UNIVERSITY OF GHANA

COLLEGE OF HEALTH SCIENCES

VIRAL AGENTS IN THE CEREBROSPINAL FLUID OF HOSPITALIZED HIV PATIENTS WITH MENINGITIS AT THE FEVERS UNIT, KORLE- BU TEACHING HOSPITAL

BY

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DEPARTMENT OF MEDICAL MICROBIOLOGY

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DECLARATION

This study was carried out at the Department of Microbiology, School of Biomedical and Allied Health Sciences and the Virology Department of Noguchi Memorial Institute for Medical Research under the supervision of Dr Theophilus Adiku and Dr Kofi Bonney.

Work from other authors that were used were duly acknowledged in the text or by reference cited. "I hereby declare that, this thesis is as a result of my own original investigation. I further certify that this thesis has not been presented to this University or elsewhere".

…… Date……

Student

(EMMANUEL FRIMPONG ADJEI)

"We hereby declare that the preparation and presentation of this thesis were done in accordance with the guidelines on supervision of thesis laid down by the University of Ghana".

…… Date…….

Principal Supervisor

(DR THEOPHILUS ADIKU)

…… Date……...

Co-Supervisor

(DR KOFI BONNEY)

DEDICATION

This work is dedicated to Jehovah and my family

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LIST OF ABBREVIATIONS

CAR- Coxsackievirus and adenovirus receptor

CDSC- Communicable Disease Surveillance Centre

CMV- Cytomegalovirus

CNS- Central nervous system

CRE- cis-acting replication element

CV- Coxsackievirus

DAF- Decay accelerating factor

DC- Dendritic cells

EBV- Epstein Barr Virus

EV- Enteroviruses

HEV- Human enterovirus

HIV- Human Immunodeficiency Virus

hPVR- Human polio virus receptor

HSV 1- Herpes Simplex Virus- 1

HSV 2- Herpes Simplex Virus- 2

IFN- Interferon

IL- Interleuken

IRES- Internal ribosome entry site

LCMV- Lymphocytic choriomeningitis virus

MHC- Major Histocompatibilty Complex

MuV- Mumps virus

NMIMR- Noguchi Memorial Institute for Medical Research

NO- Nitric Oxide

NPHEV- Non-polio human enteroviruses

NTR- Nontranslated region

ORF- Open reading frame

PABP1- Poly (A) binding protein 1

PAMP- Pathogen-associated molecular patterns

PCBP- Host poly (rC) binding protein

PCR- Polymerase Chain Reaction

PV- Polio virus

RNP- Ribonucleoprotein

rRT- PCR- Real Time Reverse Transcriptase PCR

TLR- Toll-like receptor

UG- University of Ghana

VP- Viral protein

VZV-Varicella Zoster Virus

ABSTRACT

Introduction: Meningitis is one of the leading causes of death among patients living with HIV in sub-Saharan Africa. Based on clinical presentations, the different types of meningitis may not be distinguished from each other therefore, accurate laboratory diagnosis is extremely essential. Viruses such as Enteroviruses, Mumps virus and Herpes Simplex Virus-1 are implicated in cases of meningitis.

Aim: The aim of the study was to determine the prevalence of viral meningitis among hospitalized HIV patients at the Fevers Unit (FU) of Korle Bu Teaching Hospital (KBTH).

Method: This was a cross-sectional study in which cerebrospinal fluid (CSF) specimens were collected from hospitalized HIV patients at the FU of KBTH with clinical features of meningitis but yet to be confirmed. Specimens were tested by Real-time PCR for Enteroviruses, Mumps Virus and Herpes Simplex Virus-1. Clinical and laboratory tests data on patients were analyzed and CSF parameters were also ascertained.

Results: A total of 80 CSF specimens were collected from patients. Thirty- seven (37) of the subjects were males and 43 were females. Majority (81.25%) of the patients were between the ages of 30 and 49 years. The major clinical symptoms seen in the patients were headache and fever with 35% exhibiting meningismus. Lymphocytic pleocytosis was observed in 30 while neutrophilic predominance was observed in 7 of the subjects. About two-thirds (53) of the patients had CD4 count less than 200cells/mm^3 and most were in Stage III and IV of HIV (68). Patients within normal range of glucose accounted for 71.25% while patients with elevated glucose level were 5 (6.25%). Fifty-four (67.50%) of the subjects had an elevated CSF protein level while 26(32.50%) had a CSF protein level less than 0.45. Most (68.75%) of the patients were not on ART.

The study was unable to detect Enteroviruses, Mumps virus and HSV-1 in the CSF of patients with suspected meningitis by real time PCR.

Conclusion: None of the viruses (EV, MuV, HSV-1) investigated in this study was implicated in the suspected cases of meningitis. However, lymphocytic pleocytosis, normal glucose and elevated protein levels were observed in majority of study participants

CHAPTER ONE

1. INTRODUCTION

1.1 General Introduction

Meningitis is the inflammation of the meninges and it can be caused by bacteria, parasites, fungi or viruses, but could also be drug induced. In addition to this, physical injury, subarachnoid haemorrhages, autoimmune conditions and cancers could also cause meningitis. The classical symptoms of meningitis include headache, neck stiffness, fever and change in mental status.

Viral meningitis is the inflammation of the leptomeninges as a manifestation of central nervous system (CNS) infection, with viruses being the causative agents (Gulshan, 2015). The possibility of a viral aetiology of meningitis usually arises when bacterial and fungal stains or cultures of cerebrospinal fluid (CSF) are negative. Based on clinical presentations, the different types of meningitis may not be distinguished from each other although viral meningitis is often associated with lymphocytic pleocytosis, therefore, accurate diagnosis is extremely essential (Tokuda, 2000).

The common causes of viral meningitis are Enteroviruses, Herpes Simplex Virus- 1 (HSV-1), HSV-2, Arboviruses and Mumps virus (MuV). Some other viruses that have been implicated to cause meningitis include, Human Immunodeficiency Virus (HIV), Cytomegalovirus (CMV), Varicella Zoster Virus (VZV), Adenovirus, Epstein Barr Virus (EBV), Measles and Rubella (Triant, 2003).

In the United States of America (USA), more than half (54.6%) of all meningitis-related hospitalizations are attributed to viruses (Holmquist and Elixhauser, 2008). There also a lot of admissions to hospitals in the United Kingdom due to viral meningitis (5–15 cases per 100 000 per year) (CDSC, 2000). Enteroviruses are the most implicated and are known to cause over 75

000 cases of meningitis in the USA each year and cause morbidity in adults and in children (Rotbart, 1998).

In a study conducted on a total of 183 patients with clinically suspected meningitis, 59 (32 %) had bacterial, tuberculous or cryptococcal meningitis, 39 (21 %) had normal CSF and 14 (8 %) had viral meningitis. For those with viral meningitis, a herpes virus was detected in 9 (64 %) of the total number of 14 patients; seven of the patients that had a herpes virus detected in the CSF had EBV and two had CMV. HSV- 2 and VZV were not detected (Benjamin *et al*., 2013).

Enteroviruses account for most cases of viral meningitis with the non-polio human enteroviruses (NPHEV) being the leading cause of viral meningitis. They account for 80% to 95% of all cases in which a pathogen is identified (Rotbart, 1998; Berlin *et al*., 1993; Sawyer, 1999). Most cases of recurrent viral meningitis are however caused by HSV-2. Also, in 25-50% of new HIV diagnosis, meningitis is a component of the initial viral illness. (Bruce, 2009).

Currently, there is limited published data on viral meningitis in hospitalized HIV patients in West Africa and Ghana. Information globally is also relatively sparing. Most studies on viral meningitis take into consideration the general populace and not directly dwelling on HIV and hospitalized patients. Another factor that might be the cause of this lack of extensive information could be due to the fact that most studies on meningitis especially in Sub-Saharan Africa, concentrate on bacterial meningitis.

1.2 Aim

The aim of the study was to determine the prevalence of viral meningitis among hospitalized HIV patients at the Fevers Unit of Korle Bu Teaching Hospital

1.3 Objectives

- To determine viral agents causing meningitis and the prevalence of viral meningitis in hospitalized HIV patients at the KBTH using molecular tools.
- To analyse data on demographic characteristics, clinical records and laboratory tests of hospitalized HIV patients at the KBTH with suspected meningitis.

1.4 Problem statement

Meningitis is one of the major causes of death among patients living with HIV in sub-Saharan Africa, yet there is no widespread tracking of the incidence rates of the causative agents among this category of patients (Veltman *et al*., 2014). Most studies on meningitis are usually on the general populace rather than hospitalized or HIV patients.

In Ghana, few studies have been carried out to determine the aetiological agents that cause meningitis amongst HIV patients. In the Greater Accra region, cases of clinically suspected meningitis are recorded at the Fevers' Unit of KBTH. On the average, four patients are recorded per month (personal communication, Dr Binney of the FU of KBTH). Despite these numbers of recorded suspected cases, confirmation and further identification of the aetiological agents are rarely done. Additionally, there is dearth of data on viral meningitis among HIV patients in Ghana. It is therefore necessary to investigate if the aetiological agents causing the symptoms of

meningitis in the patients are of viral origin and document relevant information to inform policy and enhance patient management.

1.5 Justification

In resource limited settings, as the case in most sub-Saharan African countries, suspected cases of viral meningitis have been recorded, but the aetiological agents in these cases are mostly not determined. (Harkim *et al*., 2000; Jarvis *et al*., 2010).

There is the need for accurate diagnosis to improve the management of viral meningitis especially in HIV patients. Accurate diagnosis will establish the viral agents in suspected cases of meningitis from other causes and in turn reduce hospitalization, medical bills and irrational use of antibiotics.

The cases of clinically suspected meningitis need to be studied in order to better inform treatment measures for the patients and also reduce the discomfort of misdiagnosis these patients experience. It is therefore necessary to carry out this study to provide relevant information which will help improve the management of meningitis in hospitalized patients, especially patients living with HIV.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Clinical presentations and cell changes in HIV positive patients

Haematological abnormalities are among the most common complications that are observed in HIV patients. These abnormalities can be seen in all the types of blood cells (Kirchhoff and Silvestri, 2008). In studies conducted in HIV positive patients with HIV negative control patients, it was observed that there was a marked difference between the two groups. Erythrocyte sedimentation rate (ESR) was significantly higher, erythrocyte count was decreased and there was a decreased haematocrit in the HIV patients. CD4 count was also decreased in the HIV positive patients as compared to the control group (Barbara and Zauli, Gloranhimi, 1990; Smith and Samadian, 1994).

