THE UNIVERSITY OF ZAMBIA

School of Medicine

LUSAKA

MMED DISSERTATION

Cytomegalovirus contribution to liver disease in HIV-patients presenting at University Teaching Hospital- Zambia

BY

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A dissertation submitted to the University of Zambia in partial fulfilment of the requirement for the degree of Master Of Medicine in Internal Medicine.

2009

DECLARATION

I declare that this dissertation represents my own work and that it has not previously been submitted for a degree, diploma or other qualification at this or another University.
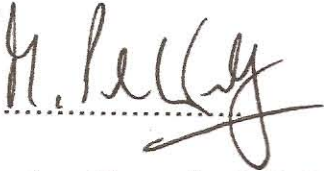
Signed..   Date.... 18th May 2010 ....

Candidate

Signed..   Date........ 18/5/10 ....

Supervisor

DECLARATION

This dissertation of Dr Clarance Chiluba is approved as fulfilling part of the requirements for award of degree of Master of Medicine by University of Zambia.

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ABSTRACT

Little research has been performed to compare Cytomegalovirus (CMV) hepatitis with the traditional viral hepatitides (Hepatitis A, B and C) in patients with Human Immunodeficient Virus (HIV) . It is a well known fact that an HIV-positive patient co-infected with HBV or and HCV has increased mortality due to liver failure. However, screening for these viral hepatitides by other researchers has not demonstrated an association with HIV, and the prevalence in HIV infected adults is similar to that in adults uninfected with HIV. In older studies, cytomegalovirus was the commonest virus found in liver biopsies in HIV-positive patients. Currently, there is a major problem in UTH with HIV positive patients presenting with hepatitis as they have a high mortality rate.

A case-control study was conducted at the University Teaching Hospital, Lusaka, Zambia to ascertain the prevalence of CMV in HIV-positive patients dying of liver dysfunction (cases) compared with those dying due to non-liver related causes (controls). I began with a study of the burden of liver disease in patients being admitted, then those who died were included in the post-mortem study. Inclusion criteria were: jaundice with other abnormal liver function tests, HIV seropositive, and aged 15 years and above. Exclusion criteria included: obstructive jaundice identified on ultrasound, haemolytic jaundice, HIV seronegative, age below 15 years and previous history of jaundice. I studied 598 potential study participants and autopsy was performed on 45 cases, and 36 controls. Liver tissue was collected for PCR diagnosis of CMV. Out of the 45 cases only one was positive for CMV and none of the controls came out positive by PCR. However, post-mortem findings suggest that bacterial infection was a dominant cause of death in HIV infected adults presenting with liver dysfunction and this will need to be looked at further.

This study showed that CMV is not the cause of liver dysfunction/hepatitis in HIV patients though it is known to cause other manifestations of AIDS such as retinitis and gastro-intestinal ulceration.

This dissertation is dedicated to my lovely wife-Brenda, and children-Misozi, Chamina and Bwalya.

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LIST OF ABREVIATIONS

AIDS	-	Acquired Immune Deficiency Syndrome
AFC	-	Adult Filter Clinic
ATT	-	Anti-Tuberculosis Therapy
CD	-	Cluster of Differentiation
CMV	-	Cytomegalovirus-
DNA	-	Deoxybonucleic Acid
DAIDS	-	Division of AIDS
EBV	-	Epstein barr Virus
GCV	-	Ganciclovir
HBsAg	-	Hepatitis B surface antigen
HBV	-	Hepatitis B Virus
HCV	-	Hepatitis C Virus
HAART	-	Highly Active Anti-Retroviral Therapy
HIV	-	Human immunodeficicient Virus
Ig	-	Immunoglobulin
MAW	-	Medical Admission virus
PCR	-	Polymerase chain Reaction
TROPGAN	-	Tropical Gastroenterology and Nutrition
TB	-	Tuberculosis
UNZA	-	University of Zambia
UTH	-	University Teaching Hospital
UK	-	United Kingdom

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6. The members of staff under UTH who were doing locum under the study in collecting data.

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CHAPTER 1

1.0 Background

Diseases of the liver are a major source of morbidity and mortality in patients with Human Immunodeficiency Virus (HIV) infection. Liver related conditions accounted for 13% of HIV-related hospitalization in the year 2000, having increased from 8% in 1996 (Gebo KA et al 2005)

It has been observed that a lot of patients present with jaundice due to liver dysfunction of unknown cause. In HIV infected adults, Deeks et al (2009) observed that liver disease is common, partly because of high rates of chronic viral hepatitis and alcohol abuse as well as long term exposure to hepatotoxic drugs, particularly antiretrovirals and herbal medication. Tsega et al (1992) had also observed that drug toxicity and viral infection, particularly hepatitis B virus (HBV) and hepatitis E virus (in pregnant women) are probably the commonest liver problems in Africa.

At the University Teaching Hospital (UTH) in Zambia, which is a tertiary hospital, during the daily audit of morbidity and mortality, the author noted that the mortality is very high indeed, possibly approaching 100% (anecdotal) in HIV patients who present with deep jaundice caused by liver dysfunction. Physicians in the Department of Medicine in UTH have seen a lot of acute liver failure in previously apparently healthy individuals not known to have any underlying liver disease. As noted by Deeks et al, many HIV infected in Zambia have a history of alcohol abuse, and some continue abusing alcohol even after knowing their HIV status and commencing Highly active Anti-Retroviral Therapy (HAART). Therefore, it becomes difficult to know if they already had liver injury due to alcohol or it has just been precipitated by other etiologies. Anecdotal reports also suggest that patients often first seek traditional

medicine before presenting to the hospital with organ failure which may be compounded by herbal medications.

The cause of hepatic failure is not thoroughly investigated in UTH due to limited resources Thus, attending doctors can only speculate the etiology based on the few labs available and the history they get from relatives or the patient. Myriad causes can contribute to hepatic failure (ie. drugs, infections and malignancies) but in the absence of solid clinical research in Zambia, even clinical suspicion may be inaccurate.

