Detection of human herpes simplex virus in clear cerebrospinal fluid by multiplex PCR at the National Reference Laboratory, Kigali, Rwanda.

BY

Ntagwabira Edouard

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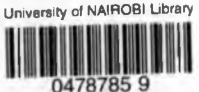
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SUPERVISORS:

Prof Omu Anzala
Chairman,
Department of Medical Microbiology
College of Health Sciences
University of Nairobi, Nairobi, Kenya

Prof. Omu Anzala (MBChB, PhD)

Dufton Mwaengo (PhD)
Lecturer,
Department of Medical Microbiology
College of Health Sciences
University of Nairobi, Nairobi, Kenya

Dufton Mwaengo (PhD)

LOCAL SUPERVISOR
Dr Odette Mukabayire
Director General
National Reference Laboratory
Kigali / Rwanda

Odette Mukabayire (PhD)

DEDICATION

This project is dedicated to my beloved family for their unconditional love and care that have always given me strength and courage to carry on.

I also dedicate it to the parents, my family and relatives who were killed in the Genocide against Tutsi 1994 and who endeavored their level best to make me whom I am today.

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LISTS OF ABBREVIATIONS

+ VE	: Positive Control
AIDS	: Acquired Immuno Deficiency Syndrome
BP	: Base Pair
CHUK	: Centre Hospital University Kigali
HUB	: Centre University University Butare
CMV	: Cytomegalovirus
CNS	: Central Nervous System
CSF	: Cerebral Spinal Fluid
DNA	: Deoxyribo Nucleic Acid
EBV	: Epstein –Barr virus
HHV	: Human herpes viruses
HHV A	: Human herpes virus A
HHV B	: Human herpes virus B
HHV-1	: Human herpes viruse one
HHV-2	: Human herpes virus two
HHV-3	: Human herpes virus three
HHV-4	: Human herpes virus four
HHV-5	: Human herpes virus five
HHV-6	: Human herpes virus six
HHV-7	: Human herpes virus seven
HHV-8	: Human herpes virus eight

HIV	: Human immunodeficiency virus
HSE	: Herpes simplex encephalitis.
HSV	: Herpes simplex viruses
IgA	: Immunoglobulin A
IgG	: Immunoglobulin G
Kb	: Kilobase
KFH	: King Faysal Hospital
KSHV	: Kaposis sarcoma associated herpesvirus
M	: Molecular weight markers
MPCR	: Multiplex Polymerase Chain Reaction
N	: Negative controls
NRL	: National Reference Laboratory
OR	: Odds Ratios
PCR	: Polymerase chain reaction
RTPCR	: Real- Time Polymerase chain reaction
SDA	: Sabouraud Dextrose Agar
TB	: *Mycobacterium tuberculosis*
TK	: Thymidine Kinase
UON	: University of Nairobi
USA	: United States of America
UV	: Ultraviolet light
VZV	: Varicella –Zoster Virus

ABSTRACT

Background: More than 80% of the clear cerebrospinal fluid (CSF) submitted to the National Reference Laboratory (NRL), Centre Hospital University Kigali and King Faysal Hospital in Kigali, Rwanda, are found to be negative for *Cryptococcus neoformans* and *Mycobacterium tuberculosis*, the only microorganisms tested for at this facility. These samples are obtained from patients with clinical suspicion of meningitis and encephalitis, which may be caused by different microorganisms including herpesviruses. The purpose of this study was to test CSF obtained from different regions of Rwanda for human herpes simplex viruses (HSV) type 1 (HSV-1) and type 2 (HSV-2) using a commercial multiplex PCR kit. The objective of this study was to determine the prevalence of HSV in clear CSF in Rwanda and to demonstrate that HSV-1 and HSV-2 can be PCR co-amplified in the same reaction tube.

Methodology: Clear CSF was obtained from patients with clinical suspicion of meningitis and encephalitis from five provinces in Rwanda. All the samples were transported to the NRL for DNA extraction and then subjected to multiplex PCR for detection of HSV-1 and HSV-2. The PCR amplicons were resolved on 2% agarose gels and bands corresponding to HSV-1, HSV-2, and the internal control identified under ultra-violet (UV) light.

Results: A total of 196 clear CSF samples were analyzed with this assay. Eleven out of 196 samples (5.6%) tested positive for *Cryptococcus neoformans* based on information supplied by medical officers where CSF originated. Of these samples, 7 (4.3%) were positive for HSV-1, 12 (6.0%) for HSV-2. 2 (1.0%) were dually infected with both HSV-1 and HSV-2. Overall, 21 out of 196 samples (10.7%) were positive for HSV nationally.

Conclusion: Multiplex PCR for diagnosis of HSV-1 and HSV-2 in clinical specimens (CSF) was found to be rapid and specific at the NRL. Demonstration of viral DNA detection by PCR is a major milestone as this provides a platform to the NRL to institute viral diagnostic procedures using PCR. The prevalence of HSV in CSF in this study is high enough (10.7%) to prompt the NRL to undertake a validation of the assay so that it can be adapted for routine HSV diagnosis in the laboratory. Once validation of the assay is done, its implementation at this referral laboratory will provide advisory leadership to physicians on whom patients can effectively benefit from available anti-HSV drugs like acyclovir, hence improving the quality of life for all Rwandese and its neighbours.

CHAPTER 1

1.0 INTRODUCTION

Human herpesviruses, including herpes simplex virus type 1 (HSV-1), and type 2 (HSV-2), cytomegalovirus (CMV), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), and human herpesvirus 6 (HHV-6) commonly cause infections of the central nervous system (CNS) and the urinary tract. Clinical signs and symptoms resulting from these six viruses are often indistinguishable from other neurological conditions[1].While clinical outcome is favourable when therapy is administered in early stages of disease; this requires early detection of herpesvirus in clinical specimens obtained from the patient. Herpes simplex viruses (HSV) persist in various cells of the host following primary infection. Infection of CNS by HSV is often associated with severe disease including encephalitis and meningitis with mortality.[2 3 4] HSV-1 is the most common cause of sporadic encephalitis in adults where the seroprevalence is widespread in the population. Meningitis resulting from infection with HSV-2 is usually self-limiting and seldom causes severe CNS symptoms.[5] Compared to HSV-1 induced encephalitis,; the symptoms associated with HSV-2 encephalitis have been described as milder, often diagnosed as Menengoencephaletis.[6] Symptoms of HSV-2 CNS infection can arise without typical genital herpes eruptions and also in patients who have never experienced genital herpes before.

Encephalitis is an inflammation of the brain, normally caused by virus infection.[7] In infected individuals with encephalitis, symptoms such as headache, fever vomiting, confusion and light-sensitiveness are common and can in severe cases also cause unconsciousness, seizures and paralysis. Meningitis, on the other hand, has bacterial or non-bacterial origin and leads to inflammation of the membranes covering the brain and spinal cord. Common symptoms for meningitis include stiff neck, light-sensitivity, severe headache, nausea, fever and mental status changes.[8] Early specific antiviral therapy will significantly reduce the mortality and serious morbidity of cases. Since the therapy must be started as possible, before the patient progresses into more lethal forms of the disease, timely diagnosis of HSV infection in the CNS must be done to enable proper treatment. With the introduction of PCR, virus DNA can be detected in the CSF and is the method most widely used for diagnosing viral CNS infections today.[9]

In Rwanda, the National Reference Laboratory (NRL) and referral hospitals routinely examine clear CSF for *Cryptococcus neoformans* and *Mycobacterium tuberculosis* in patients suspected of having CNS disease. However, the majority of clear CSF (more than 80%)

from clinically suspected cases is negative for these infections. The NRL and referral hospitals do not examine CSF for HSV infection due to lack of capacity to diagnose the virus. The purpose of this study was to use a multiplex PCR-based approach to examine the presence of HSV-1 and HSV-2 infections in clear CSF from people with clinical suspicion of meningitis and encephalitis. Results obtained in the study not only give the NRL the opportunity to provide leadership to Rwanda with respect to HSV diagnostics but also point the direction for PCR-based viral diagnostics at NRL for any other disease.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Classification of human herpes viruses

Herpes viruses belong to the herpesviridae family and are among the largest viruses known after poxviruses. Their genomes contain double-stranded DNA with the capacity to encode several hundred virus-specific proteins. Infection of susceptible cells begins with the attachment of host cell receptors to the virion envelope glycoprotein resulting in translocation of the virus particle into the cell cytoplasm. Replication of viral DNA and assembly of progeny nucleocapsid occur in the nucleus. Progeny viruses exit the cell via budding from the nuclear membrane leading to cell lysis, the main mechanism of pathogenesis for these viruses. Herpesviruses form a large group of infective agents that affect many animal species including humans. There are at least 8 known human herpes viruses (HHV) which include HHV-1 (HSV-1), HHV-2 (HSV-2), HHV-3 (VZV), HHV-4 (EBV), HHV-5 (CMV), HHV-6, HHV-7, and HHV-8 (KSHV).[10, 11]

2.2 Epidemiology of human simplex virus

Herpes simplex virus (HSV) infection is one of the most common viral sexually transmitted diseases (STD) worldwide[12] Herpes simplex virus type 2 (HSV-2) is the cause of most genital herpes and is almost always sexually transmitted. Herpes simplex virus type 1 (HSV-1) is usually transmitted during childhood via non-sexual contacts. However, HSV-1 has emerged as a principle causative agent of genital herpes in some developed countries[13]

Most HSV infections occur in women of reproductive age. The risk of maternal transmission of the virus to the foetus or neonate has become a major health concern.[14]

HSV-1 and HSV-2 each have linear, double-stranded DNA genomes of approximately 152 kbp). They belong to *Alphaherpesvirinae*, a subfamily of the *Herpesviridae* family and are characterized by a short reproductive cycle, prompt destruction of the host cell, and the ability to establish latency in sensory ganglia.[15] HSV infections are common in humans, and acquisition early in life is associated with low socioeconomic status.[16, 12, 13] Both viruses, transmitted across epithelial mucosal cells, as well as through skin interruptions, migrate to nerve tissues, where they persist in a latent state. HSV-1 predominates in orofacial lesions and it is typically found in the trigeminal ganglia, whereas HSV-2 is most commonly found in the lumbosacral ganglia. Nevertheless these viruses can infect both orofacial areas and the genital tract.[17]

In developing countries substantially higher rates of HSV-2 have been observed in sub-Saharan Africa, where prevalence in adults ranges from 30% to 80% in women. And 10% to 50% in men. More than 80% of female commercial sex workers are infected[18] Age and sex are important risk factors associated with the acquisition of genital HSV-2 infection. In fact, the prevalence of HSV infection is very low in childhood and early adolescence but it rises with age, reaching the maximum around 40 years.[14] Regarding sex, serological surveys have confirmed that infection is more frequent in women than in men in the general us population (23.1% in women versus 11.2% in men) and other countries, although in Italy, the seroprevalence is slightly higher in men (6.7%) than in women (4.9%).[19] The strongest association with HSV-2 infection appears related to the number of sexual partners.

