

HEPATITIS VIRUS B AND HEPATITIS VIRUS C CO-INFECTION AMONG HIV
PATIENTS AND DEVELOPMENT OF AN ENZYME LINKED IMMUNOSORBENT ASSAY
(ELISA) FOR DIAGNOSIS OF EQUINE PROTOZOA MYELOENCEPHALITIS

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MASTER OF SCIENCE

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ABSTRACT

Study 1: HIV patients with chronic HBV and/or HCV are more likely to die of liver disease and have a more rapid progression to Acquired Immunodeficiency Syndrome (AIDS) than patients solely infected with HIV. Blood samples were assayed for the presence of HIV 1/2 antibodies, Hepatitis B Surface Antigen (HBsAg) and Hepatitis C antibodies (HCVab). The prevalence of HBV and HCV among HIV patients was 13% and 10%, respectively. This calls for integration of HBV and HCV prevention, and treatment into HIV programs.

Study 2: The current techniques used to diagnose EPM have low sensitivities and some are expensive. To improve serologic diagnosis of EPM, a trivalent antigen (rSAG2/4D1/3D2) was expressed, purified and later incorporated into indirect ELISAs. rSAG2 and rSAG4D1/3D2 reported two and one false negative (s), respectively while rSAG2/4D1/3D2 reported none at a cut off of 15% positivity. rSAG2/4D1/3D2 could be more accurate and reliable than rSAG2 and rSAG4D1/3D2.

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DEDICATION

To my daughter Beatrice Ninsiima, who I love and miss so much!

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CHAPTER ONE. GENERAL INTRODUCTION

Liver disease caused by chronic hepatitis B Virus (HBV) and hepatitis C Virus (HCV) is emerging as a significant cause of morbidity and mortality among Human Immunodeficiency Virus (HIV) infected individuals [73]. HBV, HIV, and HCV have similar modes of transmission, and a high proportion of HIV infected adults are at increased risk of developing chronic HBV and HCV infections. This co-infection may result in serious medical complications such as increased risk for liver-related morbidity and mortality. HBV/HCV co-infections also increase the risk of hepatotoxicity from antiretroviral medication. This is particularly important in patients on protease inhibitors [61]. Patients co-infected with either HCV or HBV and take nucleoside/nucleotide reverse transcriptase inhibitors have a high risk of fatty liver disease [6]. The aim of this study was to determine the prevalence of HBV and HCV among HIV positive patients attending antiretroviral (ART) clinic at Mulago hospital in Uganda.

Sarcocystis neurona is a single celled parasite belonging to the coccidian group and is the most commonly implicated cause of equine protozoal myeloencephalitis (EPM) in horses in America. In rare cases EPM can also be caused by *Neospora caninum* and /or *Neospora hughes*. The opossums are the definitive hosts and a variety of mammals like skunks, raccoons, domestic cats and armadillos are intermediate hosts. Horses get infected by ingestion of feed or water contaminated with *Sarcocystis* sporocysts [48].

S. neurona merozoites express multiple immunogenic surface antigens (SnSAGs), four of these surface antigens have been characterized and identified as SnSAG1, SnSAG2, SnSAG3 and SnSAG4 [24, 25]. Recombinant proteins of these SAGs expressed in *E. coli* have been used in ELISA in comparison with western blot and shown to be highly accurate and reliable. Because of these findings it's reasonable to hypothesize that increased accuracy and reliability could be

attained by combining individual SAGs into a single assay. Michell *et al.*, proved this in their study in which they utilized a mixture of individual recombinant SAG protein and a collection of unique snSAG chimeras that contained fused protein domains from different snSAG surface antigens into a single recombinant protein [25, 35]. In light of this, this paper presents results generated from an approach in which a chimera of three of these surface antigens (SAG2/4D1/3D2) was evaluated as an enhancement of an ELISA utilizing the two antigens (snSAG2 and snSAG4/3) to diagnose EPM.

1.1. Problem Statement Study 1: HIV/HBV/HCV co Infection

Despite the negative impact of HBV and HCV co-infections on mortality and morbidity rates among HIV-infected individuals, detailed information on the prevalence of HCV/HIV and HBV/HIV co-infection in Uganda is lacking. Although vaccination against HBV is becoming available, integration of treatment and diagnosis in HIV programs has not been achieved. This lack of prevention strategies is partially due to high costs associated with these interventions. Information generated by this study will help inform policy makers of a baseline for HIV patients that are co-infected with HBV or HCV in Uganda. With appropriate consideration, this could be used to increase attention for integration of HBV and HCV interventions into HIV prevention and control programs.

1.2. Problem Statement Study 2: Development of an ELISA for Diagnosis of EPM

A definitive diagnosis of EPM in a live horse is quite difficult to attain because neurological signs can mimic symptoms from other diseases such as West Nile virus infection, equine herpes myeloencephalopathy, Lyme disease, Cushing disease and cervical vertebral malformation (wobblers) [21]. Besides, a simple seropositive result toward *S. neurona* antigen

does not necessarily mean the horse is infected with the parasite since the protozoan must cross the blood brain barrier into the central nervous system to cause the disease. Thus the diagnosis of EPM is handled in three parts: ruling out of other diseases, a complete neurological exam and laboratory tests. Most of the ante-mortem tests available have questionable sensitivities and specificities; western blot is one of the major tests used for detection of IgG antibodies against *Sarcocystis neurona* in cerebral spinal fluid (CSF) and serum. However, the immunoblot method has been shown to have a specificity of 44-60% and sensitivity of 87% indicating that the western blot is useful for ruling out the disease rather than diagnosing it [6]. In addition, the immunoblot method is costly and time-consuming. The use of polymerase chain reaction (PCR) is questionable due to rapid enzymatic destruction of DNA in Cerebral Spinal Fluid (CSF), and thus most samples test negative [40]. The gold standard diagnostic test for EPM is postmortem histopathology.

1.3. Main Objective

The projects were conducted to generate data that could be used in designing screening programs and diagnostic tools for the infectious diseases discussed in this paper.

1.3.1. Specific Objectives

- 1) To screen HIV positive samples for hepatitis B and C
- 2) To develop an ELISA for diagnosis of EPM

1.4. Literature Review

1.4.1. Epidemiology of HIV, HCV and HBV

1.4.1.1. Transmission of Human Immunodeficiency Virus (HIV)

The majority of HIV infections are acquired through unprotected sexual relations. In developed countries, the risk of female to male transmission is 0.04% per act while that of male to female transmission is 0.08% per act. These values are 4 to 10 times higher in low income countries [10]. Randomized controlled trials have shown that male circumcision reduces the risk of female to male sexual transmission by about 60% [29]. The World Health Organization (WHO) secretariat recommends male circumcision to be recognized as an additional intervention to reduce the risk of heterosexual transmission [74]. Vertical transmission can be *in utero*, intrapartum or via breastfeeding. The rate of transmission *in utero* is 15- 25% and the rate of transmission through breastfeeding is 20-45% [20, 79] if the mother does not receive treatment. The Joint United Nations Programme on HIV/AIDS (UNAIDS) estimates show that about 43,000 new infections occur in children through the mother-to-child route and that about 65,000 infections were averted through provision of antiretroviral prophylaxis to HIV positive pregnant mothers [67]. Transmission via blood is of great concern to IDUs, hemophiliacs and recipients of blood transfusion and blood products, and people receiving piercings, tattoos and scar rings. Potential risk of transmission by this route is minimal if sterile procedures are followed.

1.4.1.2. Transmission of Hepatitis C Virus (HCV)

Intravenous drug use is a risk factor because the users may share needles that could possibly be contaminated with HCV [46] thus an estimated 60% of intravenous recreational drug users in the United States have been shown to be infected with HCV [15]. Blood transfusion, blood products or organ transplantation prior to implementation of HCV screening in the United

States was a risk factor [19]. The risk of transmission by this route in the US is less than one per million transfused units [15]. HCV exposure via inadequately sterilized medical or dental equipment (e.g. needles or syringes, hemodialysis equipment, oral hygiene instruments, jet air guns, etc.) has been reported in some countries. This is due to loopholes in the implementation and enforcement of stringent standard precautions in public and private medical and dental facilities. Sexual transmission of HCV is considered to be rare [31]. Studies show that the risk of HCV sexual transmission in heterosexual, monogamous relationships is extremely rare or nil [70]. The risk of transmission from mother to child is about 6% in women who are HCV RNA positive at the time of delivery. In HIV/HCV co-infected pregnant women the risk of transmission of HCV to the fetus rises to 25% [15].

1.4.1.3. Transmission of Hepatitis B Virus (HBV)

In areas that are highly endemic, e.g. China, mother-to-child transmission of HBV is common [42, 39]. In areas where endemicity is intermediate, transmission is either perinatal or from person to person (horizontal transmission). Most of the HBV transmission in Africa occurs in childhood, by the horizontal rather than the perinatal route. The exact mode of transmission is not well understood. It probably involves percutaneous infection through saliva or traces of blood, as well as through unsterile needles, tribal scarification, and other possible vehicles. The route of transmission has very serious clinical implications as there is a high chance of developing chronic hepatitis if the infection is acquired vertically or at preschool age. In low endemic areas, HBV infection is acquired through unprotected sex and drug use. In the UK, the most reported risk factor in adults is injection drug use followed by sexual contact [42, 39]. Drug paraphernalia is often shared and is thus a risk; this includes needles, barrels, filters, spoons, and

water. Tattooing, body-piercing and acupuncture may also pose a risk if unsterile equipment is used.