Headache and fever are among the most frequent clinical manifestations of the HIV-positive patients suspected of having meningitis (Thinyane, Motsemme and Cooper, 2009; Herman and Fidele, 2007; Nowak, Boehmer, and Fuchs, 2003).

2.2 Viral Meningitis

Viral meningitis is the inflammation of the leptomeninges as a manifestation of central nervous system (CNS) infection and with viruses being the causative agents (Gulshan, 2015). Although based on clinical symptoms, the different types of meningitis may not be distinguished from each other, lymphocytic pleocytosis usually characterizes viral meningitis cases.

The classic triad of symptoms is, fever, headache, and neck stiffness and are collectively known as meningismus (Van de Beek *et al*., 2004). Studies also show that about 20–70% of adults with meningitis exhibit some form of altered mental status ranging from confusion to impaired level of

consciousness (Wall *et al*., 2013; Veltman, Bristow and Klausner, 2014; Domingo *et al*., 2013) These signs are not always present at the same time. Other signs and symptoms include: photophobia, phonophobia, confusion, vomiting, seizures, irritability, cranial nerve palsies, blindness and coma. In people with HIV, meningitis may present with confusion only (Wall *et al*., 2013). Irritation of the meninges such as Kernig sign (inability of leg to straighten when hip is flexed at 90 degrees) and Brudzinski sign (severe neck stiffness causes a patient's hips and knees to flex when the neck is flexed) may also be useful in predicting meningitis in patients

Neurological and neuropsychological disorders have been recorded in adults who acquire viral meningitis although it is predominant in children (Schmidt *et al.,* 2006; Lan *et al*., 2001)

Developing countries have a higher mortality rate due to viral meningitis as compared to developed countries. This could be attributed to the fact that there is delay in diagnosing and treating patients (Larvis *et al*., 2014; Luma *et al*., 2013; Christensen *et al*., 2011)

2.3 Viral meningitis in HIV patients

Meningitis is one of the leading causes of death among patients living with HIV in sub-Saharan Africa but there is no widespread tracking of the incidence rates of causative agents among patients living with HIV (Jarvis *et al*., 2010; Veltman *et al*., 2014). It is one of the most severe opportunistic infection in HIV-infected patients (Hakim *et al.,* 2000).

With increasing access to antiretroviral therapy (ART) and reduction in the incidence of the disease, HIV is now more of a chronic illness rather than an infection with high mortality. Even with the initiation of antiretroviral therapy (ART), being on the alert for meningitis is very important, because it may be the first illness that an HIV patient may develops and it may be life threatening. (Hakim, 2007).

HIV does not directly kill patients that are infected. Instead, it weakens the body's ability to fight disease. Infections which are rarely seen in the immunocompetent may be life-threatening to those with HIV. Many opportunistic infections cause diverse diseases such as pulmonary diseases, chronic diarrhea, and neurological defects. Among those diseases, meningitis associated with HIV/AIDS has an important impact considering morbidity and mortality. Meningitis can be classified according to the aetiologic agent as viral, cryptococcal, tuberculous, syphilitic, etc.

Although HIV-positive individuals are at increased risk of certain types of meningitis, typically cryptococcal and tuberculous, there is evidence to suggest that they are also likely than the general population to develop bacterial or viral meningitis (Al-Akeel, Ahmed and Syed, 2012; Gulshan, 2015; Harkim, 2000; Jarvis 2010; Thinyane, Motsemme and Cooper, 2015; Herman and Fidèle, 2007).

In a number of new HIV diagnosis, meningitis is a part of the early illness that the patient presents with. It occurs as a mononucleosis-like syndrome and it is characterized by fever, malaise, lymphadenopathy, rash, etc. Defective T cell-mediated immunity is presumed to lead to greater susceptibility to viral infections. There is also a defective B cell response in AIDS patients (Tommasini, and Fong, 1992). HIV meningitis may present with cranial nerve palsies or seizures. HIV meningitis usually tends out to be self-limiting. Studies conducted in health care systems indicate that many patients with acute HIV infected patients seek treatment because they feel ill. The acute illness that occurs before seroconversion typically lasts for a short period. It consists of rash, viral meningitis and generalized lymphadenopathy. It has been estimated that 25%-50% of those with acute HIV infection present with viral meningitis (Bruce, 2009).

There is severe immunosuppression in meningitis patients who are also HIV- positive. In a study in which 72 patients with symptoms of meningitis were recruited, the coinfection rate of HIV was 79%. The most common presenting symptoms were altered mental status, neck stiffness, headache, and fever. TB meningitis accounted for 39% of the diagnosis, followed by bacterial (27%), viral (18%), and cryptococcal (16%) (Thinyane, Motsemme and Cooper, 2015)

In a study on 284 HIV adult patients hospitalized in Soweto, South Africa, 14.1% had acute viral meningitis (Bergemann and Karstaedt, 1996).Out of 183 patients with clinically suspected meningitis recruited for a study, 14 (8 %) had viral meningitis. The patients were HIV positive (Benjamin *et al*., 2013).

2.4 Epidemiology of Viral Meningitis

Studies on meningitis especially in HIV patients are centered on other aetiological agents apart from viruses. For example, in a study by Jarvis and colleagues, 5578 lumbar punctures were performed on 4549 patients, representing 4961 clinical episodes. 2293 had normal CSF and 931 of the samples had minor abnormalities and no aetiology was identified. Of the remaining 1737 samples, diagnoses were obtained in 820 (47%). Cryptococcus accounted for 63% (514), TB accounted for 28% (227) and bacterial meningitis accounted for 8% (68). No specific tests were performed to detect viral agents (Jarvis *et al*., 2010).

There are a lot of admissions to hospitals in the UK due to viral meningitis (5–15 cases per 100 000 per year) (CDSC, 2000).

In a study conducted on a total of 183 patients with clinically suspected meningitis, 59 (32 %) had bacterial, tuberculous or cryptococcal meningitis, 39 (21 %) had normal CSF and 14 (8 %) had viral meningitis. (Benjamin *et al*., 2013).

Enteroviruses account for most cases of viral meningitis with the non-polio human enteroviruses (NPHEV) being the leading cause of viral meningitis. The NPHEV account for 80% to 95% of all cases in which a pathogen is identified (Rotbart, 1998; Berlin *et al*., 1993; Sawyer, 1999).

Enteroviruses also cause over 75 000 cases of meningitis in the USA each year and cause substantial morbidity in adults as well as children (Rotbart, 1998).

Studies conducted show that, serotypes of the Human Enterovirus B (HEV-B) species including CVB1-6, ECHO, CVA9, HEV69 and HEV73 are the most frequently implicated, which could cause sporadic cases, outbreaks or epidemics of viral meningitis worldwide. In a related study, CVB5, Echovirus 6, 9 and 30 were implicated in meningitis outbreaks, while others CV A9, B3 and B4 were found to be mostly endemic (Oberste *et al*., 1999; Oberste *et al*., 2001; Trallero *et al*., 2000; Khetsuriani *et al*., 2006; Lee and Davies, 2007; Cui *et al*., 2010).

The incidence rate of viral meningitis annually exceeds the total number of meningitis cases caused by all other etiologies combined (Irani, 2008). Often, no cause is identified for viral meningitis especially in resource limited settings due to the complex nature of the work out for the identification of viruses. For example, in a study conducted on 144 adults (HIV status unknown) with viral meningitis, only 72 had a confirmed diagnosis. The most common viruses identified were enteroviruses, accounting for 46%, followed by HSV-2 (31%), VZV (11%), and HSV-1 (4%) (Kupila *et al*., 2006).

2.5 Diagnosis and management of viral meningitis

It has been reported that many cases of meningitis and encephalitis remain undiagnosed and so this is an active area of research (National Institute of Neurological Disorders and Stroke, 2016; *Thomas, 1992*) and it is also possible that certain infections of the CNS are not always accompanied by the presence of the organism in the CSF (Read, Jeffery and Bangham, 1997). This makes accurate diagnosis key in neurological disorders. In resource-limited settings, the management of diseases is cumbersome due to limited diagnostic capabilities, fewer treatment options, and overburdened staff. In developed countries where a more exhaustive diagnostic work-up is possible, up to 50 % of cases of aseptic meningitis have been found to have a viral cause (Kupila *et al*., 2006; Logan and MacMahon, 2008).

A wide array of infectious and inflammatory diseases may mimic CNS viral infections and this also gives precedence to accurate diagnosis (Davies *et al*., 2005). The possibility of a viral aetiology of meningitis usually arises once bacterial and fungal stains or cultures of CSF are negative; however, it is important to consider other causes of aseptic meningitis. Diagnosing viral meningitis is complex because, a number of viruses can cause meningitis, each with its own distinct mode of transmission, presentation, and analyses. Secondly, the presentation of viral meningitis may only differ slightly from the other forms of meningitis. Procedures involved in viral meningitis diagnosis are therefore usually not complete and an etiological agent is rarely identified (Van de Beek *et al*., 1993).

Viral meningitis is characterized by raised CSF white cell count usually lymphocyte pleocytosis, slightly elevated protein, and normal glucose (Davies *et al*., 2005; Roa *et al*., 2013). Early viral meningitis can show a neutrophil predominance, but the differential shifts towards lymphocytes within 8 to 24 hours. Glucose is usually normal (>50 percent of serum value) but can be decreased

in some cases of enterovirus, mumps, HSV, and LCMV (Triant, 2003). Nonetheless, no combination of findings in CSF cells, protein, and glucose can rule out any of the other causes of meningitis. For example, in a study conducted on 50 patients with symptoms of meningitis, analysis revealed a substantial increase in both protein and pleocytosis level of the samples that had viral agents and samples that had other pathogens as the cause of meningitis. Elevated CSF protein level was also found in 58.3% of EV RT-PCR positive cases and 58% of EV RT-PCR negative cases. This buttressing the fact that pleocytosis, protein and glucose levels are not enough to distinguish the various types of meningitis (El Gharbawy and Barakat, 2006).

Analyses of the CSF in patients presenting with meningitis show that there is usually different values in the parameter tested for depending on the aetiological agent

Table 2.1: Reference values for CSF Biochemistry (Central Lab Biochemistry Department SOP)

PARAMETER	REFERENCE
Glucose	2.5-4.5 mmol/L
Protein	0.15-0.45g/L
Globulin	Negative

Table 2.2: Cerebrospinal fluid analysis for meningitis

Etiology	WBC Count	Protein (g/L)	Glucose (mmol/L)	CSF/Serum glucose ratio
Normal	≤5cells/mm^3 Lymphocytes	0.15-0.45	2.5-4.4	0.6-0.7
Bacterial	>100cells/mm^3 PMNs predominant	>0.45	<2.2	<0.4
Viral, ***T. pallidum***	10-500cells/mm^3 Lymphocyte predominant	0.15-0.45	2.5-4.4	0.6-0.7
Fungal, ***M. tuberculosis,*** **Lymphoma**	25-500cells/mm^3 Lymphocytes predominant	>0.45	<2.2	<0.6

Previously, a specific pathogen was found in less than 25 % of the cases of viral meningitis due to inadequate testing and limitations of the diagnostic methods until recently where there has been tremendous improvement in diagnostic methods (Irani, 2008).

The ideal method for diagnosing viral meningitis is by obtaining CSF from the patient showing clinical presentations of the disease. When obtaining CSF is difficult, viral stool culture, rectal swab or throat swab may reveal the causative viral agent. Unfortunately, isolation of an enterovirus from throat swab, stool or rectal swab is not always indicative of an infection because virus may be shed for several weeks after concurrent infection, and enteroviruses have been found in up to 10% of healthy controls during outbreaks. Limitations of viral culture for the diagnosis of viral infection include, a sensitivity of 65% to 75%, a turnaround time of between 3 to 10 days, and the high level of technical expertise needed (Rotbart, 1995). Both viral antigen search and antibody assays display low levels of sensitivity because, from the acute to the convalescence phase, there is a progressive increase in the number of neutralising antibodies.

Serological tests for enterovirus are not the best methods because there are too many serotypes for the virus. However, serological tests for certain other viruses such as mumps may be performed.

Polymerase chain reaction (PCR) is increasingly being employed as the standard for diagnosing viral meningitis. When viral cultures are negative, PCR can be employed to ascertain the result. On the average, CSF cultures are positive in only half of the cases of viral meningitis. In many of culture-negative cases, the specific viral etiology can be identified by using PCR. PCR is routinely used to diagnose EV, HSV, CMV, EBV, Mumps and other viruses that can cause meningitis.

Polymerase chain reaction (PCR) techniques can be used to amplify viral genome present in the patient's cerebrospinal fluid (CSF), even when the virus is still replicating in its early days of establishing infection. PCR is highly sensitive and specific for many infections of the CNS. It is fast, and can be performed with small volumes of CSF. The viral concentration may have a bearing on the results. Low viral concentration may give a negative result (Gorgievski-Hrisoho *et al*., 1998).

Testing may be expensive but it is a better option because it helps save cost by decreasing overall diagnostic testing and intervention. PCR of the CSF has a sensitivity of 95 to 100 %, and a specificity of 100 % for HSV, EBV and EV (Zhongliang *et al*., 2010). For example, PCR for EV was performed on CSF samples obtained from 34 patients with meningitis of enteroviral origin. Out of these 34 samples, 26 tested positive for enterovirus (Kupila *et al*., 2005).

Time of sample collection is also paramount. In the example above, most of the positives were samples collected two days after the onset of clinical symptoms. It was observed that most of the samples that gave a negative result were collected many days after onset of clinical symptoms. In a study on MuV meningitis also, it was observed that CSF from patients that had been collected after about 21 days after onset of clinical symptoms were negative for MuV PCR while those collected after a median of four days yielded a positive result (Kupila *et al*., 2005; Poggio *et al*., 2000). The optimum time span for obtaining a positive HSV-PCR has also been shown to be between 2 and 10 days after onset of the illness (Davis and Tyler, 2005).

Seasonality of some of the viruses may also come into play when diagnosing. Enteroviruses typically show late summer or early autumn seasonality (in temperate regions). The incidence of some enteroviruses implicated in viral infections has been shown to be seasonal, with a peak incidence in August of each year in the USA (Moore, 1982). Although much is not known about the seasonality in the tropical zone, mumps occurs mainly in winter or spring and in 2-7 year cycles in temperate zones (Trallero *et al*., 2000; McIntyre and Keen, 1993).

Other findings such as low concentrations of tumor necrosis factor (TNF) and lactic acid can also validate viral meningitis. Other non-microbiological tests, including the C-reactive protein, CSF glucose, CSF enzyme levels and CSF cytokine levels, are not routinely indicated because they lack sufficient sensitivity and specificity in distinguishing the diverse types of meningitis. That notwithstanding, they can also be employed.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study design

This was a cross sectional study that was based in a tertiary health facility where clinical specimens of a population of hospitalized HIV positive patients were collected. It was part of a broader study identifying possible agents causing meningitis in these patients. This current work concentrates on some viral agents that may be implicated in cases of meningitis. In total, 80 clinical specimen of cerebrospinal fluid were collected from August, 2014 to January, 2016. Initial analyses (CSF cell count and differentials) were performed. The samples were then cryopreserved in a -20^0C freezer. A volume of 200µl each of the 80 cryopreserved clinical specimens of CSF were dispensed into cryovials and transported to the Virology Unit of Noguchi Memorial Institute for Medical Research with cold chain conditions for laboratory investigations for the agents of viral origin. Information on demographic characteristics of the patients, clinical data and laboratory test results on the clinical specimens for this work were extracted from patients' folders.

3.2 Study site

Clinical specimens for this study were obtained from the Fevers' Unit (FU) of the Korle Bu Teaching Hospital (KBTH) in Korle Bu, Accra. Korle Bu Teaching Hospital is a referral hospital and the largest tertiary health facility in Ghana. It has a 2000 patient bed capacity and 17 clinical and diagnostic departments. The FU, one of the departments within KBTH is an HIV care facility which offers both outpatient and inpatient care to patients on daily basis. The inpatient facility has 24 beds.

3.3 Study subjects

Clinical specimens of cerebrospinal fluid (CSF) used in this study had been cryopreserved in a -20^{0}C freezer since January, 2016. They were collected from hospitalized HIV patients who presented at the FU of KBTH with clinical symptoms consistent with meningitis. In all, eighty (80) clinical specimens of CSF from patients suspected with meningitis were collected, preserved and used for this study.

3.4 Case Definition

3.4.1 Inclusion criteria

All adults with clinical symptoms consistent with meningitis (headache, unexplained fever, nausea and vomiting, neck stiffness, confusion, seizures, abnormal behaviour, new onset of psychiatric symptoms, altered level of consciousness, photophobia and neurologic signs) that reported to the FU of the KBTH, had a lumbar puncture performed and were hospitalized were eligible for the study

3.4.2 Exclusion criteria

I. Children less than 18 years old

II. Difficulty in getting CSF or CSF that is inadequate for all analyses

3.5 Clinical Specimen Collection

Clinical specimens were collected from a patient as soon as the patient met the inclusion criteria and an informed consent was attained. For each patient, two CSF specimens were collected by a medical doctor into two different bottles; 2ml in a sterilized Bijoux bottle and 1ml in a fluoride bottle. The CSF specimens in fluoride bottles were transported to the Central Laboratory for CSF glucose, total protein and globulin assays. The CSF in Bijoux bottles were transported to the Medical Microbiology Department, SBAHS (MD-SBAHS) for initial microbiological analysis. Specimens were then cryopreserved in a --20^{0}C freezer until they were transported to the Noguchi Memorial Institute for Medical Research (NMIMR) for further processing. Blood specimen was also collected from the subjects for haematological analyses.

3.6 Macroscopic examination of CSF

Macroscopic analyses of the CSF were performed. This included the examination of colour and turbidity, the presence of a coagulum or deposit in the CSF. Normal CSF appearance were documented as clear and colourless and abnormal CSF appearances were documented as hazy, turbid, xanthochromic, blood-stained, presence of a coagulum or deposit.

3.7 CSF White cell count

A cover slip was put on the counting chamber of the haemocytometer. The volume of 5µl CSF were dispensed unto the covered counting chamber of the haemocytometer. Cells were then counted with the aid of a microscope. White cells that fell in the 4 quadrants plus 2 quadrants in the middle of the counting grid were counted. This gave the overall number of the CSF white cell count.

3.8 WBC Differentials

Smears of CSF sediments were made on grease-free glass slides. They were air-dried, heat-fixed and subjected to Gram staining techniques. They were viewed under the microscope. Stained white cells were identified as either polymorphonuclear neutrophils (PMNs) or lymphocytes.

3.9 Biochemistry and Viral Load

Glucose in CSF, Total Protein, Globulin and Viral Load tests were performed at the Central Laboratory of KBTH. The procedures employed in performing these tests are included in Appendix B.

3.10 Extraction of Nucleic acid

The extraction and purification of nucleic acid using QIAamp Viral Mini Extraction Kit (Qiagen, Hilden, Germany) was performed. The procedures followed were in accordance with the manufacturer's instruction and were as follows;

A starting volume of 560µl of prepared buffer AVL was added to 14µl each of the CSF specimens (the buffer AVL was prepared by reconstituting 310µg of lyophilized carrier RNA with 310µl of AVE buffer). The mixtures were pulse-vortexed for 15 seconds and incubated at room temperature for 10 minutes. An additional 560µl of absolute ethanol were added to the mixture of the specimens and the AVL buffer and pulse-vortexed for 15 seconds. A total of 630µl of the solutions were then transferred to the QIAamp Mini column. This was centrifuged at 8000rpm for 1 minute.