1.0.1 CMV as a contributor to liver disease

It is known that CMV infection is often accompanied by hepatitis though there are very few studies which report the clinical presentation of CMV-associated hepatitis and other acute viral hepatitides (Kunno et al). CMV is a potential cause of liver dysfunction and failure which is not currently being screened for in HIV infected patients with liver problems in UTH. The disease in the immunocompetent has a favourable prognosis, but death has been reported in the immunocompromised (Todd, 2006). Hepatitis B is routinely tested for at UTH, but many cases of liver-failure remain unexplained (anecdotal data). It is important that we start screening for the CMV virus as earlier studies had put it second only to Mycobacterium Avium Complex (MAC) as a cause of liver failure in people with advanced HIV infection (see below). The seroprevalence of CMV, which has worldwide distribution, in our population, is not known but studies that have been done in Kenya and Brazil put the seroprevalence rate above 95% (Cunha et al, 2002, Chakraborty et al, 2003). CMV is being increasingly found as a cause of morbidity in studies in the endoscopy unit, as a cause of oesophagitis, and in the ophthalmology unit it is a frequent cause of retinitis

in advanced HIV infection (UTH Clinic 7 - anecdotal data). Thorough investigation of the causes of fulminant hepatitis is not possible in UTH both because of limited resources and because patients often present with advanced liver failure. In this case the coagulopathy makes liver biopsy unsafe and the lack of radiological facilities and skilled manpower and equipment make transjugular biopsy impossible.

Therefore I carried out a study to define the frequency of this manifestation of AIDS, and to establish the real frequency of CMV as the cause of hepatitis by doing CMV-PCR on liver tissues obtained at post mortem. This was in order to determine the contribution of CMV to liver disease in HIV population.

1.1 Statement of the problem

There are a lot of patients presenting with hepatic failure in HIV and these present a management dilemma as it is difficult to define the aetiology. This is partly because of the myriad disorders which can cause liver failure. Clinicians therefore have little to offer except supportive /symptomatic management. These patients could also have other co-morbid infections, which could also be life threatening, and some drugs might be withheld due to liver dysfunction. Mortality and morbidity is very high in this population and therefore it is important to know the commonest cause/contributing factors of liver dysfunction in this population.

1.2 Study justification

There is an increasing number of HIV infected patients presenting with jaundice with associated high mortality and morbidity. CMV is a leading cause of oesophagitis and retinitis here in UTH and it is likely that CMV could also be causing hepatic

impairment as past studies showed that it was only second only to mycobacteria (see Literature review). Routine screening for hepatic impairment does not include CMV thereby making it difficult to rule CMV out as a major factor.

This study will address the contribution of CMV to mortality in HIV jaundiced patients presenting to the medical department.

1.3 Hypothesis

Cytomegalovirus contributes to death in HIV infected patients dying of hepatic failure.

1.4 Objectives

1.4.1 Main objective

To determine the contribution of CMV to patients with HIV infection dying of hepatic failure at UTH.

1.4.2 Specific objectives

1 To determine the proportion of medical admissions attributable to HIV-related liver disease

2 To determine the mortality rate in patients admitted with HIV-related liver disease

3 To define the proportion of deaths in patients with HIV-related liver disease due to CMV by performing CMV PCR on liver samples taken at post-mortem

CHAPTER 2

2.0 Literature review

2.1 Liver disease in HIV

Since the initial description of the acquired immune deficiency syndrome (AIDS), the diverse manifestations of this disease have gained increased recognition (Guarda et al 1985). Liver involvement by CMV in AIDS usually reflects disseminated rather than primary disease. Liver disease is now known to occur frequently in patients with HIV (Reichert et al 1983, Lebovics et al 1985), with several autopsy studies reporting the presence of intra-hepatic opportunistic infections or malignancies in 33% to 78% of post-mortem examinations. (Schneiderman et al, 1997, Bach et al 1992). A study done by Schneiderman and group at San Francisco hospital, California, demonstrated that specific AIDS-related infection or malignancies were found to involve the liver in 36 of 85 patients, 11 of 26 (42.3%) biopsies and 25 of 59 (42.4%) autopsies. *Mycobacterium avium* complex (MAC) was by far the most commonly seen pathogen, found in 14 patients (8 biopsy, 6 autopsies). Seven cases of CMV hepatitis were noted histologically (2 biopsy, 5 autopsy); and in addition, CMV was cultured from one post-mortem liver in absence of microscopic finding suggestive of diagnosis. Involvement of the liver with the same opportunistic organisms and neoplasm affecting other organs has been recognised since the beginning of the AIDS epidemic. Hepatic disease may result from viral, bacterial, protozoal, or fungal infections or secondary to drugs and neoplasms.

In a separate study done by Bach et al, 1992, May, CMV and mycobacteria were the most common organisms in liver identified in biopsy and autopsy studies.

2.2Cytomegalovirus (CMV)

2.2.1 The Pathogen

CMV is a member of the B-herpesvirus group and has double stranded DNA, four open reading frames of mRNA, a protein capsid, and a lipoprotein envelope. Like other herpesviruses, CMV demonstrates icosahedral symmetry, replicates in the cell nucleus, and can cause either a lytic and productive or a latent infection. CMV can be distinguished from other herpesviruses by certain biological properties, such as host range, and type of cytopathology induced. CMV is associated with the production of characteristic enlarged cells- hence the name cytomegalovirus (Martin, 2005).

CMV was initially isolated from patients with congenital inclusion disease, and is now recognized as an important pathogen in all age groups. In addition to inducing severe birth defects, CMV causes a wide spectrum of disorders in older children and adults, ranging from an asymptomatic, subclinical infection to a mononucleosis syndrome in healthy individuals, to disseminated disease in the immuno-compromised patients. (Martin, 2005)

2.2.2 Epidemiology of CMV infection

CMV is a common infection in the general community, with 60% of adults in developed countries typically seropositive, rising to 90-100% in developing countries and in poorer socialeconomic groups within developed countries (Kaplan JE et al- 2000).

2.2.3 CMV in the immuno competent population

The most common clinical manifestation of CMV infection in normal hosts beyond the neonatal period is a heterophile antibody-negative mononucleosis syndrome. This manifestation may develop spontaneously or may follow the transfusion of leukocytes in blood products (Martin, 2005).

Although the syndrome (heterophile antibody-negative mononucleosis syndrome) occurs at all ages, it most often involves sexually active young adults. Incubation period usually range from 20 to 60 days, and the illness generally lasts for 2 to 6 weeks. Prolonged high fevers, sometimes accompanied by chills, profound fatigue, and malaise, characterise this disorder. Myalgias, headache, and splenomegaly are frequent just like in Epstein Barr Virus (EBV) mononucleosis, but in CMV mononucleosis, exudative pharyngitis and cervical lymphadenopathy are rare.

Most patients recover without sequelae, although postviral asthenia may persist for months. Rarely CMV infection is fatal in immunocompetent hosts; even when such patients survive, they can have recurrent episodes of fever and malaise that are sometimes associated with autonomic nervous system dysfunction (Martin, 2005).