1.4.1.4. Global prevalence of HBV, HCV and HIV

1.4.1.4.1. Prevalence of HIV

In 2010, UNAIDS estimates indicated that about 34 million people were living with HIV worldwide, an increase of 17% since 2001. The number of people dying of AIDS-related causes has also fallen to 1.8 million from 2.2 million due to increased access to antiretroviral drugs. In fact according to UNAIDS, 2.5 million deaths have been averted in low- and middle-income countries since 1995 due to antiretroviral therapy [54]. As of 2010, an estimated 1.2 million people were living with HIV in Uganda and 150,000 of these are children [27]

1.4.1.4.2. HIV types and subtypes

There are two types of HIV: HIV-1 and HIV-2. HIV-1 resulted from human infection by SIV that infects chimpanzees while HIV-2 resulted from infection by SIV harbored by sooty mangabeys [58]. HIV-1 is responsible for the current worldwide pandemic while HIV-2 is predominant in West Africa and rarely found elsewhere. Although HIV-2 is less pathogenic than HIV-1, both viral infections have similar clinical manifestations [14]. Both viruses are predicted to have originated from two natural reservoirs harboring the simian immunodeficiency virus (SIV).

Of the two types of HIV, HIV -1 is the most common and pathogenic. It has three groups: group M (“major”), group N (“non-M, non-O”) and group O (“outlier”) [50]. Group M is the most prevalent and accounts for about 90% of HIV/AIDS cases and is further sub-divided into subtypes A-K [76]. Group N was discovered in 1998 in Cameroon and it is extremely rare [36].

Group O is rarely seen outside West Africa and is most common in Cameroon. In August 2009, a new strain that is closely related to the gorilla simian immunodeficiency virus (SIVgorr) was discovered in a woman in Cameroon and was designated group P [35].

The first case of HIV-2 in the United States was discovered in 1987 [14]. This strain is rarely seen outside Africa. As of 2010, there are 8 known HIV-2 groups (A-H). Only two of these groups (A and B) are epidemic [14, 58]. Group A is predominant in West Africa, but it can also occur in South Africa, Brazil, India, and to a small extent Europe or USA. Group B is mainly confined to West Africa. HIV-2 A and B are closely related to simian immunodeficiency virus found in sooty mangabeys [14, 58].

1.4.1.4.3. Prevalence of HCV

The WHO estimates that 3% (170 million) of the world's population are chronically infected with HCV, two to 4 million of infected cases are in the United States, five to ten million in Europe, 12 million in India and 5.3% in Sub-Saharan Africa. Infection rates greater than 70% have been reported in IDUs and in haemophiliacs. HCV rates of 20 to 30% have been reported in haemodialysis patients [78]. The prevalence of HCV in Uganda is 1.6%.

1.4.1.4.4. Types and subtypes of HCV

Within an infected person, HCV exists as a population of closely related (quasi) species due to rapid mutations of the viral genome. Worldwide, eleven HCV genotypes have been reported, and a number of more closely related subtypes have been identified. The known genotypes have been numbered from 1 through 11, and the subtypes have been labeled a, b, and c, in order of discovery. US studies of patients with chronic HCV infection have revealed that

genotype 1 is the most common (about 70%), with subtype 1a accounting for a slightly higher proportion than 1b [15].

1.4.1.4.5. Prevalence of Hepatitis B

Two billion people are estimated to be infected with HBV worldwide; 350 million of these have chronic HBV, and 1.2 million deaths from chronic hepatitis, cirrhosis and hepatocellular carcinoma have been reported. South East and sub-Saharan Africa are highly endemic [65]. In Uganda, HBV infection is highly endemic, the highest prevalence of 23.9% has been reported in the northern part of the country. The prevalence in other parts of the country ranges from 3.8% to 10%.

1.4.1.4.6. HBV genotypes and serotypes

Hepatitis B virus (HBV) surface antigen (HBsAg), which is encoded by the HBV *S* gene, is conventionally classified into 4 serological subtypes, “adw”, “adr”, “ayw” and “ayr” [42,39]. Currently there are 8 genotypes of HBV designated A-H. These genotypes are further divided into B1-5, C1-5, D1-4 and F1-4. Genotype C has been associated with abnormal liver function tests, lower rates of seroconversion to anti-HBe, higher levels of serum HBV-DNA, cirrhosis, and Hepatocellular Carcinoma (HCC) than genotype B [42, 15]. However, infections with genotype B show a prolonged response to interferon treatment when compared to genotype C, A, and D infections. Genotype A infection is associated with better clearance of HBV-DNA than genotype D, and a higher rate of HBsAg clearance compared with genotype D. Just like HCV, HBV circulates in serum of infected individuals as a quasi-species [42, 15].

1.4.1.4.7. Global prevalence of HCV among HIV infected patients

Global estimates indicate that 170 million people are chronic HCV carriers and over 40 million people are infected with HIV [15]. Thirty percent of these HIV infected persons are estimated to be co-infected with HCV, making HIV/HCV co-infection a growing public health problem worldwide [60] (See Figure 1). In the United States, approximately 900,000 people are estimated to be infected with HIV, and 33% of these are co-infected with HCV [60]. Co-infection with HIV and HCV is above 50% among HIV-infected IDUs in the United States [31, 60]. HCV infection is one of the most important causes of chronic liver disease in the United States and progresses more rapidly to liver damage in HIV-infected persons due to increased rates of fibrosis and cirrhosis [30, 56].

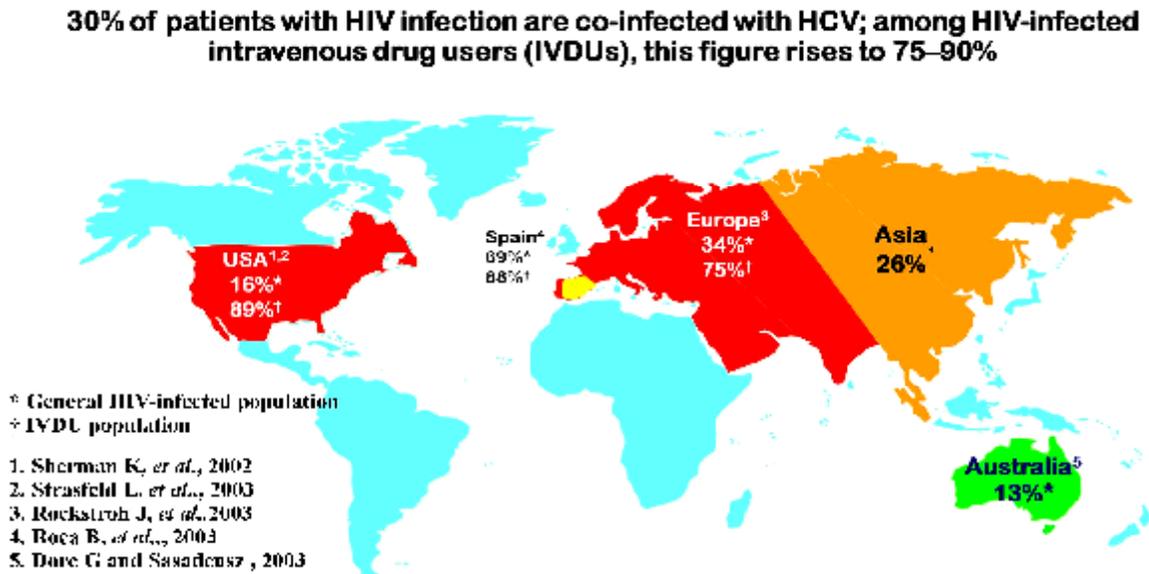


Figure 1. Map of the world showing prevalence of Hepatitis C Virus among Human Immunodeficiency Virus (HIV) patients.

The prevalence of HCV infection among HIV-infected individuals varies widely and depends on the mode of transmission. In a French cohort of 1935 HIV+ patients, a 91%

prevalence of HCV was observed among IDUs, 71%, in recipients of blood or blood products, and 7.3% in sexually transmitted HIV [57]. In smaller HIV+ cohorts of 150-300 patients, a similar pattern of HCV co-infection rate was observed [22, 49, 54]. These studies indicate that parenteral transmission of HCV is very common in HIV/HCV co-infected patients, as well as in patients infected with HCV alone, but sexual transmission is rare. In two large US HIV studies with a sample size of 1687 individuals, the overall rate of HIV/HCV co-infection was estimated at 16% [60]. Globally, HCV infection has been reported in: 72-95% of HIV positive IUDs, 1-12% of men who have sex with men (MSM) and 9-27% of heterosexuals [4].