The QIAamp Mini column was placed in a collection tube and the tube containing the filtrate was discarded. The remaining 630µl was transferred into a new collection tube and centrifuged at

8000rpm for 1 minute. A volume of 500µl of Buffer AW1 was added and centrifuged at 8000rpm for 1 minute. The QIAamp column was placed in a new collection tube and the tube containing the filtrate discarded. A volume of 500µl of Buffer AW2 was then added and centrifuged at maximum speed (13,200rpm) for 3 minutes. The QIAamp Mini column was placed in a new collection tube and centrifuged at full speed for 1 minute. The QIAamp Mini columns were placed in appropriately labeled microcentrifuge tubes and the old tube containing the filtrate discarded. The QIAamp Mini columns were carefully opened and 60µl of Buffer AVE was then added. This was incubated for 1 minute at room temperature and centrifuged at 8000rpm for 1 minute. The purified Nucleic acid solutions were stored at -70°C for further laboratory analysis.

3.11 Quantification of Nucleic acid

The quantity and purity of the nucleic acid in each CSF extract was ascertained using the NanoDrop 2000c (Thermo Scientific, Massachusetts, USA). The absorbance measurements of the nucleic acids were measured at a wavelength of 260/280nm.

3.12 Extraction Efficiency and Optimization of EV, MuV and HSV-1 PCR

Human Coxsackievirus B1 (VR-28, Strain: Conn-5), Mumps virus (VR-106, Strain: Enders), Human Herpesvirus-1 (VR-539, Strain: Maclntyre) isolates (CDC Standard positive controls) were extracted using the QIAamp viral mini extraction kit from Qiagen. Real-time RT-PCR was performed for the RNA extracts (EV and MuV) and Real-time PCR was performed for the DNA extracts (HSV-1) using the appropriate primers under optimized conditions. Detailed procedure and Master Mix can be found at Appendix C.

Positive controls (used in quality control) were used to optimize and estimate the sensitivity of the PCR. The threshold cycle (Ct) of the positive control for EV was 15.5365, MuV was 23.2317 and HSV-1 was 15.3757. The controls were used as template and run together with the samples in each of the PCR runs.

3.13 Real Time PCR for Samples

3.13.1 Enterovirus PCR

Primers and probes were synthesized by a commercial supplier (Eurofins MWG Synthesis GmbH, Germany) based on previously published sequences (Verstrepen *et al.*, 2001). The primers and probes were directed to the 5' UTR of the enterovirus genome. The reporter dye (FAM) was attached to the 5' end of the probe and the quencher dye (TAMRA) was attached to the 3' end. The primer and the probe sets used included the Forward Primer, 5'-CCCTGAATGCGGCTAATCC-3', Reverse Primer, 5'-ATTGTCACCATAAGCAGCCA-3' and a Probe, 5'-AACCGACTACTTTGGGTGTCCGTGTTTC-3'

A reaction mixture was prepared for the total number of samples (80), one negative control and one positive control. The reagent used for the PCR reaction mixture was the AgPath-ID™ One Step RT-PCR Kit (Applied Biosystems, California, USA). The cocktail mixture was dispensed into an appropriately labelled 96 - well PCR. A total volume of 1640µl reaction mixture was prepared using 1025µl of 2X RT-PCR Buffer (Ag-Path-ID™ reagent kit) 82µl of reverse transcriptase/Taq mix (25X RT-PCR Enzyme Mix, Ag-Path-ID™ reagent kit), 41µl each of type specific primer and probe set and 410µl of nuclease free water. A volume of 20µl of the cocktail reaction mixture was dispensed into appropriately labelled wells of the 96-well PCR plate.

Volumes of 5µl of the template, positive and negative controls were then added accordingly as labelled.

Amplification was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies, California, USA). Cycling conditions for all primer and probe sets consisted of a reverse transcriptase step at 45°C for 45 minutes, followed by a *Taq* polymerase activation step at 95°C for 10 minutes and then 40 cycles at 95°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension step). Data was collected at the 60°C for 1 minute (annealing and extension) step

Enterovirus Diagnosis on ABI 7300 with Ambion Agpath –ID™ One Step RT-PCR Kit

Sample Number: 82

Table 3.1: PCR Master Mix Formula for Enterovirus

Master mix reagents	**Volume(µl)**	**Unit volume(µl)**	**Total**
Nuclease Free Water	5	5	410
2X Reaction Mix	12.5	12.5	1025
Probe (FAM-TAMRA)	0.5	0.5	41
Forward Primer	0.5	0.5	41
Reverse Primer	0.5	0.5	41
25X RT-PCR Enzyme	1	1	82
		20	1640

Template volume **5**

Reaction volume **25**

3.13.2 Mumps PCR

Primers and probes were synthesized by a commercial supplier (Eurofins MWG Synthesis GmbH, Germany) based on previously published sequences (Boddicker *et al.,* 2007). The primers and probes were directed to the small hydrophobic (SH) gene. The reporter dye (FAM) was attached to the 5' end of the probe and the quencher dye (BHQ) was attached to the 3' end. The primer and the probe sets used included the Forward Primer (SH61F) 5'- GTGACCCTGCCGTTGCA-3', Reverse primer (SH147R), 5'- GTTATGATCAGAGAGAGAAGAATTAGCAATAG-3' and Probe (SH79P2) 5'-TATGCCGGCGATCCAACCTCCCTTATA-3'

A reaction mixture was prepared for the total number of samples (80), one negative control and one positive control. The reaction mixture for Mumps virus was prepared using the AgPath-ID™ One Step RT-PCR Kit (Applied Biosystems, California, USA). A 96 PCR well plate map was drawn and this served as a guide in dispensing the reaction mix into the wells. A total of 1640µl reaction mix was prepared using 1025µl of 2X RT-PCR Buffer (Ag-Path-ID™ reagent kit), 82µl of reverse transcriptase/Taq mix (25X RT-PCR Enzyme Mix, Ag-Path-ID™ reagent kit), 41µl each type specific primer and probe set and 410µl of nuclease free water.

A volume of 20µl of the reaction mix was dispensed into each well of the PCR plate and 5µl of the template was then added to each well. A volume of 5µl of nuclease free water was added to the negative control well and 5µl of the template added to the positive control well.

Amplification was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies, California, USA). Cycling conditions for all primer and probe sets consisted of a reverse transcriptase step at 45°C for 45 minutes, followed by a *Taq* polymerase activation step at 95°C for 10 minutes and then 40 cycles at 95°C for 15 seconds (denaturing) and 60°C for 1 minute

(annealing and extension step). Data was collected at the 60°C for 1 minute (annealing and extension) step.

Mumps Virus Diagnosis on ABI 7300 with Ambion Agpath–ID™ One Step RT-PCR Kit

Sample Number: 82

Table 3.2: PCR Master Mix Formula for Mumps virus

Master mix reagents	**Volume(µl)**	**Unit volume(µl)**	**Total**
Nuclease Free Water	5	5	410
2X Reaction Mix	12.5	12.5	1025
Probe (FAM-BHQ)	0.5	0.5	41
Forward Primer	0.5	0.5	41
Reverse Primer	0.5	0.5	41
25X RT-PCR Enzyme	1	1	82
		20	1640

Template volume **5**

Reaction volume **25**

3.13.3 HSV-1 PCR

Primers and probes were synthesized by a commercial supplier (Eurofins MWG Synthesis GmbH, Germany) based on previously published sequences (Weidmann, Meyer-König, and Hufert, 2003). The target for the primers and probe was the glycoprotein D (gD) gene. The reporter (FAM) and the quencher (TAMRA) dyes were attached to the 5' and 3' ends of the probe respectively.

The primer and the probe sets used included the Forward Primer (HSV1UP), 5'-CGGCCGTGTGACACTATCG-3', Reverse Primer (HSV1DP), 5'-CTCGTAAAATGGCCCCTCC-3' and Probe (HSV1P), 5'-CCATACCGACCACACCGACGAACC-3'

A reaction mixture was prepared for the total number of samples (80), one negative control and one positive control. The reaction mixtures for herpes simplex virus 1 was prepared using the AmpliTaq Gold® PCR Master Mix (Roche, California, USA). A 96 PCR well plate map was drawn and this served as a guide in dispensing the reaction mix into the wells. A total of 1640µl reaction mix was prepared using 902 µl of Universal PCR Master Mix 2x, 205µl each type specific primer and probe set and 123µl of nuclease free water.

A volume of 20µl of the reaction mix was dispensed into each well of the PCR plate. 5µl of the template was then added to each well. 5µl of nuclease free water was added to the negative control well and 5µl of the template added to the positive control well.

Amplification was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies, California, USA). Cycling conditions for all primer and probe sets were set at 95°C for 10 minutes (AmpliTaq Gold activation) then 40 cycles at 92°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension step)

Herpes simplex virus 1 Diagnosis on ABI 7300 with AmpliTaq Gold PCR Master Mix

Sample Number: 82

Table 3.3: PCR Master Mix Formula for Herpes simplex virus-1

Master mix reagents	**Volume(µl)**	**Unit volume(µl)**	**Total**
Universal PCR Master Mix 2X	11	11	902
Probe(FAM-TAMRA)	2.5	2.5	205
Forward Primer	2.5	2.5	205
Reverse Primer	2.5	2.5	205
Nuclease Free Water	1.5	1.5	123
		20	1640

Template volume **5**

Reaction volume **25**

3.14 Ethics

Ethical approval was sought from the College of Health Sciences Ethical and Protocol Review Committee for this current study (PIN: CHS-Et/M.9-P 3.1/2015-2016). An informed consent was obtained from the study participants before they were enrolled after the study had been explained to the patients in an understandable language (Refer to Appendix A). For patients with impaired mental state, written informed consent was sought from the next of kin or immediate care giver after a thorough explanation of the study. Patients were assigned study identification numbers (IDs) which were used throughout the study. Subjects as well as all other patients (including patients that did not consent) were given the standardized care at the Fevers' Unit. The CSF specimens were examined at no cost to patients. Patients benefitted from an effort at identifying the causative organisms in the CSF and this resulted in improved patient management.

3.15 Data Handling

Patients' laboratory tests were not anonymized as these were required for routine patient care.