2.2.4 CMV in Immunocompromised population

In immunodeficient patients, CMV infection has been associated with transplant complications. The transplanted organ is particularly susceptible as a target of CMV infection and thus there is a tendency of CMV hepatitis to follow liver transplantation (Martin 2005)

In HIV there are different clinical manifestations associated with CMV disease, such as retinitis, colitis and encephalitis. The virus is usually activated from the latent state (Hahn et al 1998) when the cluster of differentiation 4 (CD4) cell count in peripheral blood falls below 100 cells/μl. Cytomegalovirus gastrointestinal disease is an uncommon but serious complication of AIDS. CMV gastrointestinal disease was first reported in 1983 (Gallant et al, 1983). CMV infection, common in patients with HIV, can produce a wide range of clinical outcomes, from asymptomatic seropositivity to a fulminant, disseminated illness. CMV can produce both an acute hepatitis-like illness and biliary tract disease. Clinical illness correlates with more advanced states of immunocompromisation (Griffiths PD et al-1997)

The disease was reported in the 1980's to be one of the most frequent findings in liver biopsy in AIDS only second to *mycobacteria*. It is surprising why this has not been recognised, taking into consideration that people in Zambia still present late with CD4 counts below 100 cells/μl. The picture might be different in developed countries where access to medical services and treatment is readily available. However, in the era of HAART, it has been suggested that cytomegalovirus viraemia is not associated with an increase risk of progression to clinical cytomegalovirus disease. Deayton et al (2004) demonstrated that the presence of cytomegalovirus in the blood is independently associated with disseminated cytomegalovirus, progression to AIDS, and death. These finding are consistent with the results of the several studies completed before the advent of HAART. Bowen et al (1997) in their study done at Royal Free Hospital, London, United Kingdom (UK), reported a relative hazard of death of 1.76 per log increase in cytomegalovirus load, and Spector et al (1999) found an increase of 2.5 in the risk of death associated with a positive cytomegalovirus PCR

test. Although direct comparison of these results is not possible, they suggest that cytomegalovirus viraemia remains at least as important in terms of an increased risk of death in the era of HAART as previously reported.

Therefore, there is a need to find out the causes of jaundice due to liver failure in HIV population.

2.2.5 Diagnosis of CMV

The diagnosis of CMV infection cannot be made reliably on clinical grounds alone. Isolation of the virus or detection of CMV antigens or DNA from appropriate clinical specimens is the preferred diagnostic approach. If viral titres are high, as is frequently the case in patients with AIDS, characteristic cytopathic effects may be detected within a few days. However in some situation –such as CMV mononucleosis- viral titres are low, and cytopathic effect may take several weeks to appear. Many laboratories expedite diagnosis with an overnight tissue culture method (shell viral assay) involving centrifugation and an immunocytochemical detection technique employing monoclonal antibodies to an immediate-early CMV antigen. Isolation of virus from urine or saliva does not, by itself, constitute proof of acute infection, since excretion from these sites may continue for months or years after illness (Martin, 2005).

2.2.5.0 Techniques for analysing CMV infection

The four main ways of diagnosing CMV are by isolation of the virus, histopathology, serology and molecular.

2.2.5.1 Virus isolation

Virus isolation Virus excretion or viremia is readily detected by culture of appropriate specimen on human fibroblast monolayers (Cunha et al-2002). Culture in human embryo fibroblasts is usually slow but diagnosis can be accelerated by immunofluorescent detection of antigens in culture (Martin, 2005).

2.2.5.2 Serology

Detection of CMV antigens in peripheral-blood-leucocytes or of CMV DNA in blood or tissues may hasten the diagnosis of CMV disease in certain populations, including organ transplant recipients and persons with AIDS (Salmon-Ceron et al 1996). Such assays may yield a positive result several days earlier than culture methods. A variety of serological assays are available to detect increases in titres of antibody to CMV antigens. An increased antibody level may not be detectable for up to 4 weeks after primary infection, and titres often remain high for years after infection. For this reason, single antibody sample determinations are of no value in assessing the acuteness of infection. Detection of CMV-specific Immunoglobulin-M (IgM) is sometimes useful in the diagnosis of recent or active infection; circulating rheumatoid factors may result in occasional false-positive IgM tests.

2.2.5.3 Histopathology

The virus can be identified in tissues by the presence of characteristic intranuclear 'owl's eye' inclusion bodies.

2.2.5.4 Molecular techniques

The polymerase chain reaction (PCR), which was introduced almost 3 decades ago (1985), has revolutionalised the way DNA analyses are performed and has become a cornerstone of molecular and genetic analysis. PCR provides a rapid way of amplifying specific DNA in vitro. Exquisite specificity is conferred by the use of PCR primers designed for a given DNA sequence. The geometric amplification of the DNA after multiple cycles yields remarkable sensitivity. Therefore PCR can be used to amplify DNA from small samples, theoretically from as little as one cell. (Martin, 2005). However, the down side of this high sensitivity is false positive results from contamination. To protect against false positives from contamination, careful use of controls is required to detect contamination so that reagents can be changed and the procedure repeated.

In our analysis we are using the amplification of CMV DNA by using primers specifically designed for CMV on liver tissues (Boeckh et al 1998, Bowen et al 1997, Pellegrain et al).

2.2.6 Treatment

The first major advance in the treatment of CMV disease was the development of ganciclovir, an agent highly specific for human herpesviruses (Crumpacker 1997). Ganciclovir (GCV) is a nucleoside analogue whose activity depends on inhibition of herpesvirus DNA polymerases. It requires phosphorylation in CMV-infected cells, and most strains of CMV resistant to ganciclovir are unable to phosphorylate ganciclovir. The CMV UL97 open reading frame codes for a protein kinase capable of phosphorylating GCV in CMV-infected cells (little E et al-1992)

Though the utility of gancicovir therapy in the treatment of CMV in other organ systems is not proven by randomised controls, the established treatment of CMV hepatitis is intravenous ganciclovir (Blanshard C et al,1992, 1995).

CHAPTER 3

3-0 Clinical and Post-Mortem Procedures

3.1 Site

This study was conducted in the Department of Internal Medicine at the University Teaching Hospital (UTH), Lusaka, Zambia. UTH functions as a secondary and as a tertiary level hospital. It has a bed capacity of about 1200 and serves as the only tertiary centre for all hospitals in Zambia. The UTH department has an Adult Filter Clinic (AFC) through which every patient who is eventually admitted to medical wards has to pass. Therefore it acts as a suitable point for any data collection on the number of patients admitted to medical wards. AFC has a transit ward for patients who are admitted to Medical Admission Ward (MAW). The study was a pilot case control study.