1.4.1.4.8. HIV/HBV Co infection

In HIV-infected persons, 2-4 million are estimated to have chronic HBV co-infection [4]. At least 90% of HIV negative individuals are able to clear HBV, but HIV-infected persons have half the chance of clearing HBV without treatment. As a result, chronic HBV infection has been observed in 5-10% of HIV-infected individuals exposed to HBV [4]. Studies done in Western Europe and the US show a prevalence of chronic HBV infection of 6-14%: 4-6% of heterosexuals, 9-17% of MSM, and 7-10% of IDUs [4]. In the US, HIV/HBV co infection rates are highest among MSM and IDUs. In Asia and sub-Saharan Africa, where vertical and early childhood exposure are the most common modes of transmission, the prevalence of HBV among HIV-infected individuals is estimated at 20-30% [33].

1.4.2. Natural History of HIV

HIV infection progresses through four phases: acute, chronic asymptomatic, symptomatic and AIDS stages.

1.4.2.1. Acute phase

Following infection, the virus infects T-lymphocytes, and remains sequestered in regional lymph nodes until a threshold of replication is reached. In many cases, this takes 2-4 weeks. This is referred to as the acute phase or primary HIV infection. It is characterized by rapid viral replication accompanied by reduced number of CD4+ T cells and activation of CD8+ T cells with subsequent seroconversion. Some individuals develop an influenza-like infection while others are asymptomatic. Most common symptoms include: rash, painful enlarged lymph nodes, malaise and fever [24, 77].

1.4.2.2. Chronic phase

During the chronic phase, the virus slowly replicates in lymph nodes causing chronic swelling and pain. Persistent viral replication causes a decline in CD4+ T cell numbers. Without Antiretroviral Therapy (ART), the chronic phase may last for 3-10 years after which the patient progresses to the symptomatic stage or AIDS. Towards the end of this stage, individuals may show minor symptoms that include: occasional fevers, skin rash, herpes zoster, recurrent oral ulcerations and recurrent upper respiratory infections. If ART is given at this stage, it may help provide a better prognosis [24, 55].

1.4.2.3. Symptomatic phase

As the viral load continues to increase progressively, the immune system continues to deteriorate. The individual becomes immunodeficient which allows the overgrowth of normal flora and reactivation of old infections (e.g. herpes virus, HBV and TB). As the immune system continues to deteriorate, severe opportunistic infections set in. These are responsible for the signs and symptoms of this stage (e.g. persistent diarrhea, weight loss, Herpes zoster, acne and Candida infections) [24].

1.4.2.4. AIDS phase

The symptomatic phase eventually progresses into fully developed AIDS. This phase is associated with high viral load, severe immunodeficiency and CD4+ T cell counts of below 200 cells/ μ l. The severe immunodeficiency allows opportunistic infections and some cancers (e.g. Kaposi's sarcoma and cervical cancer) to develop. The most common infections affected by AIDS patients are: pneumocystic pneumonia, cachexia and esophageal candida [24].

1.4.3. Natural History of HCV

1.4.3.1. Acute phase

HCV at this stage is infrequently diagnosed because about 70%-80% of infected individuals are asymptomatic [45]. For those who experience symptoms, onset ranges from 3 to 12 weeks after exposure [2, 63]. Symptoms include: malaise, weakness, loss of appetite and jaundice. This stage is characterized by increased serum levels of liver enzyme alanine aminotransferase (ALT). ALT levels begin to rise 2 to 8 weeks after exposure. HCV RNA levels also rise rapidly within the first few weeks and peak before the peak of ALT and onset of symptoms [17]. The acute phase can also be self-limiting where symptoms last a few weeks and subside, and ALT and HCV RNA levels also decline. This occurs in about 15% to 25% infected individuals. Although acute HCV is severe, fulminate liver failure is rare [17].

1.4.3.2. Chronic phase

About 75% to 85% fail to clear the virus and develop chronic hepatitis [17]. This phase is characterized by persistent HCV RNA in the blood for at least 6 months after onset of acute infection. Risk factors for progression to chronic hepatitis include: age at the time of infection [7], gender [3, 37] race [59], development of jaundice [71] during acute infection, and immune

status. The chronic phase can last as long as 12 to 20 years before it progresses to liver fibrosis that will eventually lead to cirrhosis. Approximately 10% to 15% of individuals develop cirrhosis and the major risk factor associated with this is chronic alcohol use [75]. Other factors include: older age at time of infection, male gender, degree of inflammation and fibrosis, co-infection with HIV or HBV, immunosuppression, schistosomiasis infection among others [8, 9, 53]. The chronic stage is usually silent until signs of decompensated cirrhosis, end-stage liver disease, or Hepatocellular Carcinoma (HCC) begin to manifest. These signs include: ascites, varices, hepatic encephalopathy and glomerulonephritis [17].

1.4.4. Natural History of HBV

HBV infection may involve the following phases depending on the age of the patient at time of infection and genetic factors: acute phase, chronic phase or fulminate hepatitis.

1.4.4.1. Acute infection

At the acute stage of infection, the patient may show signs of fever, painful joints and hives within 3 to 6 months after infection. Acute infection is self-limiting and the majority of the patients clear the infection within 1-2 months. The signs associated with the acute phase are non-specific and may include: nausea, body aches, mild fever, loss of appetite, itchy skin and malaise [42].

1.4.4.2. Chronic infection

This phase is characterized by persistent HBV DNA for more than 6 months and hepatic inflammation. This stage has four phases: immune tolerant, immune clearance, non-replicative and reactivation phase [42]. During the immune tolerant stage, patients test negative for HBsAg, positive for HBeAg and have increased levels of HBV DNA in their blood stream. Alanine

aminotransferase (ALT) levels are usually normal. This phase is most common in children infected at birth and may last for 2-3 decades. The immune clearance stage is commonly observed in individuals that become infected during adulthood. HBeAg and HBV DNA levels decrease while ALT levels increase and necroinflammatory changes are seen. This stage is accompanied by seroconversion to anti-HBc followed by entry into the non-replicative phase. At this stage, the infection is under the control of the immune system of the patient. HBV DNA may or may not be detected in plasma, and serum ALT levels return to normal. The patient then becomes a carrier. However, some individuals show reactivation of the infection due to co-infection with another virus e.g. HIV or HCV, and other factors. Persistent inflammation and necrosis of the liver may lead to fibrosis, cirrhosis and HCC. The following symptoms will begin to manifest: ascites, varices, hepatic encephalopathy and glomerulonephritis [42]

1.4.5. Impact of HIV on the Course of HBV Infection

HIV/HBV co-infection increases the risk of cirrhosis, end stage liver disease, and death, particularly in patients with a low CD4+ T cell count [64]. Compared to patients infected with HBV alone, patients with HBV and HIV have higher rates of chronic HBV infection and higher HBV DNA levels. This indicates that in the presence of HIV, the course of acute HBV is altered and this is associated with lower rates of spontaneous clearance of HBV [25] and lower rates of clearance of HBeAg [52,61]. HIV/HBV co-infection may also reactivate HBV infection in asymptomatic carriers. In cohort studies, the risk of liver-related mortality has been reported as 2-3 times higher in HIV/HBV-co-infected patients than in HIV infected patients [11, 73]. HIV co-infection is also associated with regular bursts of hepatic transaminases, which can occur due to Antiretroviral Therapy (ART), interruption of HIV/HBV treatment, or the development of resistance to HIV/HBV treatment [16].

1.4.6. Impact of HBV on the Course of HIV Infection

There are conflicting data with respect to the impact of HBV on the progression of HIV infection. Some studies showed an increased rate of HIV progression to AIDS among individuals with markers of exposure to HBV [23], whereas other studies do not show any change in the progression of HIV disease or survival [26]. However, cohort studies indicate that HBV does not impact the progression of HIV infection [38, 40].

1.4.7. Impact of HIV on the Course of HCV Infection

The natural history of HCV infection is accelerated in patients with HIV [28]. It leads to an increased rate of progression to cirrhosis, decompensated liver disease, hepatocellular carcinoma and death [51]. For example, a Madrid study conducted between 1996 and 2000, end-stage liver disease due to HCV was the cause of 45% deaths of HIV infected patients, 80% of whom were IDUs [43].

1.4.8. Impact of HCV on the Course of HIV Infection

Hepatotoxic effects of antiretroviral therapy are more likely to develop in patients with HCV or HBV infection [12, 34, 48]. Hepatotoxic effects of antiretroviral therapy have been shown to correlate positively with fibrosis [5] in HIV co-infected individuals. HCV infection is linked with defects in cognitive and psychiatric function [18], poor quality of life and an increased prevalence of diabetes mellitus [13]. These links have the potential to influence the management of HIV infection.

1.4.9. Treatment of Viral Hepatitis in HIV co Infection

HIV patients need to be screened for HBV and HCV, and vaccinated against Hepatitis A. The aim of HBV treatment is to slow down viral replication and minimize ongoing liver damage.

The U.S. Department of Health and Human Services guidelines recommend fully active ART for HIV/HBV co-infected patients who require HBV treatment, even if they lack indication for HIV treatment [66]. Pegylated interferon (IFN) is also recommended, but there is limited data on its efficacy and safety in HIV/HCV co-infection. Scientific data indicates that long-term treatment with IFN is not feasible due to toxicity and poor tolerability [21]. Pegylated interferon and ribavirin are recommended for treatment of liver cirrhosis in HCV infection. However, patients need close monitoring for side effects since HIV/HCV co-infected patients experience rapid progression of liver fibrosis drug toxicity is common. This is also associated with poor response to treatment [44].