All documents were kept confidential and only researchers involved in the study had access to study data

3.16 Data Analysis

Clinical data and data obtained from laboratory tests were collected and recorded manually. These were entered in an Excel template. Analysis of the data was carried out using Statistical Package for Social Sciences (SPSS) V 20. Continuous data was presented as mean and standard deviation as well as range. Continuous variables were also presented using percentages and graphs.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**NC**											**PC**
B												
C												
D												
E												
F												
G												
H												

Figure 3.1: Plate map for EV, MuV, and HSV-1 Real Time PCR

NC- Negative control

PC- Positive control

- Samples

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic characteristics of study subjects

The target population for this study was hospitalized HIV patients at the FU of KBTH with clinical features of meningitis but yet to be confirmed. Eighty (80) patients were enrolled and CSF specimens were collected from these patients. Blood specimens were also collected for haematological studies. Data gathered from the folders of the 80 patients whose CSF specimens were collected include thirty- seven (37) males (46.25%) and 43 females (53.75%). Sixty-five which represents 81.25% of the patients were between the ages of 30 and 49 with only one patient being 18 years of age (Table 4.1 and Figure 4.1).

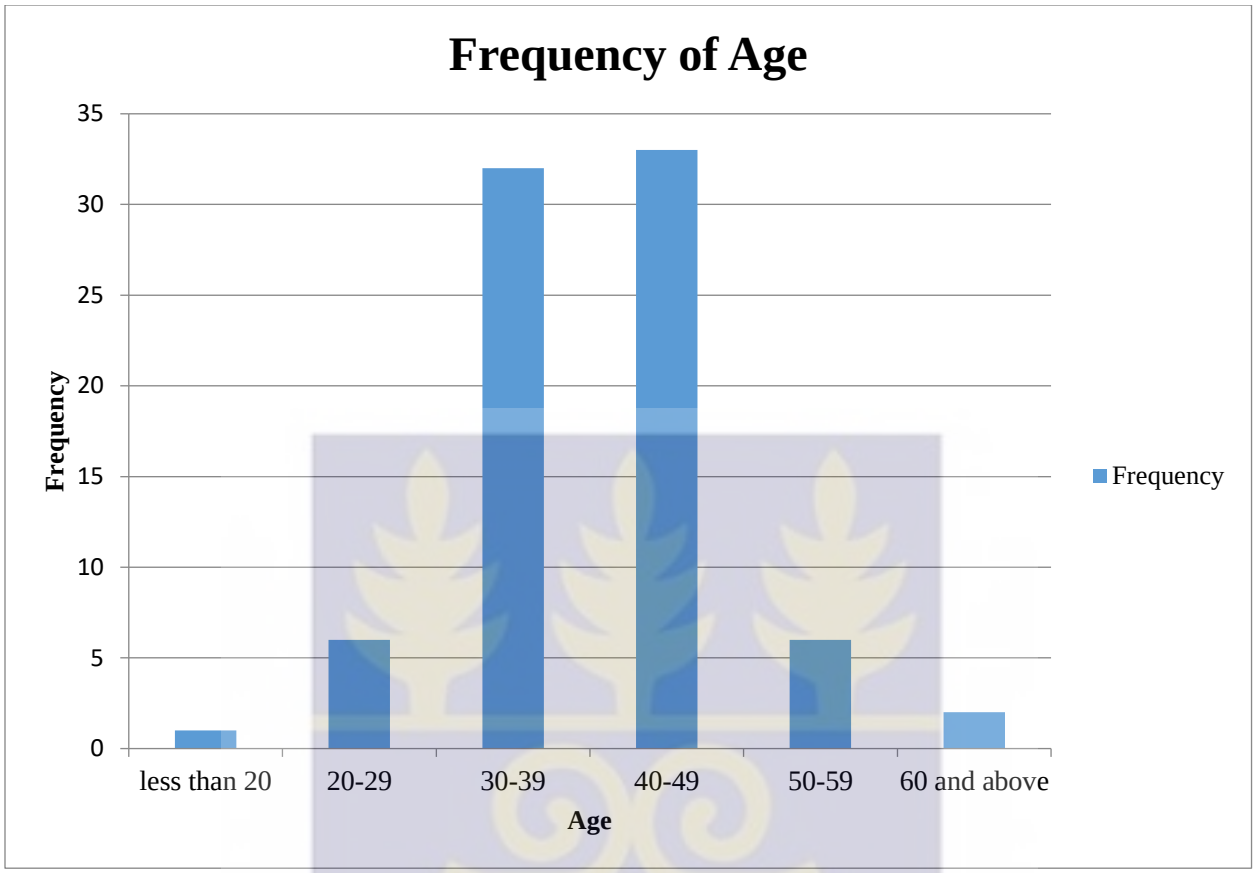

Figure 4.1: Age Distribution of Patients

Table 4.1: Demographic characteristics of subjects

Age (years)		
Mean± SD	*39.84±8.51*	
Range(min-max)	*18-65*	
	Frequency	**Percent**
Gender		
Male	37	46.25
Female	43	53.75
Education		
None	6	7.50
Primary	12	15.00
JSS	19	23.75
Middle School	16	20.00
Sec- Tech	12	15.00
Tertiary	13	16.25
Unknown	2	2.50

4.2 Clinical presentations of subjects on admission

Data on clinical presentations of subjects in this study indicates a majority of the patients had complained of headache, 63(78.75%). Thirty- seven which represent 46.25% of the patients also had fever. Nausea/ vomiting was seen in 10(12.50%), 9(11.25) had seizures, and 19(23.75%) had neck stiffness. Neurological disorder or confusion was observed in 22(27.50%) of the subjects,

meningismus **(**combination of the cardinal signs and symptoms of irritation of the meninges) in 28(35%) and tachycardia in 36(54%). Other presentations such as abdominal pains, diarrhoea, right limb weakness, cough, anorexia, weakness, chest pain, inability to walk, slurred speech, chills, fatigue, loss of appetite, general malaise, abnormal behavior, difficulty in swallowing, weight loss, neck pain, dizziness, sudden unresponsiveness, unstable gait, inability to talk, vomiting, watery stool, restlessness, palpitation, ear pain, swelling neck, sore throat were all recorded for the patients at different frequencies and degrees of severity. (Table 4.2).

Table 4.2: Clinical presentations on admission

VARIABLES	Frequency	Percent
Headache		
	63	78.75
Fever		
	37	46.25
Nausea-Vomiting		
	10	12.50
Seizures		
	9	11.25
Stiffneck		
	19	23.75
Confusion/NeuroSymptoms		
	22	27.50
Meningismus		
	28	35.00
Tachycardia		
	36	45.00

4.3 Cell counts of Participants

WBC count: A high CSF WBC count was set at more than 10 cells/mm^3 (for the purpose of this study on viral meningitis). A high WBC count was found in 17(23.75%) of the patients while a majority had CSF count lower than 10 cells/mm^3.

WBC Differentials: Results for WBC differentials was attained for 40 out of the 80 patients. Lymphocytic predominance was observed in 30(75.00) while neutrophilic pleocytosis was observed in 7(17.50%) of the subjects. Three of the patients had both neutrophilic and lymphocytic predominance.

CD4 count: The CD4 values for subjects ranged from as low as 1cells/mm^3 to as high as 979 cells/mm^3. Majority of the patients had CD4 less than 200cells/mm^3, 53(66.25%). Fourteen had CD4 greater than 200cells/mm3 which represented 17.50%

Viral load: A viral load as low as 20 copies/ml was recorded. Some patients too had characteristically high viral loads (Table 4.3).

Table 4.3: CSF Cell counts

VARIABLES	FREQUENCY	PERCENTAGE
WBC (cells/mm3)		
Mean ± SD	*14.39 ± 45.45*	
Range(min-max)	*(0-312)*	
<5	49	61.25
5 to 25	22	27.50
>25	9	11.25
CSF cell counts		
DIFFERENTIAL		
Lymphocytes	30	75.00
PMNs	7	17.50
PMNs/Lymph	3	7.50
Not done	40	
CD4 COUNT (cell/mm^3)		
Mean / SD	*1383.96 ±3285.23*	
Range(min-max)	*(1-979)*	
<200	53	66.25
>200	14	17.50
Failed	3	3.75
Not done	10	12.50
VIRAL LOAD (copies/ml)		
Mean / SD	*1045750.21±3107854.82*	
Range(min-max)	*(20-25500000)*	
<100,000	23	28.80
>100,000	38	47.50
Not detected	4	5.00
Not done	15	18.8

PMNs- Polymorphonuclear neutrophils

Lymph: Lymphocytes

4.4 Other clinical indices of participants

Antiretroviral Therapy (ART): Over two-thirds of the patients were not on antiretroviral drugs when they reported at the Fevers Unit. That accounted for 55(68.75%) of the total number of subjects selected for this study. The number of subjects on ART was 23(28.75%).

Stage of HIV: A majority (73.75%) of the subjects were at Stage IV of HIV, 11(13.75%) at Stage III and only one patient at Stage II.

Clinical outcome: Thirty (37.50%) of the patients were treated and discharged. Thirty-one (38.75%) died will undergoing treatment (Table 4.4).

Table 4.4: Other clinical indices

VARIABLES	FREQUENCY	PERCENTAGE
ON ART		
Yes	23	28.75
No	55	68.75
Unknown	2	2.50
STAGE of HIV		
II	1	1.25
III	11	13.75
IV	59	73.75
Unknown	9	11.25
CLINICAL OUTCOME		
Discharged	30	37.50
Died	31	38.75
NA	19	23.75

4.5 CSF Biochemistry of Participants

Glucose: The normal glucose level is 2.5-4.5 mmol/L (>60% of serum glucose). The Glucose level range of the participants was 1.00 to 8.80. Eighteen (22.25%) had CSF Glucose level less than 2.5mmol/L. Patients within the normal range accounted for 71.25% while patients with elevated glucose level were 5 (6.25%) .

Protein: The normal protein level in the CSF ranged from 0.03 to 9.86. Fifty-four (67.50) of the subjects had an elevated CSF protein level while 26(32.50%) had a CSF protein level less than 0.45

Globulin: A positive result for globulin test was recorded in 48(60%) of the patients while globulin test was negative for 29(36%) of the patients (Table 4.5).

Table 4.5: CSF Biochemistry

VARIABLES	FREQUENCY	PERCENTAGE
CSF- GLUCOSE (mmol/L)		
Mean / SD	*2.9025±1.22*	
Range(min-max)	*(1.00-8.80)*	
<2.5	21	26.25
>=2.5	59	73.75
CSF- PROTEIN (g/L)		
Mean / SD	*1.1498±1.56*	
Range(min-max)	*(0.03-9.86)*	
=<0.45	26	32.50
>0.45	54	67.50
CSF GLOBULIN		
Positive `	48	62.33
Negative	29	37.66

4.6 Haematological parameters of the study subjects

Haemoglobin: The haemoglobin level of most of the suspected viral meningitis patients was less than 12g/dl. This group accounted for 81.25% of the total number of patients. Fifteen (18.75%) of the patients had haemoglobin levels greater than 12g/dl.