The acquisition of genital herpes during pregnancy has been associated with spontaneous abortion, intrauterine growth retardation, preterm labour, congenital and neonatal herpes infections.[20] The risk of neonatal infection varies from 30% to 50% for HSV infections that occur in late pregnancy (last trimester), whereas early pregnancy infection carries a risk of about 1% [CDC website]. When primary HSV infection occurs during late pregnancy, there is not adequate time to develop antibodies needed to suppress viral replication before labour. Transmission of HSV from mother to foetus during pregnancy is uncommon; about 85% of perinatal transmission occurs during the intrapartum period.[21] Moreover, studies in HIV-infected pregnant women show that co-infection with HSV significantly increases the risk of perinatal HIV transmission above all in women who had a clinical diagnosis of genital herpes during pregnancy[22].

2.3 Neurological complications associated with HSV

Herpesvirus infections of the central nervous system (CNS) are a significant cause of morbidity and mortality, including long-term neurologic sequelae. Amongst the family of herpesviruses, the most significant CNS infections are due to HSV and CMV although VZV and HHV-6 are also important players as major causes of meningitis and encephalitis.[23, 24] The onset of HSV CNS infection can occur in neonates as well as older children and adults. Though CNS infections due to HSV are much less common, they are associated with significant morbidity and mortality in spite of antiviral therapy. CNS involvement in HSV infections can be categorized as neonatal HSV CNS disease when it involves neonates, and as herpes simplex encephalitis (HSE) in individuals beyond the neonatal period.

The majority of neonatal herpes simplex infection cases are caused by HSV-2[25] The risk of transmission is increased with primary maternal infection during the third trimester it can be

decreased by cesarean delivery if HSV has been isolated from the cervix or external genitalia near the time of delivery.[26] Herpes Simplex Encephalitis occurs in approximately 1 per 250,000 to 500,000 individuals per year[27] and is considered the most common cause of sporadic, fatal encephalitis[28] There is a bimodal age distribution of the disease; most cases occur in patients 6 months to 20 years of age or in patients older than 50 years (one-third and one-half of all cases, respectively)[29] HSE can occur as a primary infection (30% of cases) or it can be caused by a recurrent infection (70% of cases), and virtually all cases of HSE are caused by HSV-1.[30] If untreated, HSE mortality approaches 70%, and of those patients who do survive, significant neurologic morbidity occurs in nearly 97%[31]

2.4 Pathogenesis of HSE

Human herpesvirus types 1 & 2 enter the CNS by either viremic spread via infected cells entering the brain and meninges, direct infection from the nasal mucosa along the olfactory tract, or through spread from the trigeminal ganglia. These viruses cause a wide spectrum of clinical manifestations in the CNS of infants (encephalitis with or without disseminated visceral infection) and adults. Encephalitis is an inflammation of the brain normally caused by virus infection and is associated with symptoms such as headache, fever; vomiting, confusion and light- sensitiveness are common and in severe cases can also cause unconsciousness, seizures and paralysis[32] On the other hand, meningitis is inflammation of the membranes covering the brain and spinal cord. Common symptoms are stiff-neck, light sensitivity, severe headache, nauseas, fever and mental status charges[33].

In general, HSV-2 tends to induce meningitis while HSV-1 causes focal encephalitis[34]. In adults necrotizing encephalitis occurs in the medial temporal and inferior frontal lobes it is associated with elevated levels of cytokines and other markers of immune activation in CSF.[35] By far the majority of HSE cases where virus has been identified (outside the neonatal period) have been due to HSV-1. HSV-2 encephalitis does occasionally occur in immunocompromised adults and may be seen more frequently in those infected with the human immunodeficiency virus (HIV). Even though HSE is a rare condition, infection of the CNS often results in high mortality rates and significant morbidity in survivors especially in the acute form of the disease.[36] Symptoms of HSE include a prodrome of fever and malaise followed by headache and behavioural change sometimes associated with a sudden focal episode such as seizure, or paralysis; coma usually precedes death.

Most neonatal infections result from the retrograde spread of HSV-2 secondary to maternal genital infection or via passage of the infant through an infected maternal genital tract. Neonates may present with infection localized to the skin, eyes, and mucosa or to the CNS, or they may present with a disseminated infection[34]. The mortality rate for untreated infants who develop disseminated infection exceeds 70%. Clinical recognition leading to early specific antiviral therapy will significantly reduce the mortality and serious morbidity of untreated cases.

2.5 Prevention and Treatment of HSV infections.

In order to avoid the majority of neonatal herpes cases, identification of the *at-risk mother* is the goal. The first and most important step is the determination of the pregnant women's serostatus to establish their susceptibility to the infection during early pregnancy.[37] Women with a negative personal history of HSV and especially those with a positive history in the male partner, should be strongly advised to have no oral and sexual intercourse at the time of recurrence in order to avoid infection (in particular during the third trimester of gestation).[38]The most effective measure to prevent perinatal herpes infections is to avoid viral exposure to the neonate when primary genital herpes develops in late pregnancy whereas the risk of severe neonatal infection is small in recurrent episodes.[38] Moreover, use of condoms throughout pregnancy should be recommended to minimize the risk of viral acquisition, even it the male partner has no active lesions.[38] However, condoms are not a complete barrier for the genital region. It is recommended that prophylactic administration of acyclovir or valacyclovir in the third trimester of pregnancy should be provided to all pregnant women with frequent genital herpes outbreaks and with active genital HSV infection near term or at the time of delivery. [37, 39, 40] All pregnant women who have a suspected active genital HSV infection or symptoms of HSV infection should undergo caesarean section even though membranes may be intact.

Though advancements in antiviral therapy for HSV infections involving the CNS have led to substantially improved outcomes, the mortality and morbidity due to these infections are still unacceptably high. In patients affected by HSV, the best outcomes are seen when appropriate antiviral therapy is given prior to significant viral replication within the CNS or widespread dissemination throughout the body.[41] Early institution of therapy can substantially reduce the morbidity and mortality of mucocutaneous infections and the mortality rates of disseminated and CNS infections[42]Effective antivirals - acyclovir [2-hydrorulteoxymethyl guanine] vidarabine, and more recently the prodrugs valacyclovir (converted to acyclovir) and

famciclovir (converted to penciclovir) are available for therapeutic intervention. The first choice of antiviral drugs now available for HSV treatment is acyclovir, because it is relatively nontoxic and easy to administer and cheap. Acyclovir is phosphorylated by a virus-specific enzyme, Thymidine Kinase (TK), to acyclovir monophosphate, which is subsequently phosphorylated to acyclovir triphosphate by cellular kinases. The triphosphate form of acyclovir competes with the natural substrate dGTP for viral DNA polymerase, resulting in termination of DNA synthesis. Treatment of encephalitis with this agent reduces the mortality rate to 19% 6 months after treatment, compared with 50% among those patients treated with, vidarabine and 70% among those patients treated with placebo in prior studies[43]

Resistance of HSV to antiviral agents has not been a major limitation of therapy to date. Though reported in the laboratory, HSV resistance to acyclovir has not been a clinically important finding in immunocompetent patients, having a reported prevalence of less than 1%.[44] In immunocompromised patients, however, clinical strains of acyclovir-resistant HSV have been reported as 6%[45] with higher degrees of immunosuppression and more prolonged exposure to the agent being the most significant risk factors for the development of resistance. The most common cause of acyclovir resistance in clinical HSV strains is the deficiency or alteration of the viral thymidine kinase such that acyclovir cannot undergo the initial phosphorylation required for its activity[46] As acyclovir use for the treatment and suppression of HSV infections increases, further surveys will be necessary to monitor for increases in clinical acyclovir resistance.

A major limitation to the treatment of HSV CNS infections is the ability to diagnose the infection promptly. Though characteristic findings on magnetic resonance imaging (MRI) of the brain or electroencephalography (EEG) may be helpful, the current gold standard for diagnosis of HSV infection with CNS involvement is the detection of HSV DNA in the CSF by polymerase chain reaction (PCR). Other causes of encephalitis can mimic the MRI findings of HSV CNS infections[41] but are also non-specific[47] HSV PCR is more specific and should be able to provide rapid diagnosis; however, some laboratories are unable to perform the test in a timely fashion, and so prompt diagnosis remains a limitation to optimal therapy in many cases.

2.6 Diagnosis of HSV.

Diagnosis of genital HSV infections is often complicated because not only are non-classical presentations common, but clinical signs may also be mild and non-specific. Moreover, HSV infection is characterized by clinical outbreaks followed by asymptomatic periods within HSV transmission is possible. Therefore, it is necessary to improve the recognition and hence diagnosis of genital herpes, because a correct laboratory diagnosis is important for clinical management, counselling, treatment, management of pregnancy and assessment of the risk of transmission[14]

Conventional methods for diagnosis of HSV included virological techniques such as virus isolation from CSF by cell culture or using virus-specific antibody response approaches. These techniques have the disadvantage of being slow and insensitive. The HSV infection may be identified directly by detection of the virus or one of its components (viral antigen or DNA), or indirectly by assaying for specific serum antibodies of the viruses[14,48,49] Indirect (serological) testing can provide useful information in symptomatic patients when direct methods have yielded negative results. Although serological testing cannot reveal the onset of HSV infection or identify the locus of shedding, it allows identification of HSV infection when direct virus detection methods are not viable or when evidence of seroconversion is required[14] Enzyme-linked Immunosorbent Assay (ELISA), though not expensive, has some drawbacks like pre-exposure to viruses like HSV, CMV and EBV reduces the specificity for the episode of illness.