3.2 Prevalence Survey in UTH

Data were collected between February, 2009 and September 2009 from AFC. The data collected included:

(a) Number of patients with their demographic data admitted per day

(b) Number of patients admitted with jaundice

(c) Number of jaundiced patients who are HIV positive

For the patients who did not know their HIV status blood was collected and tested by diagnostic counselling and testing. This service is available in AFC, and it has to be done on every patient who presents to filter clinic as a standard of care. Some patient might not wish to know their results; however, documentation is made in the file for the medical personnel looking after the patient. The relevant bloods including liver function tests were obtained and sent to the laboratory.

Demography data, data on possible causes of hepatotoxicity leading to jaundice and examination finding were reviewed from the clinical file. Clinical staffs at AFC were trained on the information which was needed for this study prior to commencement to minimise on missing information and retraining was done if gaps are identified. Compiled results were recorded on Excel worksheets.

3.3 Ethical matters

This study was approved by the University of Zambia (UNZA) Research Ethics Committee bearing the Assurance No. FWA00000338, IRB00001131 of IORG0000774. The reference is 006-04-09. Informed consent was obtained from the next of kin to conduct a post-mortem. The information sheet was given and explanation was given to next-of–kin before they consented (in English, Bemba or Nyanja).

3.4 Selection of subjects

The selection of potential study participant was done following a pre-defined algorithm (Fig 2) and fulfilling the inclusion criteria.

Figure 1. Algorithm for subjects identification.
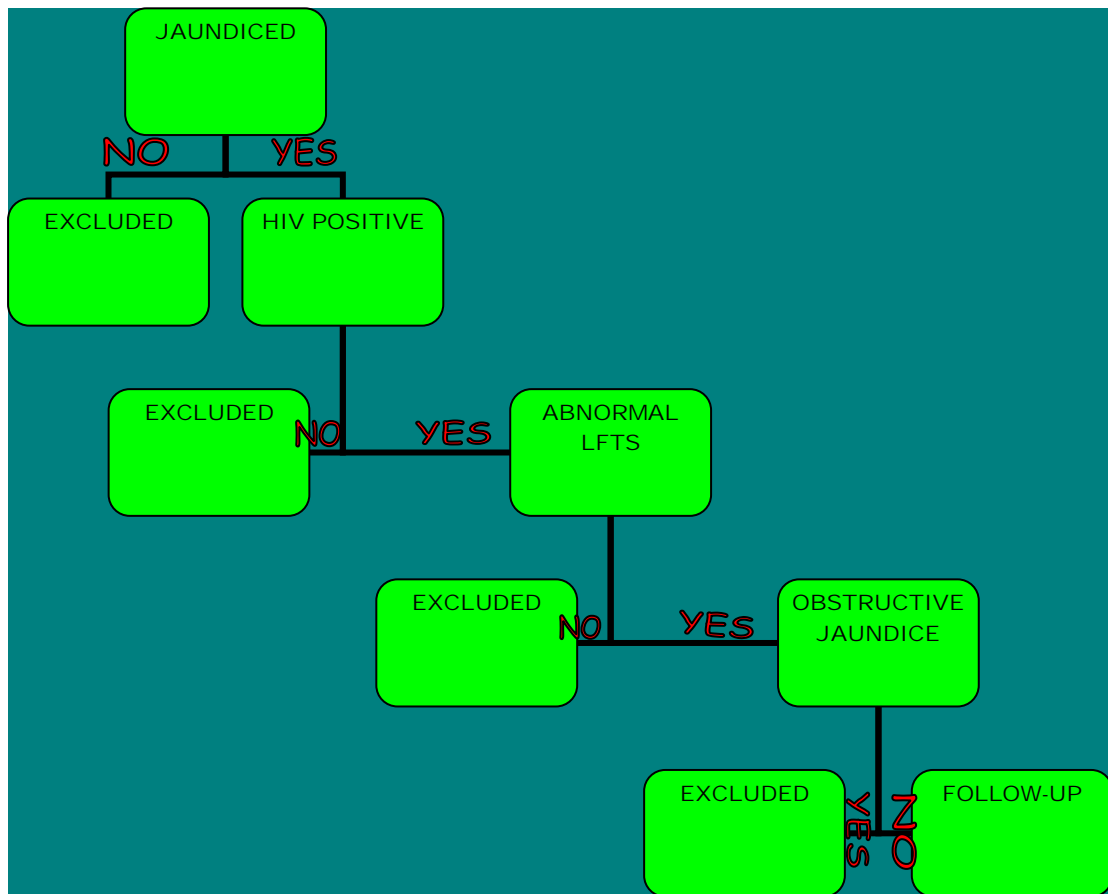
3.4.1 Inclusion Criteria

Patients presenting to UTH- medical Department with liver related diseases were identified as follows:

1 Jaundice, unless obstructive jaundice clearly identified on ultrasound

2 Abnormal liver function tests

3. Presenting with jaundice as first episode

4. HIV positive

5. 15 years and above

3.4.2 Exclusion Criteria

1 Obstructive Jaundice

2 Haemolytic jaundice

3 HIV negative

4. Age below 15 years

5. Previous history of jaundice

Those fulfilling the inclusion criteria were followed up and the further tests were carried which included:

1. Liver function tests if not already carried out

2. Ultrasound of the abdomen with special reference to the liver

3. HBsAg

4. Anti-HCV antibodies

Need another section under 3.4 describing controls and how they were selected

3.5 Autopsy

Post-mortem examinations were done on HIV infected patients dying of liver-related dysfunction. For those whose relatives declined a full post-mortem, a limited (regional, just the abdomen) autopsy or percutaneous liver biopsies were obtained if consented. Consent was obtained from the next of kin for conducting a post-mortem (see informed consent sheet in appendix). For each post-mortem examination in a patients dying of HIV-related liver disease, control samples were collected from a post-mortem examination of an HIV positive patient dying of unrelated causes, such

as neurological or cardiac disease or proven septicaemia.

To avoid any contamination of the samples being collected we used sterile gloves, sterile blades and sterile, new, biopsy needles in cases of percutaneous biopsy. The area of incision was also swabbed with methylated spirit to minimise contamination. After getting the samples from the body, samples were immediately put in sterile containers and transported to the microbiology laboratory for storage at -80°C. From each cadaver two samples were obtained, each at least 5-10g of tissue by weight. The bodies were later prepared accordingly for burial or storage if not being buried immediately by the pathologist and mortuary staff. We had made arrangement for relatives to get a written report from the pathologist, in cases of a full post-mortem, and results of CMV as indicated in the consent form.

