Serological and Molecular Epidemiology of Flaviviruses in Selected Provinces of Zambia

By

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The University of Zambia
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Declaration

This dissertation is original work of Boniface Kabungo. It has done in accordance with the guidelines for the University of Zambia. It has not been submitted elsewhere for a degree at this or another university

Candidate Name: **Boniface Kabungo**

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I, **Boniface Kabungo**, hereby certify this dissertation is the product of my own work and, in submitting it for the Degree of Master of Science in Medical Microbiology programme, further attest that it has not submitted to another University in part or full for the award of any programme.

I, **Dr. Mwaka Monze** having supervised and read this dissertation is satisfied that this is the original work of the author under whose name it is being presented.

I further confirm that the work has been completed satisfactory and is ready for presentation to the examiners.

Signature/Supervisor……

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Certificate of approval

The University of Zambia approves this dissertation of Boniface Kabungo in partial fulfilment of the requirements for the award of the degree of Master of Medical Microbiology.

Examiner's signatures Date

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Abstract

Flaviviruses may cause zoonotic or arboviral diseases which are transmitted by arthropods (mosquitoes and ticks). They are capable of spreading by human to human transmission and may result in large outbreaks. In 2010, Zambia was reclassified a low risk zone of yellow fever (YF) by World Health Organization (WHO). To date, none of the YF suspects has been confirmed as a case of YF and the epidemiology of flaviviruses has not been thoroughly investigated in Zambia. Therefore, the objective of this study was to determine the extent to which flaviviruses are causing YF like illness in selected provinces of Zambia. This cross-sectional study was conducted on 93 archived serum samples previously collected from patients presenting in selected health facilities and meeting the case definition of YF suspect from January 2014 to July 2015. Nucleotides from serum were tested by reverse transcriptase polymerase chain reaction (RT-PCR) and nested polymerase chain reaction (nPCR) for flaviviruses and hepatitis viruses respectively. Samples were also tested for YF and dengue fever (DF) antibodies using in-house Immunoglobulin M (IgM) enzyme linked immunosorbent assay (ELISA) and IgM rapid test respectively. Chi-square, two sample test of proportion and logistic regression were the statistical methods used for data analysis. Fourteen percent (13/93) of the serum samples were identified as YF IgM positive. None of the samples tested positive for dengue IgM. All 93 serum samples tested negative for the flaviviruses by RT-PCR or nPCR, whilst 8.6% (8/93) showed acute Hepatitis A and 10 % (2/20) of pooled sera was genetically confirmed for Hepatitis B virus. The median age for the group infected was 9.5 years and the uninfected group was 19 years old with Hepatitis A. Approximately 85 (91.4%) of patients had acute diseases of unknown origin. Viral hepatitis was the cause of morbidity and mortality in acute febrile jaundiced patients as observed in our study areas. Hence, the incidence of acute hepatitis A and hepatitis B was high and need to be considered if flaviviral diseases are suspected.

Dedication

This dissertation is dedicated to my wife who has always encouraged me to work hard in my postgraduate studies and financial support she offered. I also dedicate it to my daughters; Theresa, Musanya and Nelly for their moral support. To my late parents; Mr. Lotson M. Kabungo and Mrs. Paulina M. Kabungo who educated and taught me to give my best in all that I do in life however difficult.

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List of Abbreviations

BSL	Biosafety Level
CDC	Center for Disease Control and Prevention
CVs	Coefficient of Variations
DENV	Dengue virus
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DNA	Deoxyribonucleic Acid
DRC	Democratic Republic of Congo
DSS	Dengue Syndrome Shock
ELISA	Enzyme-Linked Immunosorbent Assay
HAV	Hepatitis A Virus
HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
HEV	Hepatitis E Virus
IDRS	Integrated Disease Response Surveillance
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JEV	Japanese Encephalitis Virus
JRCS	Japanese Red Cross Society
MOH	Ministry of Health
PRNT	Plaque-Reduction Neutralization Test
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SLEV	Saint Louis Encephalitis Virus
UTH	University Teaching Hospital

UTHVL	University Teaching Hospital Virology Laboratory
VHFs	Viral Haemorrhagic Fevers
WHO	World Health Organization
WNV	West Nile Virus
YFV	Yellow Fever Virus
ZIKV	Zika Virus

Chapter 1

General introduction

1.0 Introduction

1.1 Background

The genus *Flavivirus* of the family *Flaviviridae* consists of approximately 73 virus species. Several members of this family include yellow fever virus (YFV), dengue virus (DENV) and West Nile virus (WNV) that are associated with human diseases (Daep *et al*, 2014). These are zoonotic diseases that have the potential to cause human to human transmission, and can result in large disease outbreaks (Bakir *et al*, 2005; