test could not be done on 2 samples which had insufficient volumes.
**Fig 4.3**: Gender ratios and age groups for HBsAg positive patients.

HBsAg sero prevalence by age group and by gender in the age groups is shown in Table 4.1.

**Table 4.1**: Comparison of HIV prevalence and HBV prevalence in the different age groups.

<table>
<thead>
<tr>
<th>Age group</th>
<th>HIV prevalence (%)</th>
<th>HBV prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Male</td>
</tr>
<tr>
<td>18-29 years</td>
<td>12.3</td>
<td>7.9</td>
</tr>
<tr>
<td>30-34 years</td>
<td>23.6</td>
<td>18.3</td>
</tr>
<tr>
<td>35-39 years</td>
<td>26.0</td>
<td>28.4</td>
</tr>
<tr>
<td>40-44 years</td>
<td>19.6</td>
<td>23.0</td>
</tr>
<tr>
<td>45-49 years</td>
<td>10.5</td>
<td>13.3</td>
</tr>
<tr>
<td>50 years and over</td>
<td>6.8</td>
<td>9.1</td>
</tr>
</tbody>
</table>
4.3 Level of HBV exposure (Anti-HBc prevalence)

Out of the 957 samples tested, 530 samples (55.4 %) were positive for the anti-HBc marker.

4.4 combined anti-HBc/HBsAg seroprevalence

A total of 694 samples were anti-HBc or HBsAg positive, an HBV markers sero prevalence of 72.5 %.

4.5 Serology profiles and clinical stages of infection

The testing plan produced five sets of serology marker profiles as shown in Table 4.1. The table also shows the possible stage of HBV infection for individuals in each of the exhibited profiles.

Table 4.2 HBV serology marker profiles and most likely stage of infection

<table>
<thead>
<tr>
<th>HBV Serology profiles</th>
<th>Stage of HBV infection</th>
<th>Number of subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-HBc + HBsAg -</td>
<td>Anti-HBs + -</td>
<td>Resolved</td>
</tr>
<tr>
<td>Anti-HBc + HBsAg +</td>
<td>HBsAg + HBeAg + Anti-HBe -</td>
<td>Active</td>
</tr>
<tr>
<td>Anti-HBc + HBsAg +</td>
<td>HBeAg - Anti-HBe +</td>
<td>Active resolving</td>
</tr>
<tr>
<td>Anti-HBc + HBsAg +</td>
<td>HBeAg - Anti-HBe -</td>
<td>Acute resolving or chronic inactive carrier</td>
</tr>
<tr>
<td>Anti-HBc + HBsAg -</td>
<td>Anti-HBs -</td>
<td>Isolated anti-HBc, unclassified</td>
</tr>
</tbody>
</table>
4.5.1 Anti-HBc/HBsAg/anti-HBs markers profile
Four hundred and eight (408) samples were anti-HBc+/HBsAg- and were further tested for anti-HBs antibody. Out of the 408 samples 135 had isolated anti-HBc antibody, i.e. only anti-HBc positive and will be tested for occult HBV (HBV DNA in HBsAg negative individuals), 232 were anti-HBs + and classified as having resolved HBV. The anti-HBs test was not done on 41 samples which had insufficient sample volume at the time of testing.

4.5.2 Anti-HBc/HBsAg/HBe markers profile
One hundred and twenty three (123) samples were anti-HBc+/HBsAg + and were further tested for HBe markers to investigate active HBV replication. Out of the 123 samples 45 were HBeAg+ and 58 were anti-HBe +. The combined 103 samples were identified for viral load testing. Twenty (20) samples were negative for HBe markers.

4.6 Identification of samples for viral load test
A total of 238 samples were identified for HBV viral load tests on baseline and follow up samples to investigate HBV TDF/3TC resistance, evidenced by viral rebound, in patients initiating TDF/3TC combination therapy. The samples were chosen on the basis of having positive HBe markers and on possibility of occult HBV infection. Of these samples 135 had isolated anti-HBc and will be tested for occult HBV, 45 had positive HBeAg and 58 had positive anti-HBe.
4.7 HBV serology markers in Hepatitis C Virus (HCV) co-infected patients

Fig 4.3 shows the distribution of HBV serology markers in 19 patients co-infected with HCV.