2.7 Multiplex PCR.

Today, PCR is recognized as the reference standard assay method for the sensitive and specific diagnosis of CNS infections caused by HSV.[50,51, 52, 53, 54] The application of molecular biological techniques to routine clinical specimens has led to a revolution in diagnostic virology. Not only does PCR have high sensitivity but also the ability to detect non-culture viable or fastidious organisms. Such techniques offer particular advantage in the diagnosis of CNS viral infections where PCR has not only improved diagnostic sensitivity but also expanded the clinical phenotype of several virus infections. For example it is now recognized, by this technique, that both HSV-1 and HSV-2 are common causes of meningitis (HSV-2) and encephalitis (HSV-1) in different patients.[55] For example, isolation of HSV from CSF has been reported in cases of primary meningitis caused by both types of virus, i.e., HSV-1 and HSV-2[56, 57] even though in recurrent meningitis, attempts to isolate HSV in the CSF have been unsuccessful[58] In general, however, implementation of PCR for the detection

of viral DNA in CSF has resulted in considerable improvement in diagnosis as HSV DNA has been detected by PCR in several patients with primary as well as recurrent aseptic meningitis [59, 60, 61]. The use of multiplex PCR for the diagnosis of CNS disease has been well evaluated for HSV encephalitis[64, 65, 66, 67] The use of this highly sensitive technique has increased understanding of the etiological role of viruses in CNS disease. For example, it has been demonstrated that varicella-zoster virus (VZV) and HSV type 2 (HSV-2) can cause meningitic symptoms without causing concurrent skin lesions [68, 69, 70]

The development of a multiplex PCR method for a rapid and accurate detection and typing of helps simplex virus type1 (HSV-1), and type-2 (HSV-2), Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) is very important for clinical diagnosis allow the delivery of therapy as early as possible[71]

This study used a multiplex PCR commercial kit for the detection of HSV-1 and HSV-2 in CSF at the NRL in Kigali, Rwanda. Cerebrospinal fluid was collected across Rwanda from both district and referral hospitals from patients who showed signs associated with meningitis and encephalitis and who consented to participate in the study. These CSF samples were those ordinarily not tested for *Cryptococcus neoformans* and *Mycobaterica tuberculosis* on the basis of being clear as opposed to turbid. Detection of HSV by this assay will be a major contribution to the diagnostic capacity for the NRL as currently no laboratory in Rwanda does any type of HSV diagnosis.

CHAPTER 3

PROBLEM STATEMENT AND JUSTIFICATION

There are currently no diagnostic tests for HSV infections in Rwanda even though these viruses have been implicated as causative agents of meningitis and encephalitis. There are four reference laboratories in Rwanda, namely National Reference Laboratory (NRL), Centre Hospital University Kigali (CHUK), King Faysal Hospital (KFH) and Centre Hospital University Butare (CHUB), where clear CSF samples from district and provincial hospitals are submitted for further analysis. These clear CSF samples are derived from suspected cases of encephalitis and meningitis, and the expectation is that the reference laboratories will be able to advise the doctors on the etiologic agents responsible for the condition. However, the current practice in these laboratories is that the CSF samples are only examined for *Cryptococcus neoformans* and *Mycobacterium tuberculosis* as no assays are available for virus detection.

A review of the available archived data from the reference hospitals by the NRL and the Ministry of Health indicates that between 2004 and 2007 NRL processed 34 clinical specimens of clear CSF. Of these 17- tested positive for *Cryptococcus neoformans* on India Ink, one was positive for *Mycobacterium tuberculosis* while 16 samples (47%) were negative on both culture on Sabouraud Dextrose Agar (SDA) and India Ink test. Similarly, in the period 2002 to 2007, CHUK processed 4623 clear CSF samples: 499 tested positive for *Cryptococcus neoformans* on India Ink and SDA, 7 were positive for *Mycobacterium tuberculosis* using kinyoun staining, and 4117 (89%) were negative on both culture and India Ink. Between 2004 and 2007 KFH processed 545 clear CSF samples, 63 tested positive for *Cryptococcus neoformans* on India Ink and SDA, while 482(88.4%) of all samples processed, were negative on both culture and India Ink.

Based on these statistics from archived registers used by CHUK, KFH and NRL in the indicated periods through ministry of health rapport 2007- 2008 and 2009 in Rwanda, it is clear that a large number of CSF samples are negative for both *Cryptococcus neoformans* and *Mycobacterium tuberculosis*. Furthermore, clinical evaluation by attending physicians to these patients indicated that they had meningitis and/encephalitis of unknown cause. None of the samples testing negative for *Cryptococcus neoformans* and *Mycobacterium tuberculosis* were tested for a virus as a potential etiologic agent even though these CNS diseases can be associated with virus infection. Since HSV has been implicated in CNS diseases, it is

important that NRL and referral laboratories in Rwanda have the capacity to detect this virus so that it can provide results to doctors who will in turn be able to administer appropriate antiviral drugs. A clinical evaluation of the patient alone is not enough since symptoms associated with meningitis and encephalitis are similar irrespective of the causative agent. A differential diagnosis of HSV by PCR at the NRL will ensure that treatment of these CNS diseases target the right patients whose infection status with HSV has been proven, hence improve on the morbidity and mortality associated with these deadly diseases. The NRL is well equipped and has the responsibility of providing leadership in viral CNS diagnostics to the Rwandan people.

3.1 Hypothesis

Use of a multiplex PCR diagnostic method for detection of HSV-1 and HSV-2 in clear CSF of suspected cases of neurological disease at National Reference Laboratory in Rwanda will facilitate timely diagnosis and recommend appropriate treatment to infected individuals.

3.2 Objectives

3.2.1 General Objective

To determine the prevalence of HSV-1 and HSV-2 in clear CSF from patients suspected of having meningitis and encephalitis in Rwanda.

3.2.2. Specific Objectives

a. To demonstrate that multiplex PCR can detect HSV-1 and HSV-2 in clear CSF in patients suspected of having meningitis and/or encephalitis in Rwanda

b. To determine the proportion of HSV-1 and HSV-2 among clear CSF samples

c. To compare HSV infection rates versus the positives cases of *Cryptococcus neoformans* and *Mycobacterium tuberculosis* from clear CSF samples.

CHAPTER 4

4.0 MATERIALS AND METHODS

4.1 Study Area

Several hospitals in different provinces in Rwanda were requested to assist in clear CSF collection for this study. In every case, the selected hospitals had the capacity for CSF collection and diagnosis as they ordinarily serve as referral hospitals for other smaller and more remote hospitals within the province. Additionally, they were all within 2 hours (by road) to the NRL in Kigali. The selected hospitals were as follows: (1) Within Kigali City – two Referral hospitals, Central Hospital University of Kigali, [CHUK] and King Faysal Hospital [KFH])) and 3 district hospitals (Kanombe Military Hospital, (KMH), Kibagabaga Hospital and Muhima Hospital), (2) North Province (Ruhengeri and Byumba district hospitals), (3) East Province (Rwamagana District Hospital), (4) West Province (Kibuye district hospital), and (5) South Province (Kabgayi and Remera Rukoma District Hospitals). The participating hospitals were requested to save (at -20°C) clear CSF samples that they routinely collected for diagnosis of *Cryptococcus neoformans* and *mycobacterium tuberculosis* from patients suffering from meningitis and encephalitis. All stored CSF samples were subsequently collected and transported to the NRL in boxes filled with ice to maintain the cold chain. On arrival at the NRL, the samples were frozen at -70°C freezer until the time they were needed for DNA purification.

4.2 Study Design

This was a laboratory-based cross sectional study. All clear CSF collected from five different provinces were analyzed using multiplex PCR technique at the NRL in Kigali, Rwanda. Total DNA from CSF of patients with encephalitis and/or meningitis were used in PCR to determine HSV-1 or HSV-2 viral sequences. PCR amplification primers derived from both viruses, as well as internal control primers, were combined in the same tube and amplification attempted using previously optimized conditions. Since virus-specific primers target different regions of the virus genome, PCR amplicons of HSV-1 amplified a 300bp product, while those of HSV-2 amplified a 473 bp product. The internal control primers amplified a 981bp product – these primers were included to control for the integrity of the PCR reagents and the amplification efficiency which can be affected by the presence of DNA polymerase inhibitors. The PCR products were resolved by gel electrophoresis and the bands visualized under UV-light on ethidium bromide-stained gel. The molecular weight markers, included in

the kit, provided the expected positions for HSV-1 and HSV-2.

4.3 Study Samples

Clear CSF collected at various hospital sites from patients with clinical symptoms of meningitis, and encephalitis presenting between May and September 2010 were transported to NRL and stored at -20°C or -70°C until enough samples (196) were received for DNA extraction and PCR amplification.