CHAPTER 4

4.0 Laboratory methods

4.1 Sample collection

The study involved the collection of blood for liver function tests when a patient was admitted and of liver samples during autopsies. HIV tests, as earlier alluded to, were done in the AFC for all patients who needed to be followed up. Liver function tests were performed in the biochemisty laboratory of UTH. Ms Cynthia Phiri in the TROPGAN was involved in molecular diagnosis of CMV on the liver samples.

4.1.1 Sample Delivery

We collected 5mls of blood for liver function tests; 4mls was collected during autopsies for storage as serum at -80°C. The samples were delivered to respective laboratories and receipts of the samples were entered in the book for the laboratory and the principal investigator data book and signed by both deliverer and recipient.

4.1.2 Sample Receiving Procedure

TROPGAN staff on duty counterchecked the data on the samples that included unique serial number and contents in the container. If identifying data were not adequate, the samples were kept in a different compartment until verification was done in consultation with the principal investigator and involved personnel. Data was entered in the record books accordingly.

4.1.3 Sample storage

The samples in the microbiology laboratories were stored at -80°C in a dedicated compartment. The study was assigned one member of staff to be solely responsible for the samples with the principle investigator.

4.2 Methodology for detection of CMV by PCR

4.2.1 Processing for extraction of DNA

CMV is a DNA virus so DNA was extracted from the liver tissue.

Processing of the liver samples was done in an ultraviolet treated laminar flow hood. A small piece of tissue was removed for analysis and the remainder was taken back for storage at -80°C. The samples were thawed at 4° C and then placed in sterile Petri-dishes and cut up using sterile surgical blades. The liver pieces were then transferred to sterile 1.5ml Eppendorf tubes. Lysis buffer (comprising Proteinase K, Tris-chloride buffered to pH 8.0, and 10% sodium dodecyl sulphate) was added and then incubated at 60° C for 4 hours. DNA was extracted using the standard phenol-chloroform method followed by ethanol precipitation.

4.2.2 PCR analysis

In the PCR reaction we used primers (from Sigma p.l.c, Bournemouth, Dorset, UK) with the following sequences;

Forward-AGC TGA ATG ATG TGA AGC AAG

Reverse-GAA GGC TGA GTT CTT GGT AA

Which is a sequence from the early immediate gene of CMV (also known as HHV-5) as confirmed by a primer-BLAST search of the GENBANK database (.ncbi.nlm.nih.). The target sequence showed a band of 146 base pairs.

The PCR reaction profile was as follows;

Denaturing 96°- 1 min,

Cycling   94 ° C 1 min

        58°C 1min

        72°C 1min

Extension 72°C for 10mins

Hold at 4°C

25µl (half of the PCR reaction product) was subjected to gel electrophoresis using a current of 90V for 45 minutes on a 2% agarose gel containing 0.5% ethidium bromide and visualised using ultraviolet light.

4.2.2.1 Quality control

To ensure there was no contamination, we undertook the following measures,

(a) Two positive controls and three negative controls were included in every PCR run,

(b) The hood used for specimen processing was cleaned with detergent and bleach and was ultraviolet treated before and after use,

(c) All surgical blades, gloves and Petri-dishes were from sterile, unopened packets at the beginning of every procedure.

These are standard procedures in the molecular microbiology laboratories, UTH.

4.2.3 Scoring of global results

After visualization on agarose gel, the results were recorded as positive if a clear band of the correct size was visible. Where there was doubt consultation was done with other members of TROPGAN until consensus was reached or the electrophoresis was

repeated. This was recorded in the book corresponding to serial number. The principal investigator assigned serial numbers and the molecular technologist did not know which were cases or controls.

4.3 Disposal of samples

Liver and serum samples which were left after above tests have been stored for future analysis. None of the samples have been disposed.

4.4 Sample size calculation

Sample size calculations were carried out assuming 90% power at 95% confidence using plausible estimates of the frequency of CMV. If 33% of patients dying of liver disease have CMV (based on estimates in the literature) and 5% of controls have CMV as detected by PCR, then 46 post-mortem examinations are required in each group. The total number of completed post-mortem examinations was 92. Sample size calculations and analysis were completed using Stata version 10.

.

4.5 Data analysis

The primary comparison was the frequency of detection of CMV in HIV seropositive patients dying with liver disease compared to those dying of other non-hepatic manifestations of HIV or of disease unrelated to HIV. The statistical test used was a Fisher's exact test to compare the proportions with positive PCR in patients dying of HIV-related liver disease compared with other manifestations of HIV.

CHAPTER 5

5.0 Results

5.1 Prevalence and outcome of HIV related liver disease

In the eight months of the study from February, 2009 to September, 2009, 6,049 patients were admitted to Medical Admission Ward (MAW) from Adult Filter Clinic (AFC) (Figure 2 and table 1). The number of patients admitted to MAW varied between 758 and 1007 per month normally, however during industrial unrest, May to July, a drastic drop was noted. Therefore, there were about 27 to 32 patients admitted per day to MAW from AFC.

Of the 6,049 patients admitted, 767 (13%) were jaundiced. We tested every patient who came in jaundiced for HIV from AFC. Of the jaundiced patients, 598 (78% of 767; 10% of 6,049) were HIV seropositive (Fig 3and Table 2).

Expressed another way- the number of patients who were admitted to MAW with their HIV status was as follows (fig-3);

(a) 2,865 (47%) patients were HIV positive

(b) 812 (13%) patients were HIV negative

(c) 2,381 (40%) patients did not undergo HIV testing.

FIGURE 2- Admission from adult filter clinic
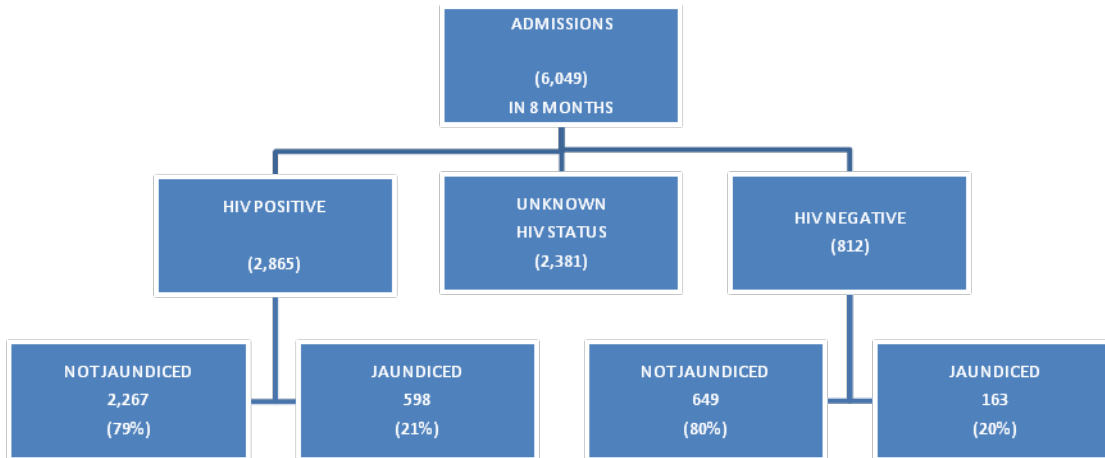
TABLE 1- Patient admitted to MAW with their HIV status month by month