Fig 4.4: HBV infection in HCV co-infected patients.
This is a study looking at the prevalence of hepatitis in the DART cohort of HIV infected patient. We tested for the different antigens and antibodies that determine the hepatitis status. Screening tests for HBsAg screening tests showed a seroprevalence of 23.5% with 59 HBsAg false positives. The confirmatory test showed HBsAg seroprevalence was 17.1% in this DART cohort of HIV-1 infected individuals. The 17.1% seroprevalence is within the HBsAg positivity prevalence in HIV infected individuals range of 9% - 100% found in other parts of Africa (Ejela et al., 2004; Mulders et al, 2004; Rouet et al, 2004). The specific antibody used in the confirmatory reagent is derived from horse serum in contrast to the goat and mouse antibodies used in the HBsAg screening test. This substitution of the specific antibody minimizes the risk of confirming false positive samples containing anti-species antibodies and use of the HBsAg confirmatory test was helpful in confirming the validity of HBsAg test results. The classification of the HBV infection, acute or chronic, could not be determined as HBsAg was not tested for in follow up samples at this stage. Chronic HBV is defined as persistent HBsAg seropositivity for at least six months (Sterling, 2003).

The 17.1% HBsAg seroprevalence in the DART cohort was higher than the 15.4% HBsAg seroprevalence reported by Tswana et al, (1996) in the general population in Zimbabwe. The difference in these proportions was not significant at 5% significance level (z_{cal} < 1.96). This should not be taken to mean that the prevalence of HBV in HIV positive individuals is the same as in the general population because figures in the general population are likely to have gone down in the last 15 years. Rates of HBV in the general population have fallen worldwide because of HBV vaccination (Murray et al, 2005). The HBsAg seroprevalence was 18.3% in males and was 15.8% in females. There was insufficient evidence at 5%
significance level ($z = 0.008$) that there is a difference in the proportions of males and females infected with HBV in this population. There also was no difference in gender proportions of HBV positive individuals in this population compared to those studied by Tswana et al (1996) (males, $z = 0.005$; females, $z = 0.007$). In our population the difference in proportions of HBV positive individuals was consistently not significant between sexes in all age groups except for the 18 – 29 age group (males, 32.3%; females 13.5%; $z = 2.00$).

The anti-HBc marker was positive in 55.4% of individuals in this population. This marker indicates that the individual has at some point been exposed to HBV. Anti-HBc seroprevalence (55.4%) was much higher than HBsAg seroprevalence (17.1%) indicating high levels of exposure to HBV in this cohort. Santos et al. (2004) also reported a higher anti-HBc seroprevalence (68%) compared to HBsAg seroprevalence (8%). In Zimbabwe, Tswana et al. (1996) reported HBsAg seroprevalence of 15.4% and anti-HBc seroprevalence of 30%. This is because anti-HBc titres increase rapidly after HBV infection but persist for many years at low titres after infection resolution and clearance of HBsAg.

The combined HBsAg and anti-HBc seroprevalence was 72.5% confirming HIV positive individuals as a high HBV exposure group and therefore at risk of co-infection with HBV. The Murex anti-HBc (total) test used could not classify positive anti-HBc individuals as acute or chronic infections because it detects combined levels of both IgG and IgM, i.e., total anti-HBc.

Isolated anti-HBc was seen in 14.1% (135 out of 408) of individuals tested for anti-HBs. Other studies have recorded 14% - 17% of HIV-positive individuals to have this serological profile (Piroth et al., 2002; Neau et al., 2004; Shire et al., 2004; Neau et al., 2005; Osborn et
al., 2007). Other studies have linked isolated anti-HBc to underlying HCV co-infection (Jilg et al., 1995; Piroth et al., 2002; Gandhi et al., 2003; Neau et al., 2004) while other studies have not confirmed the relationship between HCV and isolated anti-HBc (Davaro et al., 1996; Noborg et al., 2000). In this study, 2 out of 19 (10.5 %) HCV positive patients had isolated anti-HBc. Other explanations of isolated anti-HBc have been proposed, including early window phase before appearance of HBsAg or late acute window after disappearance of HBsAg (Kleinman and Busch, 2006), genetic variations in the S gene (Carman and Thomas 1992; Zuckerman, 2000), presence of immune complexes in which HBsAg may be hidden (Liang et al., 1990; Ackerman et al., 1994), poor laboratory detection due to low level of HBsAg and immunosuppression (Grob et al., 2000; Hu, 2002). In addition Mphahlele et al. (2006) have suggested that HIV infection may be a risk factor for occult hepatitis B.

The 135 baseline samples with isolated anti-HBc will be tested for HBV viral load to determine prevalence of occult HBV in this cohort and to investigate HBV viral rebound in follow up samples as evidence of HBV TDF/3TC resistance. The negative anti-HBs result in combination with positive anti-HBc in these samples indicates that HBV resolution stage has not been reached and so the samples may have detectable HBV DNA levels. There is evidence that HBV transmission by HBsAg negative material occurs (Allain, 2004) which confirms the presence of hepatitis B virus in such samples.