4. 4 Sample size calculation

The sample size calculation was based on routinely collected data stored in the archives of two referral hospitals: Central Hospital University of Kigali, (CHUK), King Faisal Hospital (KFH) and National Reference Laboratory. No study has been carried out on HSV using CSF samples in Rwanda. A study on HSV in Sub-Saharan Africa showed that the rate of HSV in genital ulcer was 23% in Kigali which was mainly among the HIV positive individuals (WHO report, 2001; CDC guidelines, 2002; and WHO guidelines, 2003). However, this study does not anticipate higher rates for HSV using CSF among hospital attending patients suffering from meningitis and encephalitis. The rate for HSV is anticipated to vary from 10% to 20% among these patients. Therefore, in the estimation of the sample size the study used the value of 15% with an error margin of 5%. Hence, the minimum sample size to estimate a significance difference at 5% level with 95% confidence interval, the sample size of 196 was estimated using the Kish and Leslie formula (1965)[63] where $n= [Z^2_{\alpha/2} (p) (1-p)] / d^2$ where:

Z= is the corresponding value to the 95% confidence interval = 1.96

p= expected prevalence

d= is the error margin usually taken around 5%

n= is estimated sample size

$n= Z^2_{\alpha/2} P (1-P)/d^2$

$= (1.96)^2(0.15) (0.85)/(0.05)^2 = 196$

4.5 Sampling at different hospitals

The data obtained at the two referral hospitals (CHUK and KFH) and nine district hospitals was used to determine the number of samples to be collected at various sites. A probability

proportional to size of the number of cases recorded at every facility was used. The collection was as follows:

N= 196

CHUK. and KFH = (196×45)/81 = 109

Others nine District Hospitals = (196× 36)/81 = 87

4.6 Inclusion/exclusion criteria.

All clear CSF samples with the correct volume (1.0 to 3.0 ml) collected in selected hospitals were eligible for inclusion. Clear CSF samples with volumes less than 1.0 ml were not eligible for inclusion into the study.

4.7 Duration of study

The study took five (5) months, starting from May to September 2010.

4.8 Procedure

Since three referral and nine district hospitals were involved, I visited the selected hospital sites to consult the Director Generals and research teams, before starting my research activities. The sterile tubes were distributed to the selected sites for sample collection and storage of CSF before being transported to the NRL. The objective of consultations was to organize and explain to the teams appropriate sample collection, storage and transportation. The questionnaire and Consent forms were distributed to the study team for explanation to participants about the importance of this study and to inform them that participation is voluntary without payment, not harmful and request for signature to the participants after the explanation. The stored samples at selected hospitals for the study in the provided period were transported in cold boxes with ice and well packaged to the NLR where they were stored at -20^{0}C or -70^{0}C.

Only samples with the correct volumes were accepted for the study. 232 samples were collected but only 196 samples were analysed. The facilities for this study were accepted by the Ministry of Education and NRL who covered the payment of reagent, transportation of sample, training of the study teams, and statisticians for data analysis.

In this study, only adequate clear CSF samples were transported, frozen and stored in NRL before they were used for multiplex PCR in detection of HSV types 1 and 2.

4.9 CSF collection and storage

All CSF from patients with clinical suspicion of encephalitis and/or meningitis were collected and examined for *Cryptococcus neoformans* and *Mycobacterium tuberculosis*. All adequate clear samples were eligible for inclusion in this study. Patients of all ages were eligible including those who were either positive or negative for HIV. The collection period was from May to September 2010 in all eleven (11) hospitals. Experienced medical officers, using sterile wide-bore needles, collected CSF aseptically to avoid contamination. The needle was inserted into the arachnoid space between the 4th and the 5th lumbar vertebra and allowed to collect CSF. The CSF was transferred into two dry sterilized tubes. The two tubes were labelled number 1 and 2. Tube number 1 was used for routine laboratory detection of *Cryptococcus neoformans* and *Mycobacterium tuberculosis* while tube number 2 was used to store 1.0 – 3.0 ml of CSF for this study. The handling of CSF was done in biosafety cabinets according to the World Health Organization (WHO) guidelines. All clear CSF collected from different hospital laboratories was stored at –20°C and transported in curved box with ice to NRL for multiplex PCR analysis.

4.10 Laboratory tests

4.10.1 Total DNA purification from CSF

Total DNA purification from CSF was done with a commercial DNA purification kit from South Africa (Inqababiotec, Hartfield, South Africa). Briefly, 200 µl of CSF was mixed with 800 µl of lysis buffer (ZR viral DNA Buffer) in a 1.5 ml sterile eppendorf tube. The mixture was mixed by vortexing and the tubes incubated at room temperature for 5-10 minutes. The mixture was transferred to a ZYMO-SPIN IC column in a collection tube and centrifuged at 10,000 rpm for 1 minute. The flow-through in the collection tube was discarded. Three hundred µl of DNA Washing Buffer was added to the column followed by centrifugation at 10,000 rpm for 1 minute as before. This wash step was repeated 3 times. The ZYMO-SPIN IC column was placed in a sterile 1.5 ml microcentrifuge tube and 10 µl of the DNA elution buffer added to the column and allowed to stand at room temperature for 1 minute. This was followed by centrifugation at ≥ 10,000 rpm (typically 14,000 rpm) for 1 minute to elute the DNA. The spin column was discarded and the flow-through (containing purified total DNA) was saved at -20°C until required for PCR.

4.10.2 Multiplex PCR assay

The special HSV detection kit (Seeplex HSV2 ACE) supplied by Inqababiotec, Hartfield, South Africa contains reagents that have been optimized for detection of both HSV-1 and HSV-2. The 5x HSV2 ACE PM solution is a mixture of HSV-1 and HSV-2 primers along with an internal control DNA template and the corresponding primers. The 2x Multiplex Master Mix contains the enzyme DNA polymerase, buffer with dNTPs (dATP, dCTP, dGTP, and dTTP) and $MgCl_2$. Also contained in the kit is the HSV2 ACE Marker which is used to determine the approximate size of the PCR product on agarose gels. Each PCR amplification run for the samples will include a positive control which is contained in the kit as HSV2 ACE PC. Briefly, the multiplex PCR for HSV-1 and HSV-2 was set up as follows: 4.0 µl of 5x HSV2 ACE PM was mixed with 3.0 µl of 8-Mop solution and 10.0 µl of 2x Multiplex Master Mix solution. This was followed by addition of 3.0 µl of viral DNA purified from CSF samples. For positive and negative controls, 3.0 µl of HSV ACE PC and 3.0 µl of distilled water was added in place of viral DNA respectively. The amplification profile was as follows: One cycle at 94°C for 15 minutes followed by 40 cycles of PCR each consisting of 94°C for 30 seconds, 63°C for 90 seconds, and 72°C for 90 seconds. After the last cycle, the samples were incubated for a further 10 min at 72°C to complete the extension of primers. The PCR machine was programmed to store the amplified PCR products at 4°C until removed for either analysis on agarose gel or stored at -20°C for later analysis.

4.10.3 Detection of PCR products on agarose gel

All PCR products were run on 2% agarose gel (containing Green Nucleic Acid Stain) to determine if HSV sequences were amplified in DNA derived from biological samples (CSF). Briefly, 2.0 µl of the amplified PCR products from all analyzed DNA were mixed with 6.0 µl of loading buffer and then loaded on the agarose gel. Molecular weight markers (supplied in the kit) were also loaded alongside the samples. Once the DNA samples are resolved on agarose gels the results were visualized under ultra-violet (UV) light. A picture of the green nucleic acid -stained gel was taken, labelled according to the loading programme, and saved in a folder created on desktop for raw data.

4.10.4 PCR results interpretation

The internal control DNA spiked into all samples was to help identify DNA that contained inhibitors of PCR. For this reason, only samples that amplified the 981bp band amplified by the internal control primers were eligible for interpretation with respect to whether or not they contained HSV. All samples that did not amplify the 981bp (even though they may contain

one or both HSV bands) were not valid and had to be repeated for suspicion of contamination. Only samples that amplified the HSV-1 (300bp) or HSV-2 (473bp) bands together with the 981bp band were considered for scoring or interpretation. For this reason, HSV-1 positive samples showed the 981- and the 300bp bands while those positive for HSV-2 showed the 981- and the 473bp bands. Samples with dual infections had all the 3 bands (981-, 300-, and 473bp bands).

4.10.5 Study limitations

Even if PCR is sensitive and specific it is not always able to detected HSV DNA in CSF. Sometimes it is not possible to detect HSV in the CSF sample taken at the initial time of diagnosis but it is detected later owing to the fact that individuals were recently infected and the virus has not entered the CSF. Since there were no follow up of patients, therefore, it is likely that some negative samples will turn positive later.

4.11 ETHICAL CONSIDERATIONS

This study was done using clear CSF samples from patients suffering from meningitis or encephalitis. Study team doctors obtained signed informed consent forms from patients. Approval to carry out the study was obtained from the Kenyatta National Hospital Ethics and Research Committee ref number KNH-ERC/A/466 and also Rwanda National Ethics Committee (Approval No.157/RNEC/2010). Furthermore, a recommendation letter for samples collections facilitation in selected hospital from Rwanda Ministry of Health was sought before the start of the study. Results from this study are being treated with confidentiality using laboratory codes for all samples. Authorization letter to use National Reference Laboratory for sample analysis was obtained by Director General of NRL.

CHAPTER 5

5.0 RESULTS

5.1 Multiplex detection of HSV-1 and HSV-2

A total of 196 participants from three referral and nine district hospitals from five provinces in Rwanda were enrolled for this study. The expected bands of 300-, 473-, and 981bp corresponding to HSV-1, HSV-2, and internal control respectively were observed in both the molecular weight markers and positive control samples (Fig 1). As expected, the negative control well only amplified the 981bp band indicating that there was no contamination of PCR reagents. Figure 1 is a representative gel which shows results for a section of samples that were scored positive for either HSV-1 alone (e.g. lanes 6, 7, and 14 (bottom panel)), HSV-2 alone (lanes 6-8, 10 and 12 all on top panel; and lanes 9, 10, and 15 on bottom panel), or dually infected with HSV-1 and HSV-2 (lane 9 (top panel)). An example of a sample in this gel (Figure 1) that had an indeterminate result is the sample loaded in well 5 (top panel) where a faint HSV-1 band is observed but there was no internal control band. This sample was subsequently repeated and found to be positive (data not shown). Some bands were more intense than others (e.g. HSV-2 band in lane 9 in bottom panel was more intense than all the other HSV-2 bands in this gel).

The majority of samples tested were negative for both HSV-1and HSV-2 as shown in Figure 2 below which is a representative gel. Only the internal control (981 bp) bands were amplified indicating that DNA was well extracted and PCR reactions were adequate.