Leukocyte count: The minimum leukocyte count was 1.57 x 10^9 cells/L and the highest 24.07 10^9 cells/L. Thirty- two of the patients, representing 40% had a blood leukocyte of less than 4 x10^9 cells/L, 40(50%) had between 4 and 10, and 8(10%) had greater than 10 x 10^9 cells/L.

Erythrocyte sedimentation rate: ESR was performed for 35 out of the total number of subjects. ESR values ranged from 3.70-150 mmol/L. Eight (22.85%) of the total number of patients had ESR less than 30mmol/L while 27(77.15%) had ESR greater than 30mmol/L (Table 4.6).

Table 4.6: Haematological parameters of the study subjects

VARIABLES	FREQUENCY	PERCENTAGE
HAEMOGLOBIN (g/dL)		
Mean / SD	*9.19±3.13*	
Range(min-max)	*(4.30-15.10)*	
<12	65	81.25
>=12	15	18.75
TOTAL WBC COUNT (10^9cells/L)		
Mean / SD	*5.11±3.86*	
Range(min-max)	*(1.57-24.07)*	
<4	32	40.00
4 to 10	40	50.00
>10	8	10.00
PLATELETS (/L)		
Mean / SD	*218.35±128.17*	
Range(min-max)	*(40-714)*	
<150	15	18.75
150 to 450	51	63.75
>450	14	17.50
ESR (mmol/L)		
Mean / SD	*70.11± 47.58*	
Range(min-max)	*(3.70-150)*	
<=30	8	22.85
>30	27	77.15

4.7 Drugs administered to subjects during hospitalization

The drugs administered to the patients constituted mainly of antibiotics and antifungal agents. Drugs for other conditions were also administered. In total, antifungals were administered to 41(51.25%) of the patients while 41.25% were given antibiotics. No anti-viral agents were administered to the patients (Table 4.7)

Table 4.7: Administered Drugs during hospitalization

DRUGS	Numbers	Percentage
Oral fluconazole	39	48.75
IV fluconazole	2	2.50
Oral Azithromycin	4	5.00
Septrin	10	12.50
Pyrimethamine	5	6.25
IV Metoclopramide	1	1.25
Anti Koch's	1	1.25
IV Ceftriaxone	5	6.25
IV Omeprazole	1	1.25
Oral Amoxiclav	4	5.00
Ceftriaxone	8	10.00

4.8 Measurements of the concentration and the purity of nucleic acids from clinical specimens

The absorbance measurements of all the 80 samples at a wavelength of 260/280nm yielded nucleic acids of high quality (Standard: DNA: 1.8; RNA: 2.0). Majority (96.25%) of the samples had quantities of more than 50 ng/ml. This is considered to be the minimum amount suitable for molecular studies (Psifidi *et al*., 2015). Only 3 samples had yield less than 50ng/ml and all the samples had a purity above the standard. Detailed results for all the samples can be found at Appendix D.

4.9 Amplification curve of Samples and Controls

In this study, none of the 80 suspected cases of meningitis investigated by PCR tested positive for EV, MuV and HSV-1**.** The DNA and RNA Real Time PCR were negative.

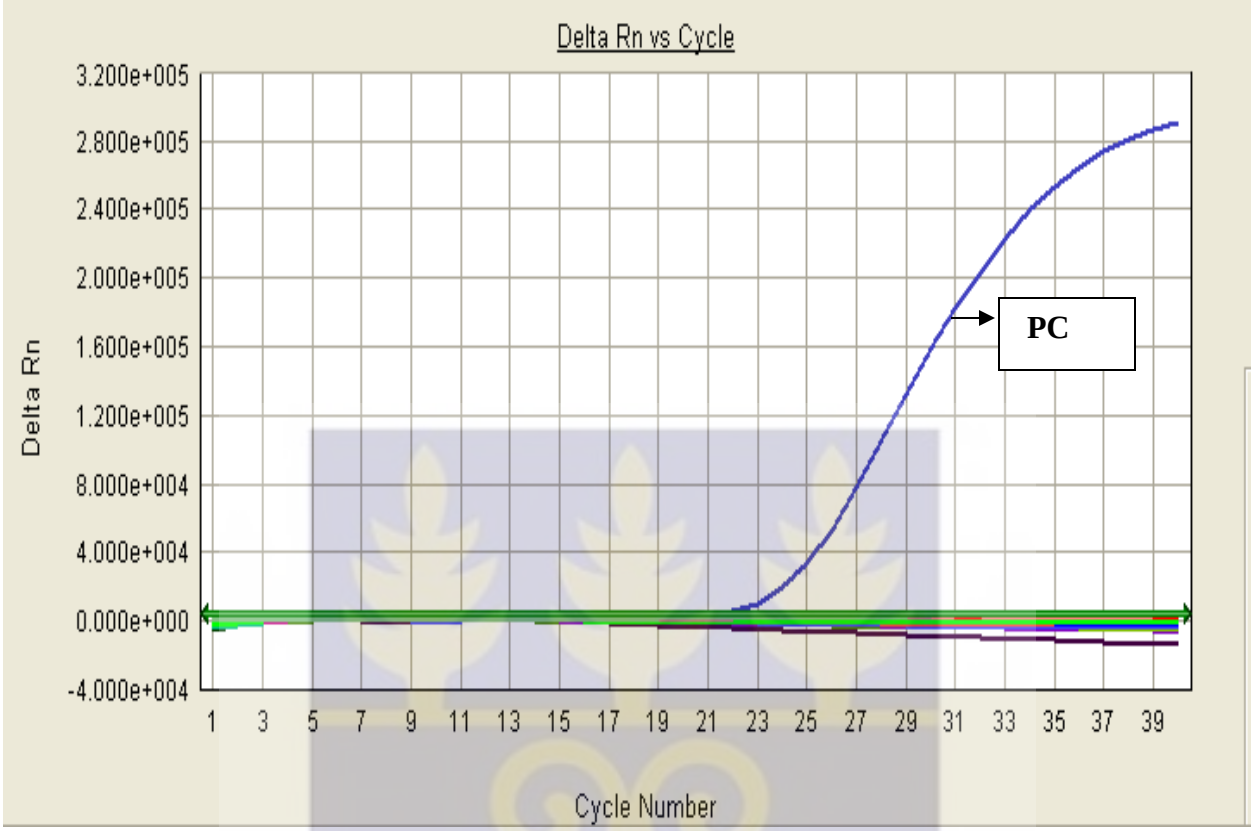

Figure 4.2: A figure representing the amplification curve of the Real-time PCR runs for the samples and controls.

The horizontal line with arrows represents the threshold. The curve that crossed the threshold represents the positive control **(PC)**. The samples and negative controls are below the threshold line (irregular lines) which represent a negative result. The amplification of the positive control depicts a successful PCR run.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Study Background

Viral meningitis is usually a benign condition that tends to be self-limiting (Hviid, Rubin and Mühlemann, 2008), however it can cause a lot of complications especially in the immunocompromised. This study sought to investigate with molecular tools, agents of viral origin in suspected meningitis cases from selected HIV patients hospitalized at a tertiary level hospital, KBTH, in Accra. The clinical specimens were suspected but not laboratory confirmed meningitis cases. This study recorded no positive signal by Real-time PCR for the viral agents (Enterovirus, Mumps virus and Herpes Simplex Virus-1) investigated. Information on demographic characteristics, clinical data and laboratory tests were extracted from patients' folders. Information captured included demographic characteristics, clinical and laboratory data. The age range of the patients whose samples were used for this study was 18-65. This is consistent with data from the surveillance report by the USA Centre for Disease Control on HIV patients' (CDC, 2015).

5.2 Clinical Presentations

Headache and fever were the most frequent clinical manifestations of the suspected meningitis patients whose clinical specimens were used in this study. This is similar to those presented in HIV patients with meningitis in other studies (Thinyane, Motsemme and Cooper, 2009; Herman and Fidele, 2007; Nowak, 2003).

Studies show that about 20–70% of adults with meningitis present with varying degrees of altered mental status ranging from confusion to impaired level of consciousness (Christensen *et al*., 2011; Schmidt *et al*., 2006; Wall *et al*., 2013; Veltman, Bristow, and Klausner, 2014; Domingo *et al*.,

2013). Data gathered from the patients with clinical specimens for this study showed a number of neurological symptoms (Table 4.2) and this gives credence to the clinical suspicion of patients as meningitis cases, which were however not confirmed in this study.

5.3 CSF Cell count and Biochemistry

In general, CSF in HIV-infected patients typically shows an increased lymphocytic pleocytosis, mildly elevated protein concentrations, and a normal glucose level (Davies *et al*., 2005; Roa *et al*., 2013). Lymphocytic pleocytosis was observed in 75.0% of the study subjects, normal glucose (71.3%) and protein level was slightly elevated in 67.5% of the study subjects. Despite these parameters that generally fit the case definition for viral meningitis, no viral agent was detected in the CSF of these patients. This may suggest that CSF cell count and Biochemistry may not be enough in predicting meningitis or even differentiating the types of meningitis.

5.4 CD4 Count of participants

Low CD4 is usually indicative of immunosuppression. The CD4 lymphocyte counts in this study show a severe degree of immunosuppression in most of the patients as was also seen in a study/results of Hakim and colleagues (Hakim *et al*., 2007). Most of the patients were at Stage III and IV (Table 4.4) of HIV and the low CD4 (Table 4.3) values may explain this level of deterioration.

Those with appreciable CD4 count and normal indices of other markers could be attributed to ART.