HBe markers were present in 103 samples and these samples were identified for viral load testing in the HBV substudy to investigate HBV TDF/3TC resistance in patients initiating TDF/3TC combination therapy. Forty five samples were HBeAg positive and 58 were anti-HBe positive. The HBeAg marker is associated with the active replication and high infectivity stage of HBV and these samples are expected to have detectable HBV DNA.
levels. The presence of anti-HBe indicates low infectivity but detectable levels of HBV DNA are expected in these samples as well. A positive anti-HBe result is associated with recovery especially if HBsAg and HBeAg are no longer detectable in the serum. Since all anti-HBe positive samples to be tested for viral load also have positive HBsAg, they are likely to have detectable HBV DNA.

The strengths of this study are its large size and detailed HBV serology results. However an important limitation is that it was a retrospective study with a selected population which means the prevalence of HBV in this cohort cannot be extrapolated to the general population of Zimbabwe. Another limitation is that HBV DNA; an important HBV marker could not be tested at this stage because of timelines and costs. In addition the study only investigated baseline serology patterns and was not able to show how the serology patterns change with intervention.
CHAPTER 6: CONCLUSION AND RECOMMENDATIONS

There was high prevalence of HBV co-infection in HIV infected individuals in the DART cohort (17.1 % HBsAg, 55.4 % anti-HBc and 72.5 % HBsAg and/or anti-HBc seroprevalence). HIV positive individuals are at risk of co-infection with HBV because of the shared routes of infection of HIV and HBV. This is supported by the high level of exposure to HBV evidenced by 55.4 % anti-HBc seroprevalence in the DART cohort compared to 30 % in the general population (Tswana et al., 1996). There is need to investigate the current prevalence of HBV in the general population as it is likely to have changed in the past 15 years. This data can then be used to determine if there is a difference in the prevalence of HBV in HIV positive individuals compared to the general population as the current data available is from different time periods.

There was evidence of active hepatitis, 103 individuals with positive HBe markers, and HBV DNA tests can be done on these baseline and follow up samples to monitor the efficacy of TDF/3TC combination therapy in suppressing viral rebound. Viral load tests can also be done on the 135 samples with isolated anti-HBc to determine the prevalence of occult HBV in this cohort.

Since this study did not show the effect of intervention on the observed HBV serology profiles, an analysis of serology markers in follow up samples is recommended. This information will be important in showing the efficacy of TDF/3TC combination therapy in HBV treatment measured by the time required from initiation to clearance of HBV serology markers. Testing for serology markers in follow up samples will also provide information on the class of HBV infections, acute or chronic, in this cohort.
REFERENCES


http://www.cdc.gov/ncidod/diseases/hepatitis/b/hebqafn.htm


Wilkins 2923-2969.


Liaw YF. 2009. HBeAg seroconversion as an important end point in the treatment of chronic hepatitis B. Hepatol Int. 3:425-433.


O’Shea RS. 2009. Liver disease related to hepatitis B remains an important public health concern and a major cause of morbidity and mortality. It also presents a common challenging problem for practicing physicians. Cleveland clinic center for continuing education publications: Disease management project.


Lamivudine (3TC) resistance in HIV-1 reverse transcriptase involves steric hindrance
with beta-branched amino acids. Proc Natl Acad Sci USA, 96.

Schutten M, Niesters HGM. 2001. Clinical utility of viral quantification as a tool for

Shaw T, Bartholomeusz A, Locarnini S. 2006. HBV drug resistance: mechanisms,


Challenge. Medical writers circle. http://www.hcvadvocate.org/hcsp/articles/Sterling-
1.html

Rossau R. 2000. Line probe assay for monitoring drug resistance in hepatitis B virus-

Stuyver LJ, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, Schinazi RF,
HEP DART international committee. 2001. Nomenclature for antiviral-resistant

Taswell HF, Czaja AJ, Nelson CA. 1985. Viral hepatitis: diagnostic testing using anti-

Hepatitis and Liver Disease: Proceedings of the International Symposium on Viral
198-200.


Vézinet FB. 2004. Intrinsic Potency of Antiretroviral Drugs Antiretroviral Drugs. 23rd
Postgraduate Education Course: Evaluation and management of treatment failure in HIV infected patients receiving antiretroviral therapy. Prague, April 30th – May 1st, 2004. ESCMID.