MONTH	FEB	MAR	APR	MAY	JUNE	JULY	AUG	SEP
ADMISSION TO MAW	758 ♀416 ♂342	899 ♀503 ♂396	906 ♀462 ♂327	760 ♀433 ♂327	210 ♀83 ♂127	617 ♀293 ♂324	1007 ♀597 ♂410	901 ♀529 ♂372
HIV POSITIVE	403 (53.2%)	447 (49.7%)	397 (43.8%)	314 (41.3%)	113 (53.8%)	416 (67.4%)	334 (33.2%)	441 (48.9%)
HIV NEGATIVE	101 (13.3%)	154 (17.1)	142 (15.7%)	98 (12.9%)	56 (26.7%)	60 (9.7%)	84 (8.3%)	117 (13%)
HIV STATUS UNKNOWN	254 (33.5%)	298 (33.2%)	367 (40.5%)	348 (45.8%)	41 (19.5%)	141 (22.9%)	589 (58.6%)	343 (38.1%)
HIV-POSITIVE JAUNDICED	98	76	101	34	25	43	118	103
HIV-NEGATIVE JAUNDICED	42	28	25	11	02	09	32	14

TABLE-2 HIV-positive patients admitted with Jaundice

MONTH	FEB	MAR	APR	MAY	JUN	JUL	AUG	SEP
HIV-POSITIVE	403	447	397	314	113	416	334	441
HIV-POSITIVE JAUNDICED	98 (24%)	76 (17%)	101 (25%)	34 (11%)	25 (22%)	43 (10%)	118 (35%)	103 (23%)

5.1.1 Mortality

Of the 598 HIV-positive patients presenting with jaundice and abnormal liver enzymes who were, 391 (65%) died-(Table 3). Death usually happened within 48 hours of admission. Most of these patients had fulminant hepatic failure. The discharges happened within 7-21 days and most of them were followed up in the out-patient clinic. These were not followed up and there is the possibility that there were additional fatalities after discharge which were not identified.

TABLE-3 Mortality in HIV-jaundiced patients

MONTH	FEB	MAR	APRIL	MAY	JUNE	JULY	AUG	SEP
TOTAL# ADMITTED	98	76	101	34	25	43	118	103
TOTAL # OF DEATHS	66 (67%)	57 (75%)	47 (47%)	28 (81%)	5 (20%)	8 (19%)	91 (77%)	89 (86%)

5.2 Autopsy data- case control study

Of the 391 patients who died, I was able to obtain permission for post mortem examination in 45. Together with controls, 81 post-mortem examinations were done which comprised 45 cases (HIV positive jaundiced patients) and 36 controls (HIV positive non-jaundiced patients with no evidence of liver disease) (Table 4).

5.2.1 Demographic data

The study had 37 males and 8 females in the cases category and 30 males and 6 females in the control category. The age range for the cases was from 21 to 65 years and for the controls was from 22 to 54 years. The mean age was 41 years and 36 years respectively.

5.2.2 Risk factors for hepatic disease

5.2.2.1 Hepatotoxic drugs (ATT, ARVs)

The prescribed hepatotoxic drugs which were generally encountered were antituberculous drugs (ATT) and nevirapine. Others like paracetamol were within the acceptable dosage and unlikely to cause hepatic damage. There were 9 study participants on ATT, 7 in the intensive phase (therefore including rifampicin and pyrazinamide) and 2 in the continuation phase. 7 of these patients were cases and 2 were controls. There was 1 patient in the controls in the continuation phase. There were 2 patients on nevirapine and two were on ARVs (unknown combination). All the four patients were cases.

5.2.2.2 Herbal medication

There were 4 cases and 1 control on herbal medications which is about 9% and 3% respectively.

5.2.2.3 Alcohol use

There were 29 (64%) case participants and 16 (43%) who had taken alcohol in the last year.

5.2.3 Clinical examination

Hepatomegaly or tenderness in the right hypochondrium was found in 17 (38%) of cases and in 5 (14%) controls. 41 (91%) cases had features of hepatic encephalopathy. The diagnosis of hepatic encephalopathy was made when some or all of four major factors were present;

- Acute hepatocellular disease
- Disturbance of awareness and mentation, which may progress from forgefulness and confusion to stupor and finally coma
- Shifting combination of neurologic signs including asterixis
- A characteristic pattern on electroencephalogram

We were unable to do electro-encephalography diagnosis and therefore we modified as shown in the table-4

TABLE-4 – Clinical stages of Hepatic Encephalopathy (Modified from Harrisons – Principle of Internal Medicine- Edition)

STAGE	MENTAL STATUS	ASTERIXIS
I	Euphoria or depression, mild confusion, slurred speech, disordered sleep	+/-
II	Lethargy, moderate confusion	+
III	Marked confusion, incoherent speech, sleeping but arousable	+
IV	Coma; initially responsive to noxious stimuli, later unresponsive	-

There was no patient among the cases who had ascites. This might signify that most cases were acute or had not decompensated.

5.2.4 Viral hepatitis

One of the cases tested positive for HBsAg among the 13 tested samples out of 45 cases. We tested five out for 45 for anti-HCV antibodies and all came out negative. It was difficult to test everyone as reagents were not readily available.

5.2.5 Postmortem results

We did 8 full post-mortem and the reports were submitted to the relatives. Regional (abdomen only) post-mortem were done in 71 cases and percutaneous biopsies were done in 2 cases (limited by the availability of biopsy needles).

In the 8 patients who underwent full postmortem, the findings attributed the cause of death to septicaemia in 5 and to disseminated TB in 3. In these 3 postmortems we found abdominal peritonitis with pockets of pus, though this patient did not have obvious evidence of peritonitis clinically. Out of the three, two had evidence of lung involvement by consolidation. The other one had evidence of bronchopneumonia and the focus of sepsis was pelvic inflammatory disease. In the disseminated TB we found caseating mesenteric lymph nodes and some pus with lung involvement in two of the three. The other case showed pleural effusion on the left with caseating abdominal lymph node and splenomegaly. This represents 11% and and 7% of the cases. These are cases where a full post-mortem was done.