5.5 Haematological parameters

Haematological abnormalities are among the most common complications of HIV and it encompasses all the types of blood cells (Kirchhoff and Silvestri, 2008). The range of haemoglobin (Hb) for males is 13.5 to 17.5g/dl while that for females is 12.0 to 15.5g/dl (Johnson, 2002). The mean haemoglobin level for the subjects was 9.19g/dl which depicts a value lower than the normal haemoglobin level of immunocompetent individuals. This is similar to the finding in a study that had a significant drop in Hb levels of 149 HIV positive patients visiting the Central Regional Hospital, Cape Coast in the Central Region of Ghana, the main referral hospital in the Municipality and region as well as an HIV Centre (Tagoe and Asantewaa, 2011).

Erythrocyte sedimentation rate determination in HIV infected patients was found to be a predictor of the development of AIDS. The normal ESR range is 0-22mm/hr for males and 0-29mm/hr for females (MedlinePlus Medical Encyclopedia, 2016). In this study, only 35 patients had their ESR ascertained. Out of this, 77.15% of the patients had an ESR above the normal range. In a study, the mean ESR of HIV+ patients was significantly higher (55.79±46.67) compared with HIV- control patients (3.68±2.90) agreeing with this study (Barbara, 1990; Smith and Samadian, 1994). The high ESR could be due to decreased erythrocyte count.

5.6 Viral Agents Investigated

Considering a wide differential diagnosis in patients presenting with symptoms of meningitis is of prime importance and that is why this study set out to investigate three different viral agents that have been documented to cause meningitis in HIV patients. The aetiological agents investigated in this study were Enterovirus, Mumps virus and Herpes Simplex Virus-1. These viruses are implicated in cases of meningitis because of their neurotropic nature.

Enterovirus infections are common in children and adults. Clinical specimens selected for this study were from adults (Table 4.1) as the broader study sought to look at infection mainly in the sexually active group. However occurrence of EV meningitis in children is known to be higher than in adults. The higher positivity in children is thought be due to absence of pre-existing immunity and easy fecal-oral transmission (Sawyer, 2001; Mulford *et al*., 2004). A study by, Ihekwaba, Kudesia and McKendrick detected EV in 40% of the CSF of adults (Ihekwaba, Kudesia and McKendrick, 2008).

In this study, none of the 80 suspected cases of meningitis investigated by PCR tested positive for EV.

Mumps meningitis is a mostly benign condition. As such, patients may not seek medical attention; and, those who do, visit primary or secondary health care centres and may not require referral to tertiary care institutions (Hviid, Rubin and Mühlemann, 2008). This may account for the result achieved in this study since all the clinical specimens used were from patients hospitalized at the FU in KBTH.

A detection rate of 6.7% MuV from 90 patients with aseptic meningitis was reported in a previous study conducted between 1994 and 1995 in Pretoria, Gauteng, South Africa (Engelbrecht, Brisley. and Taylor, 1996). Mumps is thought to be common in children as compared to adults. An example is a recent study in Malawi, where mumps vaccine coverage is reported to be minimal, 5.7% of the study subjects with suspected viral CNS infection tested positive for MuV. Antibodies are not present in children because it tends to be their first time of getting infected (Maillet *et al*., 2015). This is in contrast to adults who have active antibodies against the MuV. The bias in selection introduced in this study could have contributed to no MuV been detected. Ihekwaba and

colleagues, however found out that 7% of adults who had their CSF samples analyzed had MuV (Ihekwaba *et al*., 2008). This study reports a no detection of MuV in CSF specimens investigated by PCR.

In a study on adults with CNS infection, HSV-1 accounted for 7% of the total number of samples tested (Ihekwaba *et al*., 2008). In other studies, the prevalence of HSV-induced meningitis ranged from 0.5% to 3.9% (Tedder, *et al*., 1994; Mommeja-Marin *et al*., 2003; Benjamin *et al*., 2013). Studies conducted, especially in the Western countries have proven that HSV and EV are among the leading causes of viral meningitis and other neurological infections in adults (Kupila *et al*., 2006; Granerod *et al*., 2010).

In this study however, none of the 80 suspected cases of meningitis investigated by PCR tested positive for HSV-1

Primer-probe combinations for the EV was directed to the 5' UTR conserved sequences. This is able to detect all enterovirus strains, except for echoviruses 22 and 23, which have recently been reclassified as parechovirus because they possess a divergent genome sequence (Romero, 1999; Oberste *et al*., 1998). In this study, the test run for each PCR performed included controls of positive and negative samples and this validated each test run. Until the positive and the negative control indicators are correctly shown after each run, the test cannot be considered valid. A repeat run was carried out in instances where none or one of the control indicators showed after the test run. All the PCR test runs for this work were validated.

Most of the extracts from the specimens used in this study had a high quantity of nucleic acids when compared to the standard value needed for molecular analysis. The absorbance of the nucleic

acids extracted also yielded nucleic acids of high quality **(Result 4.8).** This points to the fact that there was nucleic acids present in the extracted materials and the extracts were of high quality and yield.

There have been studies that employed the use of Real-time PCR in the diagnosis of viral meningitis where positive results have been achieved (Sawyer, 1994; Benjamin *et al*., 2013). There have been other studies too where there was no detection of virus in the CSF using Real-time PCR (Thoren and Widell, 1994). In this study, the PCR did not detected any of the viruses tested for in the CSF of the patients.

In a study by Kupila and colleagues, CSF PCR for enterovirus was performed on samples obtained from patients with enteroviral meningitis; an enterovirus RNA was found in 26 of these samples. The researchers in that study observed that, the rate of positivity by PCR was clearly lower for CSF obtained more than two days after the clinical onset of disease. This possibly indicates that the use of CSF to detect enteroviral RNA decreases a few days after the onset of meningitis (Kupila *et al*., 2005).

In another study, the CSF from patients that had been collected 16 to 21 days after the onset of the neurological symptoms were negative for MuV PCR, while CSF from the PCR-positive patients had been obtained after an average of 4 days from the time of the first appearance of symptoms (Poggio *et al.*, 2000).

The optimum time span for obtaining a positive HSV-PCR is probably between 2 and 10 days after onset of the illness (Davis and Tyler, 2005).

Majority (87.50%) of the patients whose samples were used in this study visited the facility when they had progressed to Stage III and IV of HIV and this could be due to stigmatization or low financial background (Davis and Tyler, 2005). Most of the patients also visited the Fevers Unit many days after the onset of the signs and symptoms of meningitis. This could have also contributed to the lack of positives in the study.

There is a possibility that certain CNS diseases are not always accompanied by the presence of an organism in the CSF (Read, Jeffery, and Bangham, 1997). Indeed, it has been proposed that up to 60 percent of cases of meningitis and other CNS infections remain undiagnosed even after extensive diagnostic work up (National Institute of Neurological Disorders and Stroke, 2016; *Thomas Attlee, 1992).*

Constant freeze and thaw might have also affected the integrity of the viruses. A study showed that the in vitro sensitivity by PCR decreased about 10-fold after freeze - thawing the titrated virus dilutions a few times (Glimåker *et al*., 1993).

Studies show that toxoplasmic meningitis, tuberculous meningitis and cryptococcal meningitis are among the most common CNS opportunistic infections in patients with HIV/AIDS in sub-Saharan Africa (Thinyane, Motsemme, and Cooper, 2015; Harkim, 2000; Jarvis 2010; Gulshan, 2015). This suggests that the signs and symptoms might have been due to other aetiological agents apart from Enteroviruses, Mumps virus and Herpes Simplexvirus-1.

5.7 Limitations

Clinical and laboratory data for some patients were incomplete. This resulted in difficulties in the analyses of certain parameters.

Fewer viral agents were investigated. Many viruses have been implicated in viral meningitis but in this study, three viruses were studied.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

None of the viruses (EV, MuV, HSV-1) investigated in this study was implicated in the suspected cases of meningitis. However, lymphocytic pleocytosis, normal glucose and elevated protein levels observed in majority of study participants.

6.2 RECOMMENDATIONS

The prevalence of viral meningitis should be investigated in children since the condition is common in children.

A broader study on meningitis that considers other viruses (apart from EV, MuV and HSV-1) as well as other pathogens should be conducted

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APPENDICES

APPENDIX A

INFORMATION AND CONSENT FORM

Title: Viral agents in the cerebrospinal fluid of hospitalized HIV patients with meningitis at the Fevers' Unit, Korle- Bu Teaching Hospital

Investigator: Emmanuel Frimpong Adjei

Institution/ Organization: Department of Microbiology, School of Biomedical and Allied Health Sciences

This research aims at isolating or detecting viral agents from the CSF of HIV infected patients at the Fevers Unit of the Korle Bu Teaching Hospital. Viral meningitis is the inflammation of the meninges as a manifestation of central nervous system (CNS) infection and with viruses being the causative agents. Infections of the central nervous system (CNS) with viral agent can be self-limiting and benign, but occasionally result in severe and fatal disease especially in the immunocompromised. Cerebrospinal fluid (CSF) is required for laboratory confirmation of the disease. Early diagnosis of the disease is essential for appropriate treatment to be given to the patient and this will help prevent fatal consequences. I wish to work with you to find the prevalence and outcome of viral meningitis among hospitalized HIV patients. This would ensure that HIV patients who develop viral meningitis or who are at advanced stages of HIV/AIDS are managed well to prevent death.

All patients presenting with symptoms of meningitis (headache, seizure, altered mental state, fever and others) at the Fevers' Unit will be eligible for the study. Informed consent will be sought from the patients who are admitted to be recruited as participants or respondents if their mental state is normal or from a caregiver or next of kin if mental state is abnormal but patient meets inclusion criteria.

Possible Risks and Discomforts

The procedure for obtaining samples for laboratory tests can be associated with very rare risks including mild pain, bruising, bleeding or infection. A medical doctor will take the sample aseptically. CSF and blood loss for the study will not adversely affect your health. Any discomfort will be evaluated promptly and managed by medical officers.

Possible Benefits

As a participant, you will benefit from an effort by the researcher to identify causative organisms in the CSF. This should result in an improvement in your management. The CSF laboratory investigations will be analyzed at no cost to you.

Confidentiality

All the information collected in the study will be kept confidential. Results of investigation or other information that we collect will only be shared with medical staff taking care of you and authorized members of the research team. Any data that may be published will not reveal your identity.

Compensation

There will be no cost or payment to you for your participation in this study.

Freedom to refuse or withdraw

You should join the study only if you want to. You can ask any question. You can leave the study at any time without giving any reason and this will not affect your medical care and treatment provided.