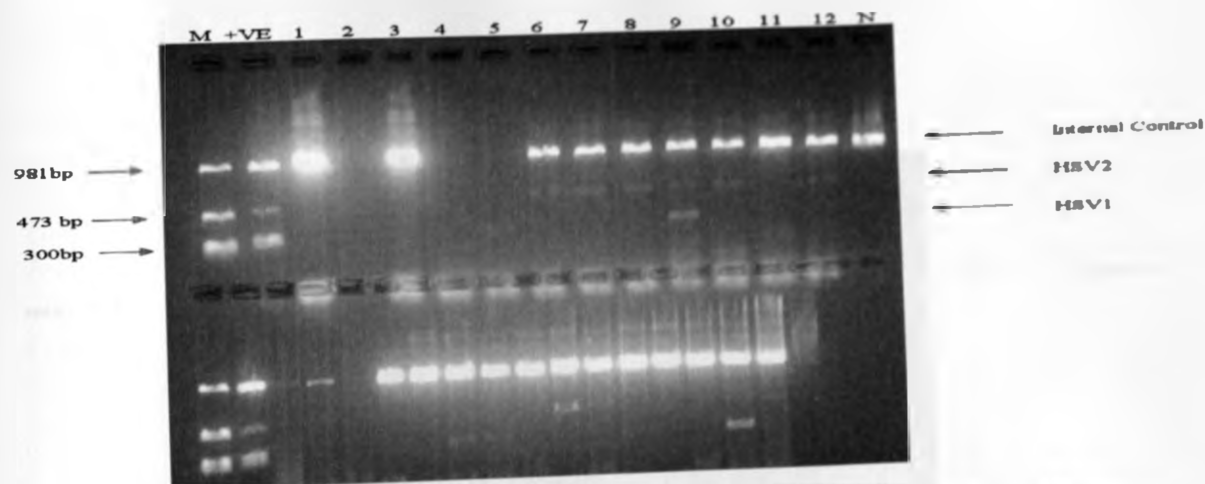

Figure 1: HSV PCR products amplified from clear CSF resolved on 2% agarose gel. First two wells (top and bottom panel) were loaded with molecular weight markers (M) and with positive control (+ve) respectively. 'N' designates negative control (top panel). All other wells were loaded with PCR products except wells 17 and 18 (bottom panel) which were empty.

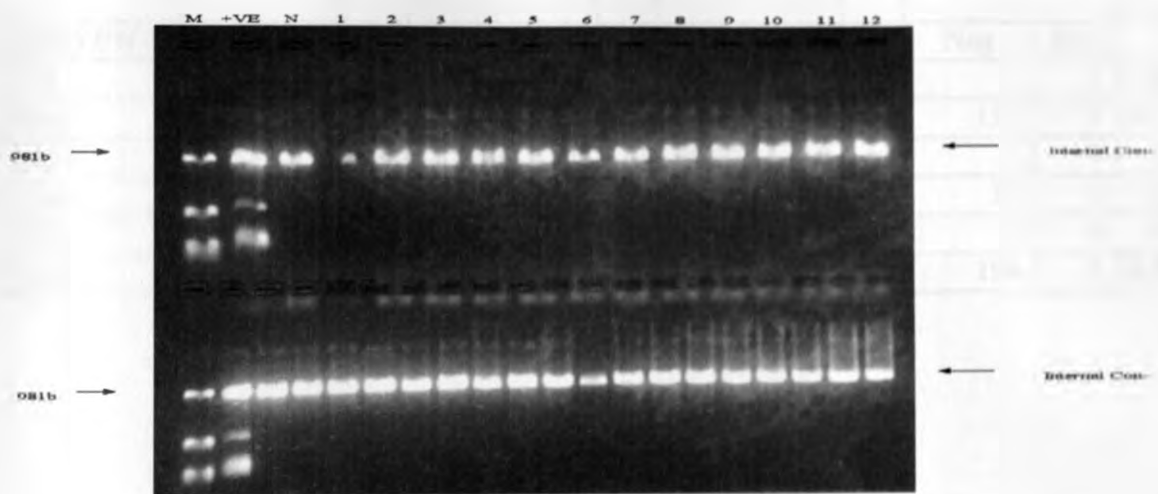

Figure 2: A 2% agarose gel showing results of the majority of samples that tested negative for HSV. Lane M is the molecular weight marker, "+ve" and N wells were loaded with positive and negative controls respectively, while wells labelled 1-12 were loaded with PCR products from different specimens.

Table 1: Infection by C. neoformans, HSV-1 and HSV-2 by provinces

	Crypto		%	HSV1		%	HSV2		%	Both HSV1 & 2		%
PROVINCE	**Neg**	**Pos**		**Neg**	**Pos**		**Neg**	**Pos**		**Neg**	**Pos**	
East	7	0	0	7	0	0	7	0	0	7	0	0
Kigali	128	11	7.9	133	6	4.3	127	12	8.6	137	2	1.4
North	7	0	0	7	0	0	7	0	0	7	0	0
South	35	0	0	34	1	2.9	35	0	0	35	0	0
West	8	0	0	8	0	0	8	0	0	8	0	0
Total	**185**	**11**	**5.6**	**189**	**7**	**3.6**	**184**	**12**	**6.1**	**194**	**2**	**1.0**

5.2 Proportion of HSV-1 and HSV-2 compared to C. neoformans and M. tuberculosis in clear CSF samples

Data on *Cryptococcus neoformans* was obtained from hospitals that participated in sampling of clear CSF. In all Rwandan hospitals, testing for *Cryptococcus neoformans* is routinely done using India ink. Culture of these microorganisms on SDA media is also performed but only in referral hospitals and at the NRL. *M. tuberculosis* is done by Ziehl-Nelson staining technique.

A summary of data obtained for *Cryptococcus neoformans* from different provinces that participated in this study is shown in Table 1 above. Only 11 of 139 clear CSF samples (7.9%) from Kigali City Province were positive for *Cryptococcus neoformans* – samples from all other provinces had no *Cryptococcus neoformans*. When the entire sample size (196) for the study is considered, this translates to only 5.6% of samples as *Cryptococcus neoformans* positive. For Kigali City Province, the prevalence of *Cryptococcus neoformans* (7.9%) and HSV-1 or HSV-2 (4.3% and 8.6% respectively) for the same population is not statistically different (Figure 3).

When all samples were analyzed by multiplex PCR, a total of 7 samples were positive for HSV-1. Of these, 6 were from Kigali City Province while one was from South Province. The total number of CSF samples from these two provinces was 174 (139 from Kigali City and 35 from South Province). This represented a prevalence of 4.0% of HSV-1 for these two provinces alone and 3.6% when all the five Rwandan provinces are considered (Table 1). On the other hand, 12 out of 38 CSF samples were positive for HSV-2 alone in Kigali City Province while two samples from Kigali City Province were positive for both HSV-1 and HSV-2 (1%). This study, therefore, identified a total of 21 out of 196 CSF samples (10.7%) as being HSV positive. The majority of CSF samples were negative for HSV as shown in Figure 2 and summarized in Table 1. Of all the clear CSF collected and analyzed, no *M. tuberculosis* infection was detected (data not shown).

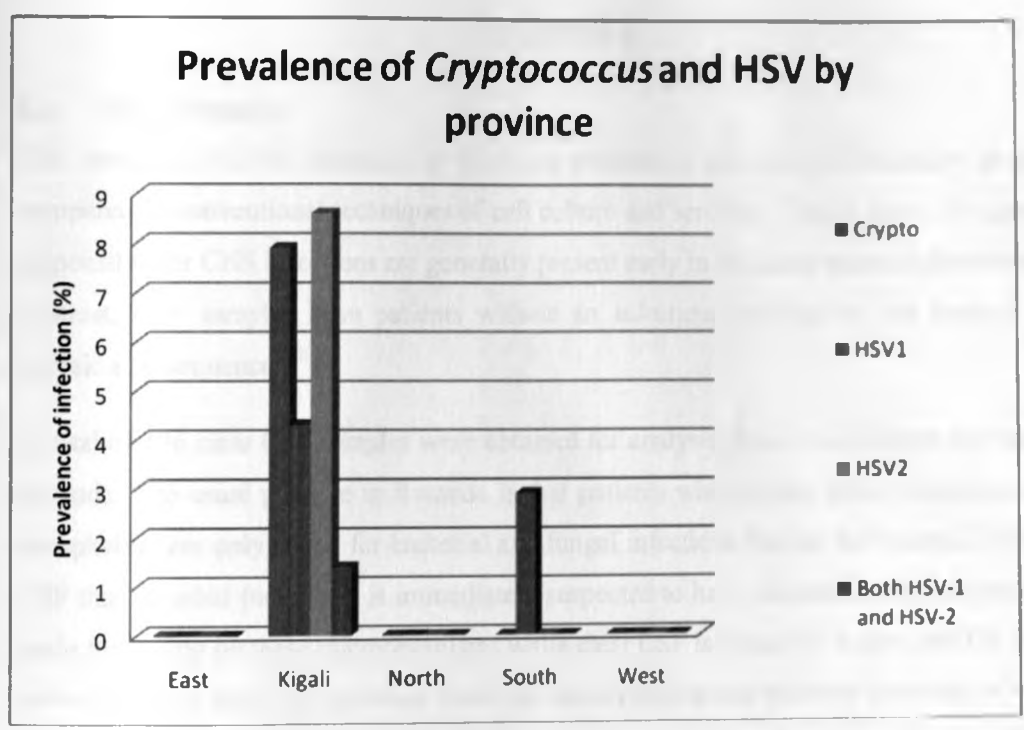

Figure 3: Summary on the prevalence of *Cryptococcus neoformans* and HSV by province. The y-axis shows the prevalence of *Cryptococcus neoformans* and HSV while the x-axis shows the provinces within Rwanda where clear CSF samples were collected. The bars are shown in colour as follows: Blue – Proportion of *Cryptococcus neoformans*, Red – Proportion of HSV-1, Green – Proportion of HSV-2, and Purple – Proportion of samples with dual infection (both HSV-1 and HSV-2).