1.4.10. Epidemiology of EPM

A retrospective analysis of equine data from 2000-2005 at equine diagnostics in Lexington, Kentucky revealed a seroprevalence of 58% in the US [38]. Other studies have shown an exposure rate of 33-89% in horses located in geographical regions where the opossum is present: Pennsylvania (~ 45%)[3], Oregon (45%)[5], Ohio (53.6%)[42], Colorado (33.6%)[46], Michigan (60%)[41], California (27%), Florida (28%), Oklahoma (89.2%)[2], Missouri (54%)[49], Montana (0%)[49], and New Zealand (0%)[49]. A seroprevalence rate of 35% was reported in South America [12, 15]. The seroprevalence rate for *N. hughesi* is quite low; however a study done in California showed a rate of 37% [49]. Risk factors for the disease include: age, presence of opossums on the farm, presence of woods on the farm, seasonal effect e.g. duration of winter [43]. Young horses below five years of age are four times more likely to develop EPM than horses between 5 and 20 years of age. Also, young horses less than 3 years that are used for training or showing are at an increased risk to develop EPM because of the stress associated with these activities [43, 40]. Although a high percentage of horses has been

reported to have previously been exposed to *S. neurona*, few horses develop clinical disease. In fact, an estimate of the incidence in one study was reported at 1% of all horses or less each year [40]. A United States Department of Agriculture (USDA) study documents an incidence of 0.14% [36] in the US.

1.4.11. Life Cycle of *S. neurona*

The opossum is the definitive host and a several other animals including armadillo, skunk, raccoon, and domestic cats are intermediate hosts. The prevalence of the parasite in the definitive host is reported to be 18-25% [16]. Since the opossum is a scavenger it will feed on intermediate host carcasses and if these intermediate hosts are infected with encysted stages, then the opossum will ingest these cysts. Intermediate hosts become infected by ingesting sporocysts in contaminated food or water; the sporocysts then excyst in the gut and release sporozoites. The sporozoites invade the vasculature and are carried to the muscle tissue to form muscle cysts known as sarcocystis (Number 1 in Figure 2). *S. neurona* intermediate hosts include: the nine banded armadillo, the striped skunk, the raccoon, and domestic cats [7-10]. A *S. neurona* prevalence of 59% has been reported in raccoons [10].

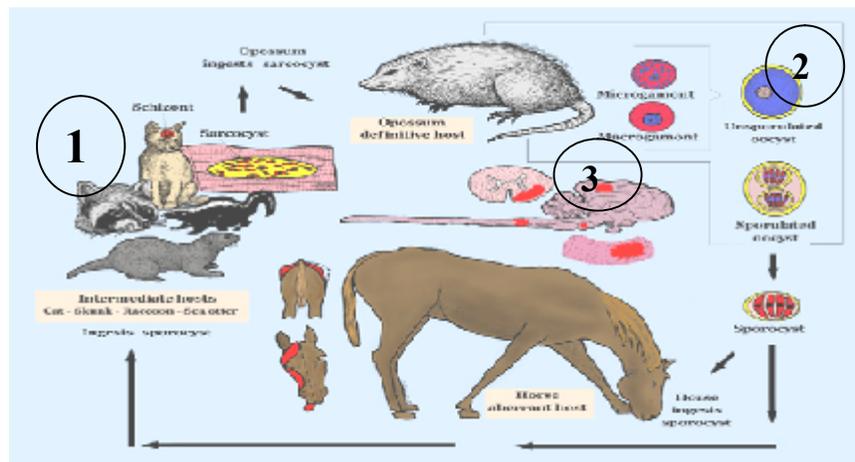


Figure 2. Life cycle of *S. neurona*: Courtesy of the Ohio State University and Tim Vojt [40].

Inside the definitive host the organism undergoes sexual reproduction (number 2 in Figure 2) to produce large numbers of sporocysts which are shed in the feces and are infective to the intermediate hosts. The intermediate host will develop clinical disease when the protozoan invade the nervous system. The Sarcocysts are the infective stage for the definitive host so animals that do not form Sarcocysts e.g. horses are aberrant intermediate hosts [40]. In the horse, ingested sporocysts pass into the epithelium of the small intestines where they excyst to release sporozoites that enter the blood stream. The sporozoites replicate in the endothelial cells of the blood vessels to produce tachyzoites which migrate to the Central Nervous System (CNS). In the CNS, the tachyzoites slowly replicate asexually in neurons and microglia cells causing gradual destruction of the nervous tissue (number 3 Figure 2). This damage is responsible for the clinical signs of EPM, which include: ataxia, lack of coordination, muscle atrophy, paralysis of muscles of the eyes, face or mouth and others [14, 40].

1.4.12. Diagnosis of EPM

EPM is quite challenging to diagnose because there is no test or group of tests that can accurately diagnose the infection. Confirmation of a suspected case is attained through a combination of: neurological examination, blood and Cerebral Spinal Fluid (CSF) serology, cytological examination of CSF, examination of cervical vertebral radiographs, a myelogram, response to treatment and postmortem examination [40]. Some authors have reported the use of complete and differential cell counts or serum chemistry [14]. These assays do not produce consistent results but they may help rule out other infections. The ratio of IgG concentration in serum and CSF can be used in conjunction with the albumin quotient to evaluate the integrity of the blood brain barrier. Elevated albumin concentration in CSF and /or AQ indicate that blood brain barrier permeability is likely or the sample is contaminated with blood [40]. The western

blot is the most used ante mortem assay to detect IgG antibodies against *S. neurona*. ELISAs based on the snSAGs have been developed and showed to be highly specific and sensitive. IFA has a specificity and sensitivity of 100% but this assay is known to cross react in horses exposed to *S. fayeri* [7]. PCR has been developed to detect *S. neurona* but it has poor clinical sensitivity since most samples test negative. Because most merozoites don't enter the CSF and the DNA is destroyed by enzymatic action in CSF [40]. The only accurate test for EPM is a post-mortem autopsy that reveals multifocal randomly distributed hemorrhagic lesions in the brain or spinal cord. In sub-acute infection, the lesions show areas of discoloration (pale to dark) [14].

1.4.13. Surface Antigens of *S. neurona*

S. neurona surface antigens are orthologues of the surface antigens (SAG) and SAG-Related sequences (SAG/SRS) family surface proteins in *Toxoplasma gondii* [27]. These SAG/SRS antigens are developmentally regulated and have been shown to have sequence similarity to *T. gondii* surface antigens, TgSAG1 or TgSAG2, that are involved in receptor-ligand interactions with the host and modulation of the host immune responses [25]. The *S. neurona* surface antigens have been designated SnSAG1, SnSAG2, SnSAG3 and SnSAG4. All these SAGs are membrane associated and are displayed on *S. neurona* merozoite surface. SAG1 and SAG2 are the two most immunodominant antigens that were previously known as Sn30 and Sn16, respectively [28]. Immunofluorescence labeling of all the four SAGs reveals that they are present throughout intracellular development [25]. SAG1 is reported to be highly immunogenic as revealed by a cDNA library immunoscreening that identified SnSAG1 gene in 22 of the 25 reactive phage clones [25]. The basis of naming SnSAG1 through SnSAG4 was as a result of their chronological identification and abundance [25]. Given that they are displayed on the surface, are highly immunogenic and are expressed throughout the development of the protozoa,

the SAGs are promising targets for therapeutic intervention (e.g. Vaccine development and diagnostics) in EPM.

1.4.14. Treatment and Prevention of EPM

The US Food and Drug Administration (FDA) approved a combination therapy of 1 mg/kg of pyrimethamine and 20 mg/kg of sulfadiazine for 30 days or 120–150 days for the treatment of EPM. If the CSF remains positive and/or the horse continues to show clinical signs, then the treatment period is extended [40]. Other drugs include ponazuril and nitazoxanide. Ponazuril is an anticoccidial triazine-based compound, and it is well tolerated with few complications, and no significant elevations of serum chemistry values or changes of complete blood counts have been observed [22, 29, 35]. Nitazoxanide (NTZ) is a broad-spectrum drug and works against bacteria, protozoa, and helminths. It has been shown to kill *S. neurona* in cell culture. In a clinical trial, the treatment of horses of EPM with NTZ showed an efficacy of 63% [39]. Protection of feed and bedding supplies from exposure to opossums or other small wildlife has been reported to reduce the incidence of EPM on the farm [40]. Currently there is no effective vaccine for use against EPM.

**CHAPTER TWO. THE PREVALENCE OF HEPATITIS B AND C AMONG PERSONS
LIVING WITH HIV/AIDS ATTENDING ANTIRETROVIRAL THERAPY (ART)
CLINIC AT MULAGO NATIONAL REFERRAL HOSPITAL IN UGANDA**

2.1. Introduction

Liver disease caused by chronic hepatitis B Virus (HBV) and hepatitis C Virus (HCV) is increasingly being implicated as the major cause of morbidity and mortality among Human Immunodeficiency Virus (HIV) infected individuals [73]. The three viruses have common routes of transmission, so co-infections are very common. In fact, a 91% prevalence of HCV among HIV patients has been observed among IDUs. About 5% to 10% of HIV patients are reported to harbor persistent serum HBsAg [4].