TABLE-5 Postmortem findings

CASES	FINDINGS
1	Intra-abdominal pockets of pus
2	Intra-abdominal pockets of pus with lung consolidation(pneumonia)
3	Bronchopneumonia
4	Pelvic inflammatory disease
5	Intra-abdominal pockets of pus with lung consolidation
6	Caseating mesenteric lymph nodes with pus covering peritoneum
7	Caseating mesenteric lymph nodes with pus covering the peritoneum and turbecles affecting the lung.
8	Caseating mesenteric lymph nodes with pus covering the peritoneum and turbecles affecting the lung.

5.2.6 CMV-PCR

PCR was performed on all liver tissue obtained. In every PCR run (primers from Sigma p.l.c, Bournemouth, Dorset, UK) we had 3 positive controls and 2 negative controls for proper validation of results. The positive control had a target sequence of 146 base pairs. Out of 45 cases only one came out positive and the rest were negative. In the controls, all the 36 samples were negative (Table 6).

TABLE-6

	PCR-CMV POSITIVE	PCR-CMV NEGATIVE
CASES	1	44
CONTROLS	0	36

P=1.00 by Fisher's exact test .

CHAPTER 6

6.0 Discussion

6.1 Number of admission

The University Teaching Hospital (UTH) in Zambia, a Sub-saharan country, high number of admission has shown by the data makes it a suitable place for conducting a research in HIV-related field. In the 8 months we had seen more than 6000 patients and they were 598 potential study participants.

6.2 Number of HIV jaundiced patients

We had seen about 598 HIV positive jaundiced patients in the 8 months of the study.

6.3 Risk factors for developing liver dysfunction (hepatotoxicity , herbal medication, alcohol use)

The commonest risk factor for liver dysfunction was alcohol followed by herbal medication, antituberculosis (ATT) drugs, and nevirapine in that order. Alcohol will causes alcoholic hepatitis occasionally, but in our study it will be unusual. This is because most patients had not been well in the last 2-3 weeks prior to admission.

We don't have local statistics on drug-induced hepatic injury, but according to the study in USA by Ishak et al it accounted for approximately 2 to 5 percent of hospitalizations for jaundice, 10 percent of cases of hepatitis in all adults and more than 40 percent of cases in adults older than 50. The commonest causes of drug induced hepatitis among the prescribed medication in patients presenting at UTH are antituberculosis drugs. The antiretroviral drugs are not mainly associated with drug induced hepatitis.

The use of herbal remedies must be considered as a possible etiology in any setting of clinical manifestations of liver injury. Herbal remedies use worsened with the

introduction of Chinese medication and also complicated by traditional healers who claim to have a cure for AIDS. The information on the use of these herbal medications is sometimes difficult to get as most would not want to review as it is associated with witch-doctors. Therefore, it is important to find a way of asking so that patients do not feel stigmatize (Kaplowitz et al 2001). Continued use of the herbal (offending product) greatly increases morbidity once hepatotoxicity has developed [Cole et al-2003, Zimmerman et al 1995]. Non-specific constitutional symptoms of toxicity often occur and frequently the patient will increase the use of the herbal preparation to help "treat" the symptoms.

As in other cases of elevated liver enzymes, all other causes of liver disease must be ruled out. There are no specific tests or markers to confirm a diagnosis of drug-induced liver injury(Lucena et al 2001, Maria et al 1997). Proving that a drug causes liver injury relies on both chronological and clinical criteria (Larrey et al 2002, Cole et al 2003, Benichou et al 1990,1993).

6.4 Liver enlargement /right hypochondria tenderness

38 percent of the 81 study participants had evidence of hepatomegaly/right hypochondria tenderness. This represents a small proportional with what is expected because HIV-positive patients usually present with hepatomegaly of non clinical significance. However, some of the finding could be due to extensive necrosis of the liver as evident on autopsy.

6.5 Hepatic encephalopathy

91 percent of the 45 case study participant had evidence of hepatic encephalopathy. This only shows how extensive the liver was damaged. With the modification to the criteria, its possible that some cases could have metabolic acidosis to septicaemia and

not due to liver decompesation as a specific entity. This is supported by cases where autopsy demonstrated septicaemia, where clinical diagnosis had indicated hepatic encephalopathy.

6.6 Ascites

We didn't have any case of ascites clinically, though it was noted on post-mortem some (8) had evidence of pus in the abdominal. The absence of ascites also just demonstrate how acute some cases were.

6.7 Viral hepatitides

The prevalence of viral hepatitis is no different in HIV-positive and ,HIV-negative patients in our population- (Kapembwa k,2008, Oshitani et al 1995). It is known that coinfection with HIV and HBV is complicated because the rate of grade 3-4 hepatotoxicity to HIV antiretrovirals is increased substantially (J Infect Dis fold (Lancet 2002;360:1921). It has also been observed that HIV-HCV coinfection is associated with a 3 fold increase in rates of progression to hepatic failure (Clin Infect 2001;33:240, Lancet 1997;350:1425, Dorucci et al 1995) In our study we don't think the high mortality in the HIV positive patients is due to the traditional viral hepatitides (hepatitis A,B and C) as evident by the percentage of patients who tests negative and thereby making most cases unexplained.

6.8 Post Mortem findings

Full post-mortems which were done in 8 (18%) cases had attributed the cause of cases. Septicaemia could contribute mostly to deaths in the HIV population. Most of the patients usually seek proper medical advice late and presents with complication. Probably what we see is the end stage of a process which starts localised then disseminating. Translocation of bacteria cannot be ruled out completely in patients with liver dysfunction. . Disseminated tuberculosis is another likely diagnosis in our patients with liver dysfunction (Sinkala, 2008). There was no evidence of liver granuloma but the disseminated picture could have affected the liver. Disseminated tuberculosis was shown in the past to be the high ranking cause of liver dysfunction and is still a probable cause. Therefore it should be entertained in cases of liver dysfunction in HIV population.

6.9 CMV-PCR

It is evident that CMV is not the cause for the liver dysfunction in our population and this entails that we look for other etiologies. Only one case came out positive for CMV out of the 45 cases. The confusion arose when we considered the other studies which had shown probable CMV infection in HIV-positive liver biopsies mostly by characteristic cell appearance ('owls' with inclusion bodies)- (Gallant JE et al-1992). It would be interesting for us to do histopathology on our liver samples and see if this would correspond with previous findings.