CHAPTER 6

6.0 DISCUSSION

The detection of CNS infections by PCR has provided a new tool of laboratory diagnosis compared to conventional techniques of cell culture and serology. Target genes of organisms responsible for CNS infections are generally present early in the acute phase of the disease; in contrast, CSF samples from patients without an infectious etiology do not contain these nucleic acid sequences[62]

A total of 196 clear CSF samples were obtained for analysis from five different provinces in Rwanda. The usual practice in Rwanda is that patients who present with meningitis and/or encephalitis are only tested for bacterial and fungal infections but not for viruses. Typically, CSF that is turbid (not clear) is immediately suspected to have bacterial infections and is set aside for testing on these microorganisms while clear CSF is tested for fungal and TB, but is never tested for virus. In literature, there are several papers that describe detection of viruses associated with neurological conditions such as meningitis and encephalitis Specifically, HSV has been associated with these neurological conditions even though there is currently no diagnostic test for them in Rwanda. To gain an understanding of the prevalence of HSV in clear CSF in Rwanda, several technicians were trained on the collection, handling, and storage of CSF in five different provinces included in this study. The samples were collected and stored at -20C before transportation in ice to the NRL, Kigali for analysis.

In the present study, both HSV-1 and HSV-2 were detected in clear CSF indicating that some of the neurological conditions from individuals where the samples were collected could be attributable to HSV, even though other viral agents may be responsible. The prevalence of HSV-1 was much lower than that of HSV-2 (Table 1) which is consistent with observations by other groups. Only two individuals were dually infected with both viruses from Kigali City Province. One observation made in the data (Figure 1) is that some bands (for both viruses) were more intense than others. Since the test applied here is only a qualitative test (i.e. to indicate presence or absence of HSV DNA), it is likely that band intensity reflected either on the abundance of HSV in the sample relative to others or different efficiencies of PCR amplification. A quantitative assay (such as real-time PCR) will have to be applied to determine the relative amounts in different CSF samples perhaps in a future study.

It is well known that CSF may contain inhibitors that can partially or completely block DNA polymerase activity and cause false negative results. Due to the clinical and therapeutic

implications of a false-negative PCR result, identification of inhibited PCRs is a priority. Therefore, when PCR is employed for diagnostic purposes, it is imperative to adopt adequate controls for assessing sample suitability for PCR. In the present multiplex PCR assay, occasional false-negative results for HSV DNA amplification could lead to unidentifiable false-negative results if internal control primers were not included in the assay. Thus, failure of DNA extraction or failure to remove any inhibitors of DNA amplification may be avoided by the present assay. Apart from the ability to detect two different viruses in the same reaction tube, the inclusion of a positive control sample (which is added at the stage of setting up PCR in place of CSF-derived DNA sample) is important in ensuring specificity of amplified HSV DNA from biological samples. Reactions that fulfilled the requirement for positive control but had a negative control that failed to either amplify the expected internal control band or had more than one band were repeated before individual samples could be scored as either being positive or negative for HSV.

It is interesting that HSV was detected only in Kigali City and South Provinces. Perhaps it is not too surprising though, as the majority of CSF samples analyzed were from these provinces since of 196 clear CSF collected, a total of 174 (or 89%) were from Kigali City and South Provinces. Since 20 of 21 HSV positive CSF were from Kigali City Province alone, the prevalence of HSV in this province is 14.3%, a figure much higher than the national average of 10.7% obtained in this study. Indeed, all the other 3 provinces had no detectable HSV in CSF. This discrepancy can be explained in part due to the low number of samples analyzed. Another possible explanation for the high prevalence of HSV (particularly for Kigali City Province) is the fact that I personally supervised the collection, storage, and transport of all CSF obtained at the Centre Hospital University Kigali (CHUK) site which is just next to the NRL where I work.

As shown in Figure 1 and 2, demonstration of HSV detection with multiplex PCR from CSF in Rwanda is a major finding. Even though there are no laboratories in Rwanda that attempt to detect HSV as possible causative agents of encephalitis and meningitis, the practice in many countries that have not adopted PCR techniques is an invasive and expensive brain biopsy specimen procedure which has been previously used as a benchmark for the laboratory diagnosis of HSV encephalitis, meningitis and other CNS diseases. The NRL is very well equipped and has dedicated sections for nucleic acid (DNA or RNA) purification, PCR set up, and amplification and detection. This partitioning of sections is important in avoiding PCR contamination since this is a very sensitive technique for DNA detection. In

the course of carrying out these studies, contamination was not detected – all the negative controls in gels amplified the expected internal control band of 981 bp and the positive controls and markers appeared as expected. Because of the necessity for dedicated sections/areas for DNA extraction, PCR set up, and amplification and detection to avoid contamination, clearly there is need to forward CSF from patients suspected of CNS disease such as meningitis and encephalitis to the NRL for HSV detection by multiplex PCR since the NRL has the capacity for this specific diagnosis.

The prevalence of *Cryptococcus neoformans* was 7.9% out of 128 samples and 11 (5.6 %) out of 196 samples collected from five provinces. based on data obtained from hospitals where clear CSF was collected for all the 196 samples from five provinces There was additional data on *Mycobacterium tuberculosis* for a section of patients (21% of 196), whose physicians requested the test. For those tested, all of them were negative to TB. These observations indicate that these microorganisms can still be detected in clear CSF, hence the need to analyze all clear CSF for *Cryptococcus neoformans* infection.

This observation indicates that these individuals could benefit from treatment with drugs such as acyclovir if the attending physician requested the HSV test.

Routinely, of all CSF samples received by the NRL from regional hospitals in Rwanda, only about 20% regularly test positive for *Cryptococcus neoformans* and/or *Mycobacteria tuberculosis*. The majority (about 80%) of CSF samples are not tested for viruses such as HSV. It is quite likely (based on results from this study) that a huge fraction of these samples are positive for HSV. Since both encephalitis and meningitis are serious neurological diseases, it will be important for NRL to routinely analyze these samples for HSV, since unlike most viruses; these can effectively be treated with drugs.

In conclusion, the multiplex PCR assay presented in this study can provide an early, rapid, reliable non-invasive diagnostic tool allowing antiviral therapy to be initiated on the grounds of a specific HSV diagnosis. Since many clinical feature/symptoms associated with meningitis and encephalitis are similar, differential diagnosis with this assay will enable specific detection of HSV, hence avoiding unnecessary hospitalization and ensuring that these patients are initiated on antiviral therapy for HSV. However, additional studies are required in order to validate this assay with well-defined specimens that have been characterized by other methods. Validation and implementation of this assay at the NRL will also be critical in assessing its clinical significance and its utility in monitoring treatment

efficacy with antiviral drugs. Demonstration of the utility and importance of this assay in disease diagnosis and in monitoring treatment efficacy will undoubtedly influence policy at the national level which is consistent with the NRL vision 2020.

6.1 RECOMMENDATION

Results from this study indicate that detection of HSV by multiplex PCR in clear CSF at the NRL is possible. Sample processing (DNA extraction) using this kit was easy and DNA amplification and detection on agarose gels was easily accomplished at the NRL without contamination. The NRL has capacity to perform molecular diagnosis of HSV in CSF samples submitted by both District and Provincial hospitals and provide leadership in advising doctors on the management of patients suspected of CNS diseases. The prevalence for HSV in Kigali City Province was 14.3% which is well above the required 10% by the NRL for validation of test so that it can be routinely done at the laboratory. Validation and implementation of this assay at the NRL will provide Rwandan people with a less invasive procedure, compared to brain biopsy used before, for diagnosis of HSV. Additionally, since drugs for HSV are available, routine use of this method will ultimately reduce morbidity and mortality for Rwandan people.

REFERENCES:

1. Baskin, H.J., Hedlund, G., 2007. Neuroimaging of herpesvirus infections in children. Pediatr Radiol 37:949- 963

2. Baskin, H.J., Hedlund, G., 2007. Neuroimaging of herpesvirus infections in children. Pediatr Radiol 37:949- 963

3. Minjolle, S., at al ., 2002. Detection of herpesvirus genomes by polymeras chain reaction in cerebrospinal fluid and clinical findings. J. Clin. Virol. 25: S59-S70

4. Ružek, D., Piskunova, N., Žampachová, E. 2007. High variability in viral load in cerebrospinal fluid from patients with herpes simplex and varicella-zoster infections of the central nervous system. Clin. Microbiol. Infect. 13: 1217-1219

5. Momméja-Marin, H., 2003. Herpes Simplex Virus Type 2 as a cause of Severe Meningitis in Immunocompromised Adults. Clin. Infect. Dis. 37:1527-1533

6. Omland, L.H., Vestergaard, BF., Wandall, JH., 2008. Herpes simplex virus type 2 infections of the central nervous system: A retrospective study of 49 patients. *Scand. J. Infect. Dis. 40: 59-62*

7. Sindic, C.J.M., Van Antwerpen, M.P., Goffette, S., 2003. Clinical relevance of the diagnosis of neurological infectious diseases. *Brain Res.Bull. 61: 299-308*

8. http://mesh.kib.ki.se/swemesh/show.swemeshtree.cfm, Mesh_No= C10.228.228.553 & tool= karo MeSH Tree location for Meningoencephalitis. *Access date 080513*

9 DeBiasi, R.L., at al ., 2002. Use of PCR for the diagnosis of herpesviru infections of the central nervous system. *J. Clin. Virol.* 25: S5-S11

10. Baskin, H.J., Hedlund, G., 2007. Neuroimaging of herpesvirus infections in children. *Pediatr Radiol* 37: 949-963

11. Bergström, T. 1997. Virusinfektioner – klinik, diagnostik och behandling. Editor: Cederholm, M. Glaxo Wellcome AB, Mölndal. Pp: 12-13

12. Xu F, Lee FK, Morrow RA, Sternberg MR, Luther KE, Dubin G, Markowitz LE. Seroprevalence of herpes simplex virus type 1 in children in the United States. *J. Peds.* 2007;151:374–377.

13. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, Nahmias AJ, Berman SM, Markowitz LE. Trends in herpes simplex virus type 1 and type 2 seroprevalence in the United States. *JAMA.* 2006;296:964–973.