HBV is a small hepatotropic DNA virus (of the family *Hepadnaviridae*) that causes liver damage. Its genome is quite unique because the DNA is partially stranded and contains four overlapping open reading frames. The virion measures about 42 nm and it has an outer envelope containing hepatitis B surface antigen (HBsAg) proteins. This surface protein encloses the nucleocapsid that carries the viral genome. Hepatitis B e antigen (HBeAg) is a nonstructural protein of HBV that is a marker of active viral replication [42]. Hepatitis C virus (HCV) is an enveloped virus of the family *Flaviviridae* family. The HCV genome is a single-stranded, positive-sense RNA. The virion structure consists of an envelope glycoprotein which surrounds the core protein. The core protein in turn encloses the genetic material [19]. The HIV genome is made up of two copies of single-stranded RNA enclosed in a conical capsid. The capsid is in turn surrounded by a lipid envelope. Attached to the envelope are glycoproteins (gp41) that provide docking sites for glycoprotein 120 (gp120).

2.1.1. Question to be Answered

What is the prevalence of viral hepatitis B and C among HIV+ patients visiting antiretroviral therapy (ART) clinic at the Mulago referral hospital?

2.1.2. Specific Objectives

- 1) To screen serum samples for viral hepatitis B surface Antigen (HbsAg), viral hepatitis C antibody (HCVab) and HIV 1&2 antibodies.
- 2) Establish a baseline of HIV/HCV/HIV co-infected persons in Uganda.

2.2. Materials and Methods

2.2.1. Study Location

This study was conducted at the Mulago National Referral hospital using serum samples provided by the Mulago-Mbarara Joint AIDS program (MJAP). The Mulago National Referral Hospital is the largest hospital in Uganda located in the northern part of the capital city Kampala.

2.2.2. Study Design

This was a retrospective, case series study in which blood samples from suspected HIV positive patients were assayed for the presence of HIV 1 and 2 antibodies, Viral Hepatitis B Surface Antigen (HBsAg) and Viral Hepatitis C antibodies (HCVab). The total number of samples tested was 100; 47 of these were from males and 53 from females.

2.2.3. Sample Collection and Analysis

Blood samples were collected by venipuncture from HIV+ patients on antiretroviral therapy (ART) and processed to harvest plasma. Stored plasma at -20°C was thawed and screened for the presence of HIV 1 & 2 antibodies, HBsAg and HCV antibody using

chromatography immunoassay (CIA) rapid test kits according to manufacturer's instructions and Uganda Ministry of Health algorithm (for HIV testing). MJAP obtained these blood samples for general patient care, and the patients consented to having their blood used for further testing. The resulting waste from the testing procedure was disposed of through the Mulago Hospital waste disposal system.

2.3. Results

Of the HIV + (N=100) samples tested; 10 tested positive for HCV, and 13 samples tested positive for HBV. The number of female patients that test positive for both HBV and HCV was slightly higher than that for male patients (refer to figure 4 and Table 1). However, we were not able to establish whether there was any correlation between HBV and/or HCV infection and gender since the chi-square values were invalid due insufficient sample size. The raw data is described in Figure 3.



Figure 3. Pie charts showing the frequency of HBV (13%) and HCV (10%) among HIV patients in Uganda (N=100).

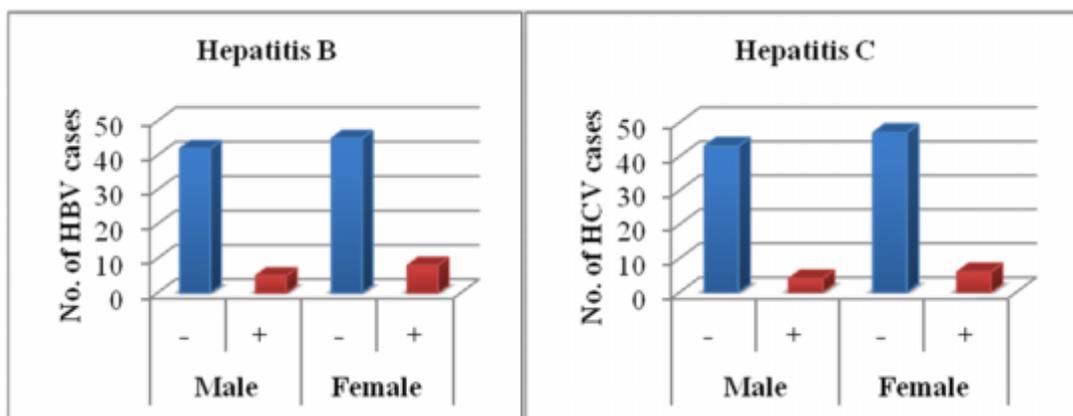


Figure 4. Bar graphs showing the frequency of Hepatitis B (left graph) in HIV+ males and females, and the frequency of Hepatitis C in HIV+ males and females (right graph).

Table 1. Showing number of cases with HIV and/or hepatitis B and C co-infection

Number of (HIV/HBV)+		Number of (HIV/HCV)+		Number of (HIV/HBV/HCV)+	
Female	Male	Female	Male	Female	Male
8	5	6	4	1	0

2.4. Discussion

Serological results from this study indicate that approximately 23% of HIV seropositive patients were exposed to hepatitis B and C. A study done at the same facility found a 3.3% HCV prevalence among HIV patients [72]. Another study performed in 2000 at the Joint Clinical Research Centre (JCRC) an antiretroviral therapy (ART) clinic in Uganda got a 2.8% HCV prevalence among HIV patients [62]. Our study reports a 10% HCV prevalence.

Among IDUs, the prevalence of HCV in HIV+ patients has been reported between 70% and 90% [60]. Because intravenous drug use is very rare in Uganda, this is rarely a risk factor for transmission of HCV thus the prevalence of HCV is often low. This correlates with studies done in Western Europe and the US that observed a prevalence of 7.3% (HCV) in sexually transmitted HIV [60]. Londenyo *et al.*, 2000 [41] in South Africa observed a HBV/HIV co-infection rate of

41%. Similar studies in Kenya reported equally high figures [49]. Among HIV-positive persons studied from Western Europe and the US, chronic HBV infection has been reported in 6-14% patients [4]. Here we report a prevalence of 13% (HBV/HIV) in Uganda.

In our study Hepatitis B and C viral loads were not performed due to limited time and resources; however, having these data would help clarify the actual prevalence of viral hepatitis exposure and chronic status. Some reports indicate cross reaction of the chromatography immunoassays (CIA) with other antigens and/or antibodies from other infectious diseases (e.g. malaria) which may give false positives [1]. We did not have a comparable group of HIV-uninfected patients to help account for confounding variables like the presence of other infections or sexually transmitted disease that would affect disease progression. Also, this study did not put baseline CD4 T cell counts under consideration; this could limit the validity of antibody responses measured and increase false-positives.

2.5. Conclusions

In this study, the number of HIV/HBV and HIV/HCV co-infected females was slightly higher than the number of co-infected males. The interpretation of this result is constrained because the chi-squares were invalid. This could be avoided if a large sample size had been used. Compared to the two studies done in Uganda, our study shows a much higher prevalence of HCV (10%) among HIV patients. These data are not very conclusive either since we did not quantify HCV DNA which would help differentiate carrier patients from those with the infection. This could have resulted into an overestimation of the infection.

HCV/HBV/HIV co-infections are becoming increasingly common. This raises the need to devise systems that incorporate HCV and HBV screening services, and treatment programs

into HIV prevention and control programs. This cannot be successfully achieved unless a nationwide survey of all HIV patients is performed to determine the infection burden of HIV/ HBV and/or HCV and risk factors associated with this co-infection. Such data are very useful in conducting health policy assessments, setting research priorities and designing screening programs.

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**CHAPTER THREE. DEVELOPMENT AND EVALUATION OF A *SARCOCYSTIS*
NEURONA ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) BASED ON THE
TRIPLE DOMAIN ANTIGEN rSAG2/4D1/3D2**

3.1. Introduction

S. neurona is a single celled apicomplexa parasite of the coccidian group and sarcocystidae family. It is the most common cause of EPM. Only asexual stages have been reported in the horse and are confined in CNS. The SAG proteins used in this study are expressed on the surface of merozoites. The function of the SAGs is still under investigation but some reports say they could be used for attachment to the host cell. In this project a chimeric antigen made of three surface proteins of *S. neurona* merozoites was used to develop an ELISA.

3.2. Broad Objective

To develop and evaluate a *S. neurona* Enzyme Linked Immunosorbent Assay (ELISA) using a trivalent antigen.

3.2.1. Specific Objectives

- 1) To express the recombinant protein SAG2/4D1/3D2;
- 2) To purify the recombinant protein;
- 3) Evaluate the ability of the recombinant to detect antibodies against *S. neurona*.