Contact

This proposal has been reviewed and approved by the University of Ghana Medical School Ethical and Protocol Review Committee which ensures that the participants of the study are protected from harm. If you have any concerns about your right as a participant, you can contact the chairman of the Ethical and Protocol Review Committee of the College of Health Sciences, Korle-Bu, Accra.

CONSENT FORM

The above document describing the benefits, risks and procedures for the research titled**, "Viral agents in the CSF of hospitalized HIV patients with Meningitis at the Fevers' Unit, Korle-Bu Teaching Hospital**" has been read and explained to me/caregiver/next of kin. I/caregiver have read and understood the above information. I/caregiver have been given opportunity to ask questions about the research, and have received satisfactory answers. I/caregiver agree to participate in this study as a volunteer.

…….. ……

Date Signature/Thumbprint of patient/caregiver

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual

…….. ……

Date Signature/Thumbprint of Witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Signature of Researcher

APPENDIX B: CSF Biochenistry and Viral load tests

CSF Biochemistry

The colour, turbidity, presence of a coagulum or deposit of collected CSF were noted and documented. Normal CSF appearance was documented as clear and colourless, abnormal CSF appearance was documented as hazy, turbid, xanthochromic, blood-stained, presence of a coagulum or deposit. Blood stained specimen were centrifuged at 5000rpm for 5 minutes and reported as clear supernatant plus red cell deposit, persistent colouration with or without red blood cells. Xanthochromic, hazy, turbid or cloudy, coagulum, fibrin clot were centrifuged at 5000rpm for 5 minutes and the supernatant was used.

Globulins

0.5ml of Pandy's reagent was dispensed into a clean dry glass test tube. 2 or 3 drops of CSF specimen was added to the tube and mixed well. The set up was observed for an immediate turbidity which indicates a positive reaction or a clear solution which signifies a negative reaction.

Total protein

0.8ml of 3%Trichloroacetic acid (TCA) was pipetted into three test tubes; the Reagent Blank, Standard and Test. 0.2ml of standard specimen and CSF specimen were pipetted into the

Standard tube and Test, respectively. The solution was mixed well and incubated at room temperature for 10 minutes. A spectrophotometer was zeroed with distilled water and the absorbance of the standard was read against the reagent blank and the test at 670nm.

Glucose

0.1ml Glucose oxidase was pipetted into three test tubes; Reagent Blank, Standard and Test. 0.001ml of distilled water, peroxidase and CSF specimen were pipetted into Reagent blank, Standard and Test, respectively. The contents of the three test tubes were mixed well and incubated for 15 minutes at room temperature or 7 minutes at 37˚C. The absorbance of standard and sample were measured against reagent blank at 505nm. Glucose concentration of CSF specimen was calculated using the formula below;

Glucose= (Absorbance of Sample/Absorbance of Standard) ×Conc. of Standard (mmol/L)

Viral load specimens and processing

About 4ml of blood was phlebotomized from the patients and transferred into an EDTA vacutainer. The blood specimen was centrifuged at 1000rpm for 1minute. The plasma was separated from the corpuscles and transferred into a cryotubes and stored at -80˚C. Plasma was thawed before use.

The viral loads of the participants were estimated using the COBAS®AmpliPrep/COBAS®TaqMan®HIV-1Test, Version 2.0. 850µL plasma for each specimen was processed in the COBAS®AmpliPrep instrument by a generic silica-based capture technique to get a processed specimen that contained the HIV-1 RNA.

The processed specimens were then added to amplification mixtures in amplification tubes and heated to anneal downstream primers to the HIV-1 target RNA as well as to HIV-QS RNA to form a DNA strands complementary to the RNA target.

The reaction mixtures were heated using Thermal Cycler in the automated equipment to reverse transcribed and amplified target DNAs and cooled to denature RNA:cDNA hybrid. Double stranded DNAs were produced by using Thermus species Z05 DNA polymerase in the presence of Mn2+ and excess deoxynucleotides triphosphates. Automatic repeats of the process doubled the amount of double-stranded DNA. DNA strands containing deoxyuridines were destroyed by AmpErase enzyme leaving DNA containing deoxythymidine.

HIV-RNAs were detected using real-time PCR technology where dual-labelled fluorescent probes allowed for real-time detection of accumulated PCR products by monitoring the emission intensity of fluorescent reporter dyes that were released during the amplification process.

APPENDIX C: Quality control and optimization of PCR

The reaction mixtures for Enterovirus and Mumps virus were both prepared using the AgPath-ID™ One Step RT-PCR Kit (Applied Biosystems). A 25µl reaction mix was prepared using 5µl of RNA, 12.5µl of 2X RT-PCR Buffer (Ag-Path-ID™), 1µl of reverse transcriptase/Taq mix (25X RT-PCR Enzyme Mix, Ag-Path-ID™), 0.5µl each type specific primer and probe set and 5µl of nuclease free water. Primers and probes were synthesized by a commercial supplier (Eurofins MWG Synthesis GmbH, Germany) based on previously published sequences (Verstrepen *et al.*, 2001, Boddicker *et al.*,2007). Amplification was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies, USA). Cycling conditions for all primer/probe sets consisted of a reverse transcriptase step at 45°C for 45 minutes, followed by a *Taq* polymerase activation step at 95°C for 10 minutes and then 40 cycles at 95°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension step). Data was collected at the 60°C for 1 minute (annealing and extension) step.

Enterovirus primers and probe

Primer and probe sequences used in the enterovirus real-time RT-PCR assay

Forward primer 5'-CCCTGAATGCGGCTAATCC-3'

Reverse primer 5'-ATTGTCACCATAAGCAGCCA-3'

Probe 5'-AACCGACTACTTTGGGTGTCCGTGTTTC-3'

Master mix reagents	Volume(µl)
Nuclease free water	5
2x Reaction Mix	12.5
Probe (FAM-TAMRA)	0.5
Forward Primer	0.5
Reverse Primer	0.5
25x RT-PCR Enzyme	1
Template	5
Total	**25**

Mumps primer and probe

Forward primer 5'- GTGACCCTGCCGTTGCA-3'

Reverse primer 5'- GTTATGATCAGAGAGAGAAGAATTAGCAATAG-3'

Probe 5'-TATGCCGGCGATCCAACCTCCCTTATA-3'

Master mix reagents	Volume(µl)
Nuclease free water	5
2x Reaction Mix	12.5
Probe (FAM-BHQ)	0.5
Forward Primer	0.5
Reverse Primer	0.5
25x RT-PCR Enzyme	1
Template	5
Total	**25**

The reaction mixtures for herpes simplex virus 1 was prepared using the AmpliTaq Gold® PCR Master Mix (Roche). A 25µl reaction mix was prepared using 5µl of DNA, 11 µl of Universal PCR Master Mix 2x, 2.5µl each type specific primer and probe set and 1.5µl of nuclease free water. Primers and probes were synthesized by a commercial supplier (Eurofins MWG Synthesis GmbH, Germany) based on previously published sequences (Weidmann, Meyer-König, and Hufert, 2003). Amplification was performed on the Applied Biosystems® 7300 Real-Time PCR instrument (Life Technologies, USA). Cycling conditions for all primer/probe sets were at 95°C for 10 minutes (AmpliTaq Gold activation) then 40 cycles at 92°C for 15 seconds (denaturing) and 60°C for 1 minute (annealing and extension step)

HSV 1 primers and probe

Forward primer 5'- CGGCCGTGTGACACTATCG-3'

Reverse primer 5'- CTCGTAAAATGGCCCCTCC-3'

Probe 5'- CCATACCGACCACACCGACGAACC-3'

Master mix reagents	Volume(µl)
Universal PCR Master Mix 2X	11
Probe	2.5
Forward Primer	2.5
Reverse Primer	2.5
Nuclease free water	1.5
Template	5
Total	**25**

APPENDIX D: Quantity and purity of nucleic acids

The absorbance measurements of the nucleic acids at a wavelength of 260/280nm yielded nucleic acids of high quality/purity (DNA: 1.8; RNA: 2.0). Most of the extract had a quantity of 50 ng/ml or more of nucleic acid which is considered to be the minimum amount suitable for molecular studies (Psifidi *et al.*, 2015).

SAMPLE ID	NUCLEIC ACID CONCENTRATION (ng/µl)	PURITY (260/280nm)
004	86.9	3.19
005	69.6	3.22
006	75.8	3.23
007	69.4	3.27
008	74.0	3.15
011	74.6	3.26
012	64.2	3.12
013	71.7	3.21
014	75.1	3.15
016	74.2	3.12
017	82.6	3.15
018	74.7	3.15

019	69.8	3.16
020	86.4	3.22
021	65.2	3.17
022	79.4	3.23
025	72.8	3.18
027	84.2	3.23
029	87.5	3.17
030	73.7	3.19
031	80.4	3.23
032	77.2	3.17
033	75.0	3.37
034	96.6	3.22
035	83.8	3.28
037	14.3	2.69
041	89.6	3.20
043	93.0	3.15
045	101.3	3.18

046	20.5	3.12
047	86.0	3.24
048	91.6	3.35
049	93.4	3.24
050	18.1	2.72
051	94.9	3.15
052	91.6	3.22
053	55.5	3.05
054	94.8	3.25
057	90.9	3.27
059	93.2	3.33
060	94.4	3.15
062	93.3	3.21
063	96.5	2.90
064	100.8	3.26
065	91.3	3.19
066	53.2	3.12

067	62.6	3.20
068	96.8	3.24
069	92.8	3.20
070	99.1	3.28
071	86.4	3.09
072	94.0	3.17
073	57.1	3.10
075	76.3	3.24
076	73.2	3.31
077	63.0	3.17
078	87.7	3.11
079	83.5	3.08
080	92.1	3.05
081	66.7	3.21
082	87.7	3.35
083	82.9	3.29
084	90.3	3.03

085	63.0	2.08
086	81.3	3.26
087	75.8	3.26
088	82.4	3.17
089	85.0	3.26
090	72.5	3.02
091	62.6	3.21
092	84.5	3.20
093	87.7	3.25
095	82.5	1.94
097	73.8	3.31
098	86.5	3.23
099	101.4	2.75
101	83.0	3.23
102	75.8	3.23
103	69.7	3.25
104	94.8	3.23