14. Cusini M & M. Ghislanzoni. 2001. The importance of diagnosing genital herpes. J Antimicrob Chemother. 47 Suppl T1:9-16. Review

15. Whitley RJ, Roizman B. Herpes Simplex Viruses. In: Richman DD, Whitley RJ. Hayden FG, editors. *Clin. Virol.* ASM Press; Washington, D.C.: 2004. pp. 375–401.

16. Nahmias AJ, Lee FK, Beckman-Nahmias S. Sero-epidemiological and - sociological patterns of herpes simplex virus infection in the world. *Scand. J. Infect. Dis.* 1990;69(Supplement):19–36.

17. Kriebs JM. Understanding herpes simplex virus: transmission, diagnosis, and considerations in pregnancy management. *J Midwifery Womens Health.* 2008:53:202–208.

18. Weiss H. Epidemiology of herpes simplex virus type 2 infection in the developing world. *Herpes.* 2004;11:24A–35A.

19. Suligoi B, Cusan M, Santopadre P, Palù G, Catania S, Girelli G, Pala S, Vullo V. HSV-2 specific seroprevalence among various populations in Rome, Italy. The Italian Herpes Management Forum. *Sex Transm Infect.* 2000;76:213–214.

20. Brown ZA, Selke S, Zeh J, Kopelman J, Maslow A, Ashley RL, Watts DH, Berry S, Herd M, Corey L. The acquisition of herpes simplex during pregnancy. *N Engl J Med.* 1997;337:509–515

21. Enright AM, Prober CG. Neonatal herpes infection: Diagnosis, treatment and prevention. *Semin Neonatol.* 2002;7:283–291.

22. Chen KT, Segú M, Lumey LH, Kuhn L, Carter RJ, Bulterys M, Abrams EJ., New York City Perinatal AIDS Collaborative Transmission Study (PACTS) Group Genital herpes simplex virus infection and perinatal transmission of human immunodeficiency virus. *Obstet Gynecol.* 2005;106:1341–1348.

23. Koskiniemi, M., T. Rantalaiho, H. Piiparinen, C. H. von Bonsdorff, M. Farkkila, A. Jarvinen, E. Kinnunen, S. Koskiniemi, L. Mannonen, M. Muttilainen, K. Linnavuori, J. Kolski, H., E. L. Ford-Jones, S. Richardson, M. Petric, S. Nelson, F. Jamieson, S. Blaser, R. Gold, H. Otsubo, H. Heurter, and D. MacGregor. 1998. Etiology of acute childhood encephalitis at The Hospital for Sick Children, Toronto, 1994-1995. Clin. Infect. Dis. 26:398-409.

24. Studahl, M., T. Bergström, and L. Hagberg. 1998. Acute viral encephalitis in adults—a prospective study. Scand. J. Infect. Dis. 30:215-220.

25. Kimberlin DW, Lin CY, Jacobs RF, Powell DA, Frenkel LM, Gruber WC, Rathore M, Bradley JS, Diaz PS, Kumar M, Arvin AM, Gutierrez K, Shelton M, Weiner LB, Sleasman JW, de Sierra TM, Soong SJ, Kiell J, Lakeman FD, Whitley RJ., The National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group Natural history of neonatal herpes simplex virus infections in the acyclovir era. *Pediatrics.* 2001;108:223–9.

26. Brown ZA, Wald A, Morrow RA, Selke S, Zeh J, Corey L. Effect of serologic status and cesarean delivery on transmission rates of herpes simplex virus from mother to infant. *J.A.M.A.* 2003;289:203–9.

27. Puchhammer-Stockl E, Presterl E, Croy C, Aberle S, Popow-Kraupp T, Kundi M, Hofmann H, Wenninger U, Godl I. Screening for possible failure of herpes simplex virus PCR in cerebrospinal fluid for the diagnosis of herpes simplex encephalitis. *J. Med. Virol.* 2001;64:531–6.

28. Olson LC, Buescher EL, Artenstein MS, Parkman PD. Herpesvirus infections of the human central nervous system. *N.E.J.M.* 1967;277:1271–7.

29. Whitley, R., et al. 1991. A controlled trial comparing vidarabine with acyclovir in neonatal herpes simplex virus infection N. Engl. *J. Med.* 324: 444 – 449

30. Nahmias AJ, Whitley RJ, Visintine AN, Takei Y, Alford CA., Jr. Herpes simplex virus encephalitis: laboratory evaluations and their diagnostic significance. *J. Infect. Dis.* 1982;145:829–36

31. Kimberlin DW. Management of HSV encephalitis in adults and neonates: diagnosis, prognosis and treatment. *Herpes.* 2007;14:11–6.

32. http://mesh.kib.ki.se/swemesh/show.swemeshtree.cfm.Mesh_No=C10.228.228. 553&tool =karo MeSH Tree location for Meningoencephalitis.

33. DeBiasi, R.L.2002. Use of PCR for the diagnosis of herpesvirus infections of the central nervous system. *J. Clin. Virol. 25: S5-S11*

34. Stahl HD, et al. 2000.; Detection of multiple viral DNA species in synovial tissue and fluid of patients with early arthritis. *Ann Rheum Dis 2000; 59:342–6.*

35. Tedder, D. G., 1994. Herpes simplex virus infection as a cause of benign Recurrent lymphocytic meningitis. *Ann Intern. Med.* 121:334–338.

36. Wolontis et al, 1977; Real-Time Polymerase Chain Reaction Detection of Herpes Simplex Virus in cerebrospinal Fluid and Cost Savings from Earlier Hospital Discharge *J. Molecular Diagnostics, Vol.* 7, No. 4, October 2005

37. Baker DA. Consequences of herpes simplex virus in pregnancy and their prevention. *Curr Opin Infect Dis.* 2007;20:73–76.

38. Sauerbrei A, Wutzler P. Herpes simplex and varicella-zoster virus infections during pregnancy: current concepts of prevention, diagnosis and therapy. Part 1: herpes simplex virus infections. *Med Microbiol Immunol.* 2007;196:89–94.

39. Andrews WW, Kimberlin DF, Whitley R, Cliver S, Ramsey PS, Deeter R. Valacyclovir therapy to reduce recurrent genital herpes in pregnant women. *Am J Obstet Gynecol.* 2006;194:774–781

40. Sheffield JS, Hollier LM, Hill JB, Stuart GS, Wendel GD. Acyclovir prophylaxis to prevent herpes simplex virus recurrence at delivery: a systematic review. *Obstet Gynecol.* 2003;102:1396–1402.

41. Whitley RJ, Corey L, Arvin A, Lakeman FD, Sumaya CV, Wright PF, Dunkle LM, Steele RW, Soong SJ, Nahmias AJ. Changing presentation of herpes simplex virus infection in neonates. *J. Infect. Dis.* 1988;158:109–16

42. Domingues, R. B. et al 1997. Advantage of polymerase chain reaction in the diagnosis of Herpes simplex encephalitis: presentation of 5 atypical cases. Scand. *J. Infect. Dis.*29:229–231

43. World Health Organization. Guidelines for the management of sexually transmitted infections, revised version. WHO/RHI/01.10 2003. Geneva: WHO, 2003

44. Christophers J, Clayton J, Craske J, Ward R, Collins P, Trowbridge M, Darby G. Survey of resistance of herpes simplex virus to acyclovir in northwest England. *A.A.C.* 1998;42:868–72.

45. Stranska R, Schuurman R, Nienhuis E, Goedegebuure IW, Polman M, Weel JF, Wertheim-Van Dillen PM, Berkhout RJ, van Loon AM. Survey of acyclovir-resistant herpes simplex virus in the Netherlands: prevalence and characterization. *J. Clin. Virol.* 2005;32:7–18.

46. Gilbert C, Bestman-Smith J, Boivin G. Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. *Drug Resist.* 2002;5:88–114.

47. Sainio K, Granstrom ML, Pettay O, Donner M. EEG in neonatal herpes simplex encephalitis. *Electroencephalography & Clinical Neurophysiology.* 1983;56:556–61.

48. Reil H, Bartlime A, Drerup J, Grewing T, Korn K. Clinical validation of a new triplex real-time polymerase chain reaction assay for the detection and discrimination of Herpes simplex virus types 1 and 2. *J Mol Diagn.* 2008;10:361–367.

49. Coyle PV, Desai A, Wyatt D, McCaughey C, O'Neill HJ. A comparison of virus isolation, indirect immunofluorescence and nested multiplex polymerase chain reaction for the diagnosis of primary and recurrent herpes simplex type 1 and type 2 infections. *J Virol Methods.* 1999;83:75–82.

50. Aslanzadeh, J., et al. 1993. Use of polymerase chain reaction for laboratory diagnosis of herpes simplex virus encephalitis. Ann. *Clin. Lab. Sci.* 23:196–202

51. Cinque, P., at al , 1998. Herpes simplex virus infections of the central nervous system in human immunodeficiency virus infected patients: clinical management by polymerase chain reaction assay of cerebrospinal fluid. *Clin. Infect. Dis. 27:303–309.*

52. Koskiniemi, M., at al , 1996. Herpes encephalitis is a disease of middle aged and elderly people: polymerase chain reaction for detection of herpes simplex virus in the CSF of 516 patients with encephalitis. J. Neurol. Neurosurg. Psychiatry 60:174–178.

53. Madhavan, H. at al 1999. Detection of herpes simplex virus (HSV) genome using polymerase chain reaction (PCR) in clinical samples. Comparison of PCR with standard laboratory methods for the detection of HSV. *J. Clin. Virol. 14:145–151.*

54. Mitchell, P. at al, 1997. Laboratory diagnosis of central nervous system infections with herpes simplex virus by PCR performed with cerebrospinal fluid specimens. *J. Clin. Microbiol. 35:2873–2877.*

55. Stahl HD, et al. 2000. Detection of multiple viral DNA species in synovial tissue and fluid of patients with early arthritis. *Ann Rheum Dis 2000; 59:342-6.*

56. Nowak, D. A., R. Boehmer, and H. H. Fuchs. 2003. A retrospective clinical, laboratory and outcome analysis in 43 cases of acute aseptic meningitis. Eur. J. Neurol. 10:271-280

57. O'Sullivan, C. E., A. J. Aksamit, J. R. Harrington, W. S. Harmsen, P. S. Mitchell, and R. Patel. 2003. Clinical spectrum and laboratory characteristics associated with detection of herpes simplex virus DNA in cerebrospinal fluid. Mayo Clin. Proc. 78:1347-1352

58. Bachmeyer, C., A. de la Blanchardiere, J. Lepercq, R. Dhote, G. Grateau, M. Detilleux, M. Tournaire, and B. Christoforov. 1996. Recurring episodes of meningitis (Mollaret's meningitis) with one showing an association with herpes simplex virus type 2. J. Infect. 32:247-248.