3.4. Materials and Methods

3.4.1. Study Location

This project was performed at the University of Kentucky Equine research center in Dr. Daniel Howe's laboratory. This laboratory has cloned and transformed these *S. neurona* surface antigens into *E. coli* cells. This laboratory also works closely with the Equine Diagnostic

Solutions (EDS) laboratory to continually evaluate the accuracy of these ELISAs. The EDS is a commercial laboratory that obtained a license (from the University of Kentucky research foundation) to use the rSAGs for diagnosis of EPM.

3.4.2. Steps in Recombinant Protein Generation

First, the protein was expressed in BL-21 codonplus cells using Isopropyl β -D-1-thiogalactopyranoside (IPTG). The expressed protein was purified and quantified. In final procedures, ELISAs were run to evaluate the ability of rSAG2/4D1/3D2 to detect antibodies against *S. neurona*.

3.4.3. Recombinant Protein Expression, Purification and Quantification

3.4.3.1. Preparation of Starter Culture

The starter culture was prepared by streaking out rSAG2/4D1/3D2 (from a glycerol stock) on LB agar plate. The agar plate was incubated overnight at 37°C. The following day, a single colony was picked and inoculated into 3 ml of LB containing carbenicillin (CARB) and chloramphenicol (CAM) to a final concentration of 1:1000 and 1:2000, respectively. The inoculum was incubated at 37°C in an incubator shaker for 3 hours. It was then stored at 4°C overnight.

3.4.3.2. Preparation of an Induction Culture and Induction of Expression

The induction culture was prepared by inoculating 21 ml (in a 250 ml flask) of LB broth (containing CARB and CAM to a final concentration of 1:1000 and 1:2000, respectively) with 210 μ l of starter culture. The flask was placed in an incubator shaker until the optical density (O.D₆₀₀) was 0.6. Two ml of the culture was placed into a 15 ml tube and IPTG at a final concentration of 1:1000 was added to the remainder of the culture to induce expression. The

flask together with the 15 ml tube was left in the incubator shaker for 3 ½ hours to allow expression to take place. The induced culture was transferred into a polypropylene centrifuge tube and centrifuged at 12,000 RPM for 15 minutes to collect a pellet. The supernatant was drained off and the tube was stored at -20°C overnight.

3.4.3.3. Recombinant Protein Extraction and Purification

The pellet from the above procedure was resuspended in a mixture of 4 ml of BugBuster (Novagen: United States), 4 µl of benzonase and 50 µl of lysozyme (20 mg/ml; Thermo Fisher Scientific) and left to digest for 20 minutes on a rotary mixer. The mixture was then pelleted at 12,000 RPM for 20 minutes at 8°C and the supernatant was saved for Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The pellet was resuspended in 4 ml of BugBuster and 20 µl of lysozyme and the mixture was incubated at room temperature for 10 minutes. In subsequent steps, the pellet was treated twice with BugBuster at a dilution of 1:10. The generated inclusion body pellet was left to dissolve in 2 ml of equilibration/wash buffer containing 6 M urea (pH 7.0) overnight on ice. Protein purification was done on the Talon resin (Becton, Dickson Biosciences; United States) followed by electrophoresis in an SDS-PAGE gel. The concentration of the purified protein was determined by colorimetric assay (Coomassie Plus Protein assay reagent, PIERCE).

3.4.4. Serum Samples

The positive control serum sample was from two clinically affected horses that were confirmed with EPM. The negative control serum sample was from a pre-infection weaning foal that was used in a *S. neurona* research trial. The 13 EPM samples used to evaluate this ELISA showed disparate reactivities with the two rSAGs (i. e. rSAG2 and rSAG4D1/3D2) that are

currently used at the EDS laboratory. The EDS laboratory obtained a license from the University of Kentucky Research Foundation group to use these antigens to diagnose EPM but with time differences in antibody detection have been documented.

3.4.5. Enzyme Linked Immunosorbent Assays (ELISAs)

3.4.5.1. Determination of rSAG2/4D1/3D2 Concentration to Use in ELISAs

After expression and quantification of the rSAG2/4D1/3D2, we had to determine the appropriate concentration to coat ELISA plates. Four medium-binding ELISA strips were incubated overnight at 4°C with 75 µl of the purified antigen solution diluted to: 0.25 µg/ml, 0.5 µg/ml, 0.75 µg/ml, and 1.0 µg/ml in each respective strip. Each column in the table below represents a strip and each cell represent a well. For strip 1, each well was coated with 75 µl of 0.25 µg/ml of rSAG2/4D1/3D2; for strip 2, each well was coated with 75µl of 0.50 µg/ml of rSAG2/4D1/3D2; for strip 3, each well was coated with 75 µl of 0.75 µg/ml of rSAG2/4D1/3D2, and for strip 4, each well was coated with 75 µl of 1.00 µg/ml of rSAG2/4D1/3D2. The strips were kept at 4°C overnight.

Table 2. Illustrating the layout of ELISA strips

Strip 1 0.25 µg/ml	Strip 2 0.5 µg/ml	Strip 3 0.75 µg/ml	Strip 4 1.0 µg/ml
Positive serum	Positive serum	Positive serum	Positive serum
Positive serum	Positive serum	Positive serum	Positive serum
Negative serum	Negative serum	Negative serum	Negative serum
Negative serum	Negative serum	Negative serum	Negative serum
Blank	Blank	Blank	Blank
Blank	Blank	Blank	Blank

The following morning, the strips were rinsed three times with PBS containing 0.05% Tween 20 (PBST) and blocked for 1.5 hrs at room temperature (RT) with 200 μ l (each well) of PBS containing 1% Tween 20, 5% Normal Goat Serum (NGS), and 1% nonfat dry milk. Primary sera (i.e. positive and negative serum samples) was diluted to 1:250 in PBS containing 0.1% Tween 20, 0.5% NGS, and 1% nonfat dry milk. 75 μ l aliquots of each antibody mixtures were added to duplicate wells of the strips (as shown above) and incubated for 1 hour at 37^oC. The wells were rinsed five times with PBST and incubated for 1 hour at 37^oC with 75 μ l (each well) of horseradish peroxidase (HRP)-conjugated goat anti-horse immunoglobulin G (IgG) secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) diluted to 1:5000 in PBS containing 0.1% Tween 20, 0.5% NGS, and 1% nonfat dry milk. The wells were then rinsed five times with PBST. The chromogenic substrate TMB (3, 3', 5', 5',-tetramethylbenzidine; Pierce, Thermo Fisher Scientific, Inc.) was added to each well and the color was allowed to develop for 10 minutes. The reaction was stopped with 75 μ l (each well) of 2 M H₂SO₄, and the optical density at 450 nm (OD₄₅₀) was measured in an E_{max} microplate reader (Molecular Devices.) The signal-to-noise ratio was calculated by dividing the average O.D of the positive serum sample by the average O.D of the negative serum sample for each concentration of the antigen. The concentration with highest ratio was used to coat ELISA plates to evaluate the rSAG2/4D1/3D2 ELISA.

In the next step, we evaluated the rSAG2/4D1/3D2 ELISA in comparison to rSAG2 and rSAG4D1/3D2. A 96-well microtitre plate was portioned into three sections (four rows of wells per section) and each section was coated with these antigens: rSAG2, rSAG4D1/3D2 and rSAG2/4D1/3D2 respectively. The ELISA was run as described above using the 13 EPM

positive serum samples as the primary antibody and HRP-conjugated goat anti-horse IgG as the secondary Ab.

3.5. Results

3.5.1. Recombinant Protein Expression and Purification

The recombinant protein was expressed in BL-21 codonplus *E. coli* cells and purified on the Talon resin. After purification 10 μ l of the protein were mixed with 10 μ l of sample buffer and run on a 12% SDS-PAGE gel. The resulting gel was visualized in the chemiluminescence imager (FluoroChem M.) and is shown in figure 3. The thicker band of rSAG2/4D1/3D2 is present in the induced sample (lane I) and column load (lane CL) but missing in the uninduced sample (UI.) The purified protein (lane P) band was observed at the same position as the thick band in the induced sample, further confirming that expression of rSAG2/4D1/3D2 was successful.

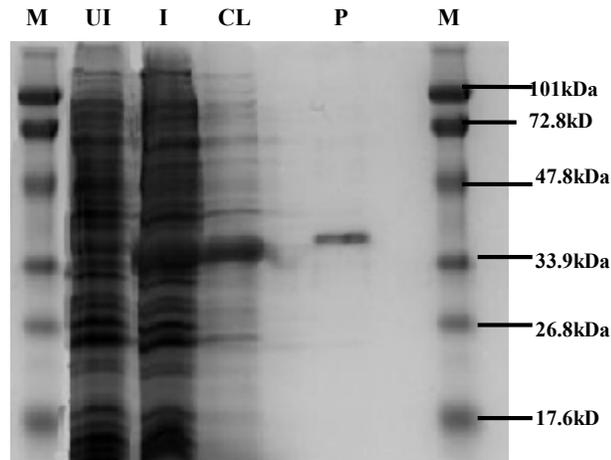


Figure 5. 12% SDS-PAGE showing results of expression and purification of rSAG2/SAG4D1/3D1. 10 μ l of the induced (I), uninduced (UI), column load (CL) and purified (P) samples were mixed with sample buffer and heat treated before loading 10 μ l on the gel. Note the thicker band in the induced sample that is missing in the uninduced sample.