The probable causes would be mainly attributed to Tuberculosis and Septicaemia as evident in the post-mortem findings.

I have suggested an algorithm for investigation of such patients if resources permitted.

FIG 3 Algorithm for evaluating HIV-infected patients with acute hepatocellur pattern.
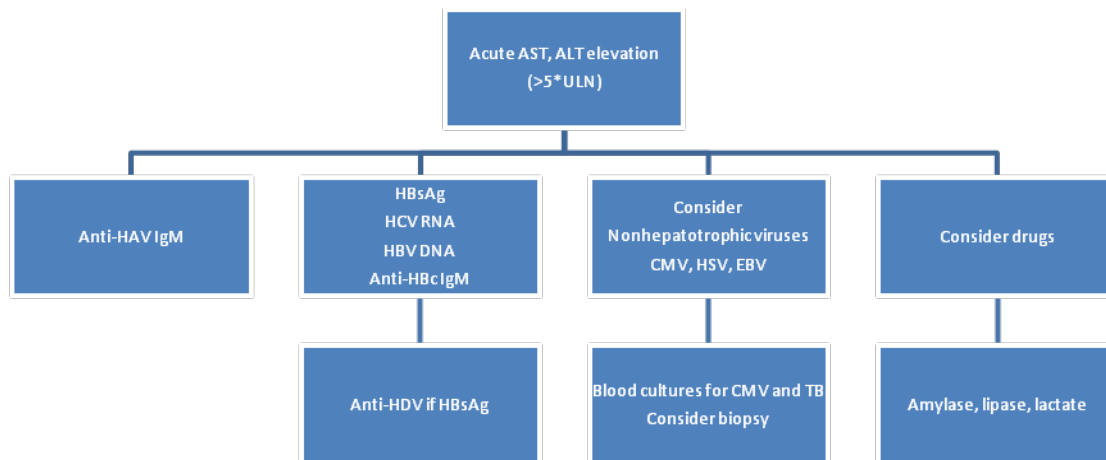
CHAPTER 7

7.1 Conclusion

CMV is not a cause of jaundiced caused by liver dysfunction as evident by this study. Therefore it will be imperative for us to address this issue and do further tests on the remaining samples considering the high mortality in this group of patients. There could be myriad causes of liver dysfunction , however from this study it seems TB and septicaemia would be topping the lists and they should be considered and excluded by doing appropriate tests. The viral hepatitis should also be investigated as shown by Kapembwa et al-2008, though they don't account for most cases.

7.2 Limitations

Lack of adequate funding had precluded us from doing appropriate studies to do necessary tests to properly ascertain the severity of liver dysfunction ie International Normalised Ratio. The other tests which should have been done are CD+4 count (assessing the immunity) and routine histopathogical examination of the liver specimens.

Viral hepatitis serology was not done in all the cases and this, of course, would have affected the results.

7.3 Recommendations

Since the management of jaundiced caused by liver dysfunction in HIV infection is complex, and following up the treatment algorithms (Figure-3) will be costly to resource constrained countries like Zambia, it will be important to follow up tests on the samples still remaining. Bearing in mind that there is a dearth of knowledge on this issue, it will be important for us to narrow down on the infective causes by

following up the algorithm- (Fig 3).

There is also need to continue educating our people on hepatotoxic drugs which can mostly be avoided. Alcohol and herbal medication being among the avoidable etiologies for liver dysfunction.

There is also need to continue educating Healthcare workers caring for such patients to be conversant with common causes of liver injury, diagnostic modalities, and treatment interventions. As we might be aware that the very medications that improve life expectancy in HIV infected patients may alter hepatic metabolism and produce direct and indirect liver injury, therefore every healthcare provider should meticulously countercheck the medicine they are prescribing.

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INFORMATION SHEET

POSTMORTEM CONSENT FOR THE NEXT OF KIN IN THE CMV-LIVER

I am sorry to hear that your relative has passed away. We too are worried that we are losing too many people and are striving to find what else we can do to improve the health care we offer. Our request is that you go through this information sheet and possibly give an informed consent for us to conduct a post-mortem/ percutaneous liver biopsy.

 STUDY

This information is being provided to you regarding the CMV-liver study to enable you to give informed and voluntary consent to participate in this study.

Kindly read it carefully or let someone else read it to you before you sign the consent form. If there is anything you do not understand, ask that it be explained to you before you sign the consent form.

Introduction

It has been shown that people who are infected with HIV are susceptible to a lot of infection because of the low immunity (ability to combat disease). In Zambia, we have noted that we are losing a lot of patients who are HIV positive coming with liver problems. We have failed to establish the main cause for this as so many things can contribute: infections and both prescribed and non prescribed medication. With the limited resources in our health care system it is very difficult and expensive to run through all the investigation we could do. The infection we want to look for is a virus called Cytomegarovirus (CMV) which can cause liver problems/ infection. In order to prove this, we intend to get liver samples and test for this virus on the sample. If it is

found that it is the CMV which is causing liver problems then we will be able to introduce a new treatment and possibly prevent premature loss of life.

Benefits and Risks

There are no risks to the family or the deceased as no more will be done than the normal procedure for post-mortem. The immediate benefit to the family is that the post-mortem will be expedited and family will be well informed about the cause of death after all the tests have been done. We encourage you to come back to us and ask for a report. The benefits are immeasurable as doctors will be able to understand what is causing death in people presenting with yellowing of eyes due to liver failure. This will definitely improve our understanding of disease process and management, and possibly save lives.

Should you have any questions, please contact Dr. Chiluba Clarance on 0977-434763.

Confidentiality

 All the information gathered on this study will only be used in privacy and known by the members of this research team and you as a relative/next of kin. The identity of persons will not be disclosed to any one outside the research team.

<div align="center">**Appendix 2**</div>

Consent form

CONSENT FOR POSTMORTEM

I give consent to have the deceased have liver samples be taken at the time of full post-mortem/ abdominal post-mortem/ percutaneous liver biopsy (delete unapplicable) for the purpose of taking part in the CMV-liver study. I give consent indicating voluntary and informed consent as next of kin to the deceased. I may withdraw my consent at anytime without penalty or loss of benefits or treatment to which I am entitled. Doctors may choose not to proceed to collect specimen or use such specimens for study without my consent.

I understand what the study is all about and what is expected of me if i participate in this study.

Interviewee's name:………………………………………………………………….

Signature/Thumb print;…………………………………………………………………

Phone number/ contact address…………………………………………………………

Date ………………………………………………..

Witness's name:…………………………………………………………………….

Signature …………………………………………………………………………

Date…………………………………………………………………………..

Should you have any question, contact the following;

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