59. Aurelius, E. 1993. Herpes simplex encephalitis. Early diagnosis and immune activation in the acute stage and during long-term follow-up. Scand. J. Infect. Dis. Suppl. 89:3-62.

60. Chin, R. H., B. C. Ross, K. I. Taylor, A. P. Yung, and P. D. R. Johnson. 1996. Chronic meningitis due to herpes simplex virus in an immunocompetent host. Eur. J. Clin. Microbiol. Infect. Dis. 15:650-653.

61. Read, S. J., and J. B. Kurtz. 1999. Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay. J. Clin. Microbiol. 37:1352-1355

62. Skoldenberg et al.,1975; Real-Time Polymerase Chain Reaction Detection of Herpes Simplex Virus in Cerebrospinal Fluid and Cost Savings from Earlier Hospital Discharge *J. Molecular Diagnostics, Vol. 7, No. 4, October 2005*

63. Sibai BM, Gonzalez-Ruiz A: Severe preeclampsia-eclampsia. In young primigravid women : Subsequent pregnancy out come and nemote prognosis. Am Journal obstet Gynaecol 1986, 155: 1011-6

64. Guffond T, Dewilde A, Lobert P-E, Caparros-Lefebvre D, Hober D, Wattre P. Significance and clinical relevance of the detection of herpes simplex virus DNA by the polymerase chain reaction in cerebrospinal fluid from patients with presumed encephalitis. Clin Infect Dis. 1994;18:744–749.

65. Koskiniemi M, Piiparinen H, Mannonen L, Rantalaiho T, Vaheri A. Herpes encephalitis is a disease of middle aged and elderly people: polymerase chain reaction for detection of herpes simplex virus in the CSF of 516 patients with encephalitis. J Neurol Neurosurg Psychiatry. 1996;60:174–178.

66 Lakeman F D, Whitley R J. the National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group. Diagnosis of herpes simplex encephalitis: application of polymerase chain reaction to cerebrospinal fluid from brain-biopsied patients and correlation with disease. J Infect Dis. 1995;171:857–863.

67. Mitchell P S, Espy M J, Smith T F, Toal D R, Rys P N, Berbari E F, Osmon D R, Persing D H. Laboratory diagnosis of central nervous system infections with herpes simplex virus by PCR performed with cerebrospinal fluid specimens. J Clin Microbiol. 1997;35:2873–2877.

68. Echeverria J M, Casas I, Tenorio T, de Ory F, Martinez-Martin P. Detection of varicella-zoster virus-specific DNA sequences in cerebrospinal fluid from patients with acute aseptic meningitis and no cutaneous lesions. J Med Microbiol. 1994;43:331–335.

69. Schlesinger Y, Tebas P, Gaudreault-Keener M, Buller R S, Storch G A. Herpes simplex virus type 2 meningitis in the absence of genital lesions: improved recognition with use of the polymerase chain reaction. Clin Infect Dis. 1995;20:842–848.

70. Tedder D G, Ashley R, Tyler K L, Levin M J. Herpes simplex virus infection as a cause of benign recurrent lymphocytic meningitis. Ann Intern Med. 1994;121:334–338.

71. Nugueira ML, Ferreira PC, et al. Detection of herpes virus DNA by the polymerase chain reaction (PCR) in vitreous samples from patients with necrotising retinitis. J clin. Pathol 2001,;54;103-6.

APPENDIX

Research Questions

Patient code

Hospital name……………………………………………

Date of collection ………………………………………….

Sex

Age

Patient administrative Residence:

Province…………………………

District……………………………

Sample aspect

Clear

Bloody

Volume:

Adequate:

Inadequate: ☐

Encephalitis suspected Clinical symptoms:

Headache:

Yes ☐

NO ☐

Fever

YES ☐

NO ☐

Vomiting

YES ☐

NO ☐

Convulsions

YES ☐

NO ☐

Photophobia

YES ☐

NO ☐

Unconsciousness

YES ☐

NO ☐

Seizures

YES

NO

Paralysis

YES

NO

Meningitis suspected common symptoms

Stiff neck

YES

NO

Photophobia

YES

NO

Severe headache

YES

NO

Nausea

YES

NO

Fever

YES

NO

Mental status changes

YES

NO

Laboratory results from selected hospital.

Cryptococcus Neoformans.

Negative

Positive

Mycobacterium tuberculosis.

Negative

Positive

Multiplex PCR Results from Study.

HSV1

Negative

Positive

HSV2

Positive

Negative

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P O. Box 20723, Nairobi.
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP", Nairobi.
Email: KNHplan@Ken.Healthnet.org

Ref: KNH-ERC/ A/466

16th April 2010

NTAGWABIRA Edouard
Dept.of Medical Microbiology
School of Medicine
University of Nairobi

Dear Edouard

RESEARCH PROPOSAL: "IMPLEMENTATION OF PCR DIAGNOSIS METHOD FOR DETECTION OF HERPES SIMPLEX VIRUS(HSV) AT THE NATIONAL REFERENCE LABORATORY IN RWANDA" (P360/12/2009)

This is to inform you that the KNH/UON-Ethics & Research Committee has reviewed and **approved** your above revised research proposal for the period 16th April 2010 to 15th April 2011.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimens must also be obtained from KNH/UON-Ethics & Research Committee for each batch.

On behalf of the Committee, I wish you a fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of the data base that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH/UON-ERC

c.c. Prof. K. M. Bhatt, Chairperson, KNH/UON-ERC
The Deputy Director CS, KNH
The Dean, School of Medicine, UON
The HOD, Records, KNH
Supervisors: Prof. O. Anzala, Dept.of Medical Microbiology, UON
Dufton Mwaengo, Dept.of Medical Microbiology, UON

REPUBLIC OF RWANDA/REPUBLIQUE DU RWANDA

NATIONAL ETHICS COMMITTEE / COMITE NATIONAL D'ETHIQUE

Telephone: (250) 55 10 78 84

E-mail: rnec@moh.gov.rw

Web site : www.rnec.moh.gov.rw

Ministry of Health
P.O. Box. 84
Kigali, Rwanda.

FWA Assurance No. 00001973
IRB 00001497 of IORG0001100

No. 157/ RNEC /2010

January 09, 2010

Dear Eduard NTAGWABIRA
Principal Investigator

Your research Project: **Development of PCR Diagnostic Method for Detection of Herpes Simplex Virus (HSV) in Rwanda** ,has been evaluated by the Rwanda National Ethics Committee.

		Involve in the decision		
			No (Reason)	
Name	**Institute**	**Yes**	**Absent**	**Withdrawn from the proceeding**
Dr. Justin Wane	King Faisal Hospital, Kigali HOD Laboratory	X		
Prof. Emmanuel Bajyana	Immunologist, faculty of sciences (NUR)		X	
Dr. Emmanuel Nkeramihigo	Senior Lecturer, National University of Rwanda. Faculty of Medicine	X		
Dr. Dariya Mukamusoni	Director of Nyamata Hospital	X		

Dr Juliet Mbabazi	King Faisal Hospital, Kigali Ag. Chief Executive Officer	X		
Prof. Alexandre Lyambabaje	National University of Rwanda	X		
Mrs. Françoise Uwingabiye	Lawyer Musanze		X	
Dr. Eugène Rutembesa	National University of Rwanda	X		
Sr. Domitilla Mukantabana	Kabgayi Nursing and Midwife school		X	
Mrs Rosette Murigande	Well Spring Academy Nyarutarama		X	

After reviewing your protocol, during the RNEC meeting of 09th January 2010, where the quorum was met, **we hereby provide approval for the above mentioned protocol**
Please note that approval of the protocol and consent form is valid for **12 months**.

You are responsible for fulfilling the following requirements:

1. Changes, amendments, and addenda to the protocol or consent form must be submitted to the committee for review and approval, prior to activation of the changes.

2. Only approved consent forms are to be used in the enrollment of participants

3. All consent forms signed by subjects should be retained on file. The RNEC may conduct audits of all study records, and consent documentation may be part of such audits.

4. A continuing review application must be submitted to the RNEC in a timely fashion and before expiry of this approval.

5. Failure to submit a continuing review application will result in termination of the study.

Sincerely,

RWANDA NATIONAL ETHICS COMMITTEE DATE 09/01/10 COMITE NATIONAL D'ETHIQUE

Dr. Justin Wane
Chairperson, Rwanda National Ethics Committee.

Date of Approval: January 09th, 2010
Expiration date: January 08th, 2011

C.C.
- Hon. Minister of Health.
- The Permanent Secretary. Ministry of Health

REPUBLIC OF RWANDA

Kigali, 05 MAR 2010
N°20/ 539 /P.S.I.1.1/2010

MINISTRY OF HEALTH
P.O. BOX 84 KIGALI
www.moh.gov.rw

TO WHOM IT MAY CONCERN

Dear Sir/Madam,

RE: Facilitation for Samples collection

This is to certify that Mr. Ntagwabira Edouard, an employee of the Ministry of Health at the National Reference Laboratory (LNR). He is doing Masters Degree in Medical Microbiology (MSc), with specialization in Medical Microbiology. He has reached the stage research of this study and his research will be on Implementation of PCR Diagnostic Method for Detection of Herpes Simpiex Virus (HSV) at National Reference Laboratory in Rwanda.

He is visiting your site to collect CSF samples, which will assist him in his dissertation.

Any facilitation rendered to them to this purpose will be highly appreciated.

Thank you for your cooperation

MINISTERE DE LA SANTE

Dr. Agnes BINAGWAHO,
Permanent Secretary, Ministry of Health