3.5.2. Determination of rSAG2/4D1/3D2 Concentration to Use in ELISAs

The table below shows OD₄₅₀ readings of an ELISA performed when the strips were coated with different concentrations of the recombinant protein. Using these data, the signal-to-noise (S/N) ratio was calculated to determine which concentration produced the strongest signal. From the table, we note that the strongest signal was produced by 1 µg/ml concentration. Thus this concentration was used in the evaluation step.

Table 3. Results of an ELISA determining the correct concentration of rSAG2/4D1/3D2

SAMPLE (O.D ₄₅₀)	0.25 µg/ml	0.5 µg/ml	0.75 µg/ml	1 µg/ml
POSITIVE	0.531	0.729	1.332	1.637
POSITIVE	0.439	0.705	1.461	1.743
NEGATIVE	0.026	0.040	0.060	0.019
NEGATIVE	0.071	0.012	0.044	0.053
BLANK	-0.004	0.006	-0.001	-0.020
BLANK	0.004	-0.006	0.001	0.020
S/N*	1:10.1	1:27.6	1:26.9	1:46.9

*S/N= average O.D of positive serum/average O.D of negative serum

3.5.3. Evaluation of the rSAG2/4D1/3D2 ELISA

To evaluate the use of rSAG2/4D1/3D2 chimera for antibody detection, the chimeric antigen was incorporated into standard indirect ELISAs alongside rSAG2 and rSAG4D1/3D2 using sera from 13 confirmed cases of EPM. The samples were run in duplicates and the results are shown in the table below. The ODs highlighted in pale blue showed differences in antibody detection for the three rSAG antigens.

Table 4. Evaluation of rSnSAG single antigen and chimeric ELISA

Plate 1 OD ₄₅₀	rSAG2 (1:175)				rSAG4D1/3D2 (1:700)				rSAG2/SAG4D1/3D2 (1:26.4)			
	1	2	3	4	5	6	7	8	9	10	11	12
A	3.725	3.539	0.45	0.366	0.6975	0.4385	1.5075	2.6505	1.9855	1.3725	1.8645	1.3635
B	2.271	1.722	2.069	1.278	3.6505	2.6805	3.3995	3.6035	3.4575	2.9275	3.5925	3.1065
C	2.348	2.167	1.785	1.569	0.9215	0.7655	3.4335	3.5455	1.3825	0.9605	3.4395	2.9635
D	2.572	2.273	0.91	0.618	3.7065	3.4105	1.1475	1.0305	3.1305	2.8645	1.0765	0.8935
E	2.043	2.123	1.416	0.675	0.9625	0.8115	3.7835	3.7155	1.8195	1.5925	3.8205	3.4595
F	1.947	1.774	0.43	0.334	0.9965	0.7505	1.2255	0.8885	1.8225	1.4035	0.9985	0.6175
G	0.111	0.108	2.953	1.402	0.0675	0.0535	1.7965	1.2585	0.1495	0.0765	1.6125	1.4005
H	3.529	3.546	0.004	-0.004	3.6805	3.5575	0.0045	-0.0045	3.5845	3.3725	0.0035	-0.0035

Samples showing disparate antibody reactivity
 Negative control samples
 Positive control samples
 Blanks

For instance, highlighted samples in row “A” were from the same case but the ODs for rSAG2 are very low compared to the ODs for the chimeric antigens. The same scenario is observed for samples in row “F”.

Each sample OD was then compared with the positive and negative controls within their group to calculate a percent positive (PP) value by using this formula;

$$PP (\%) = [OD (\text{sample}) - OD(\text{NC})] / [OD(\text{PC}) - OD(\text{NC})] \times 100 [51],$$

where PP is the percent positivity of each sample, NC is the average negative control OD, and PC is the average positive control OD. The PP value represents the sample’s measured percent of the positive control; it gives a relative OD value thus eliminating errors that would arise when an absolute OD is used to determine a positive or negative result. Figures 4 to 6 below represent the data generated from these comparisons. Overall, rSAG2 produced the lowest detection power compared to the other two antigens.

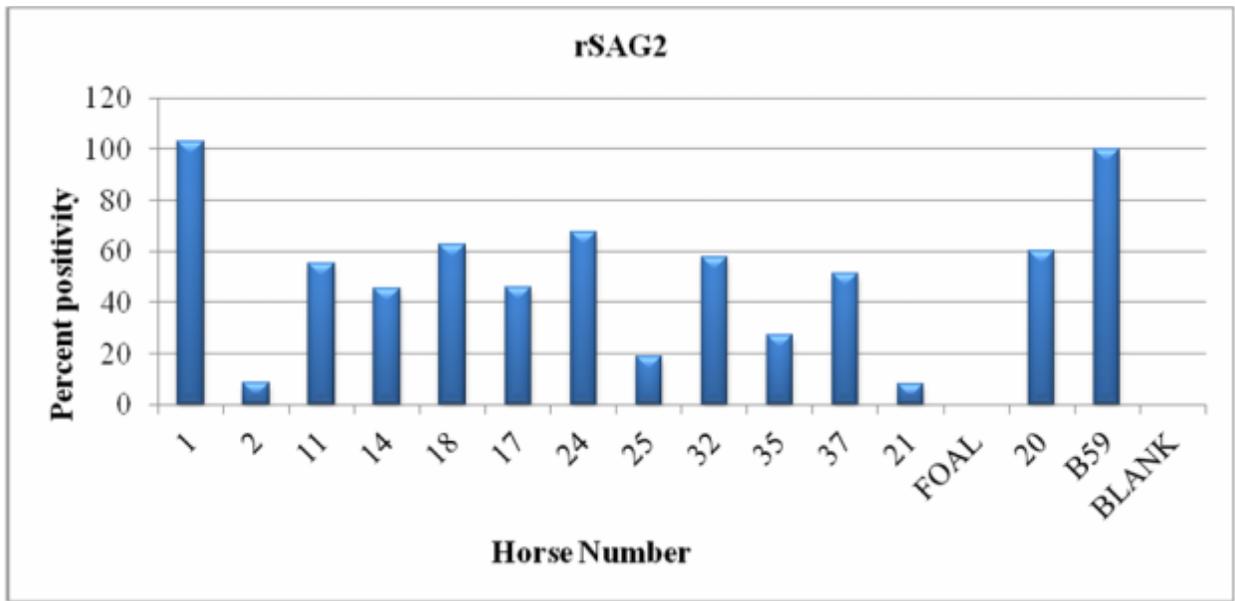


Figure 6. Percent Positivity of rSnSAG2 single antigen. FOAL is the negative control and B59 is the positive control. Samples 2 and 21 would be considered negative at 15% PP cut off.

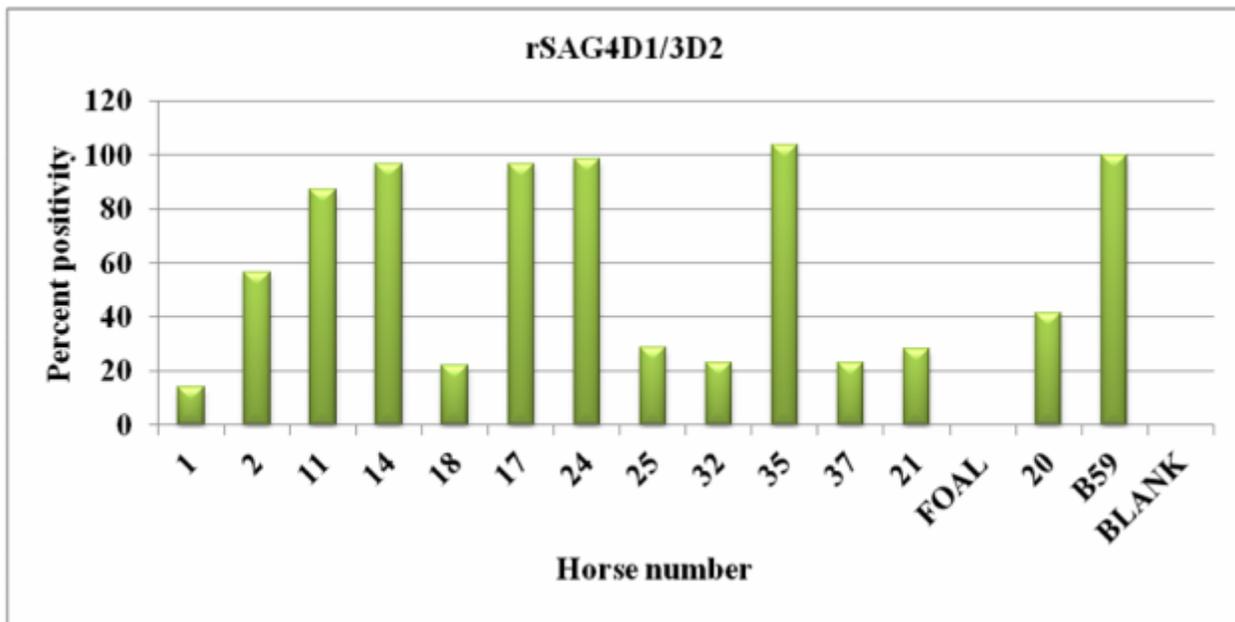


Figure 7. Percent positivity of rSAG4D1/3D2 double domain antigen. FOAL is the negative control and B59 is the positive control. Sample 1 would be considered negative at 15% PP cut off.

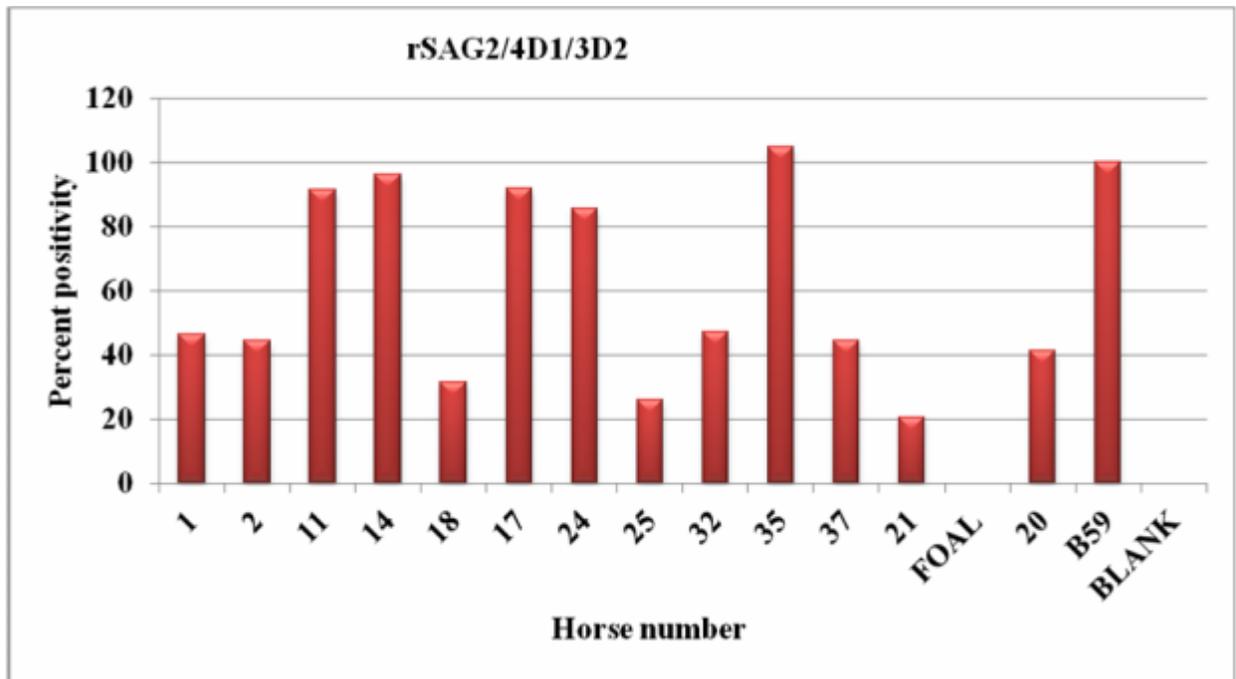


Figure 8. Percent positivity of rSAG2/4D1/3D2. This antigen showed increased detection of antibodies against *S. neurona*. FOAL is the negative control and B59 is the positive control. Note: most of the samples gave signal strengths greater than 15% Positivity cut off.

3.6. Discussion

The SnSAG merozoite surface antigens have been proven to be good candidates for serologic diagnosis of *S. neurona* infection especially when incorporated into ELISAs [35]. ELISAs based on recombinant forms of SnSAG2, SnSAG3, and SnSAG4 have been shown to be accurate and reliable for detection of antibodies against *S. neurona* [23, 35]. Michelle *et al.*, 2011 [35] have created polyvalent assays consisting of mixtures and chimeras of rSnSAG2, rSnSAG3, and rSnSAG4 into single ELISAs. These polyvalent assays have performed accurately compared to original ELISA assays. For instance, a sensitivity of 90.5% and specificity of 100% was reported for a cocktail of rSAG2, rSAG4D1 and rSAG3D2 at 15% positivity compared to rSAG2 that produced a sensitivity and specificity of 85.7% and 100% respectively at 15% positivity [35].

These differences could arise from the antigenic diversity that exists in various strains of *S. neurona*. For example, the major surface antigen SAG1 has been reported to be absent in some *S. neurona* isolates; this same antigen is reported to be a poor candidate for use in serological diagnosis of EPM [24]. However, based on the data generated from *S. neurona* strains isolated from horses, opossums, raccoons and cats, the three SAG antigens (i.e. SAG2, SAG3 and SAG4) are highly conserved and exhibit little or no polymorphism [50]. Serum antibody analysis of horses challenged with *S. fayeri* (which is a closely related protozoa that utilizes the horse as its intermediate host) demonstrated that anti-*S. fayeri* sera do not react with the SAG1, SAG2, SAG3 and SAG4 antigens [44]; this further demonstrates that the rSAGs are highly specific.

Compared to western blots; these ELISA assays are easy to use, less expensive and none labor intensive. Besides, they provide a quantitative output in the form of a percent positivity value and/or an end-point titer, and can be completed in a short period of time. A large number of samples can be handled at the same time and use of the surface antigens rules out the need for propagation of parasites in tissue culture. Other tests developed to detect *S. neurona* antibodies in serum or CSF include a *S. neurona* direct-agglutination test (SAT) and indirect fluorescent antibody (IFA) test. Unfortunately, there are limitations associated with these assays e.g. as mentioned above the need for propagation of parasites in tissue culture makes the IFA technique cumbersome and expensive, and the use of whole parasites can increase the risk of false-positive results due to cross-reactivity with closely related pathogens [23].

3.7. Conclusions

All the samples used to evaluate this assay were from horses confirmed with EPM but the single domain antigen rSAG2 and the double domain antigen rSAG4D1/3D2 gave signal detections below 15% positivity. For instance rSAG2 reported two samples with PPs of 8.71 and

7.95, and rSAG4D1/3D2 reported only one sample with 14.26% positivity. Since the PP value represents the sample's percent of the positive control, this clearly indicates rSAG2 alone cannot produce reliable results.

One other thing to note in this study is, the trivalent antigen detected all the 13 samples with signal strengths greater than 15% cut off. In fact, all the PP values were above 20% positivity. This is consistent with the results generated by Michelle et al., 2011 who combined the three individual rSAGs into one ELISA assay and got high sensitivity and specificity. This clearly indicates that the trivalent antigen rSAG2/4D1/3D2 will greatly improve the accuracy and reliability of these ELISA assays.

The rSnSAG ELISAs, are key tools in understanding the pathogenesis of *S. neurona* infection since they can be used to track changes in antibody titers throughout the course of disease progression. The use of polyvalent antigens in ELISA will help evade the antigenic diversity that has been observed in *S. neurona*. These assays can also be used to analyze specific antibody isotype responses against SnSAGs to determine whether there is a difference in humoral responses between horses that are seropositive but not infected, and horses infected with EPM. These data will be very useful in understanding the immunology of the infection and in designing reliable diagnostic tools.

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CHAPTER FOUR. GENERAL DISCUSSION AND CONCLUSIONS

4.1. Role of Rapid Laboratory Testing

Rapid, accurate and accessible diagnosis is the key to proper management of infectious diseases. Laboratory testing is very useful in assessment of treatment failures, in assessment of patients with severe infections, confirmation of epidemics and continuous monitoring of prevalence. In rural Africa where rapid diagnosis is not available and laboratory facilities are poorly equipped, malaria is treated on presentation with fever. This leads to wastage of expensive drugs, potential of exposure to toxicity and exertion of unnecessary drug pressure to the new organisms by exposing them to sub-therapeutic drug levels [2].

In the treatment of HBV, the use of antiviral therapy is limited because the outcomes associated with such treatment are not properly understood. Seroconversion from HBeAg-an indicator of active infection to anti-HBe positive status is used to indicate response to treatment. However, some individuals with active HBV, HBeAg may not be detectable because of a mutation in the precore gene. For such people, response to treatment can only be monitored through HBV-DNA detection [1]. This scenario clearly emphasizes the importance of laboratory diagnosis in management of infectious diseases.

One aspect of laboratory diagnosis that is usually less emphasized is exclusion. Majority of EPM cases are confirmed by exclusion of other infections. Although laboratory diagnosis is sensitive, it is also not very specific. Thus solely relying on detection of the causative agent may result into over-diagnosis. From the public health perspective, over-diagnosis may increase perceived levels drug resistance, in malaria situations it may cause misperceptions about the efficacy of anti-malarial drugs especially when these drugs appear to “cure” other infections mistaken to be malaria [2].

The use of parasite based techniques can be very helpful in differential diagnosis to exclude the causative agent and give opportunity to treat other infections, resulting into improved case management. The use of parasite based techniques also guides prescription of more specific treatment, which is most likely to result into a better compliance to treatment because fewer drugs are prescribed and thus taken [2].

4.2. General References

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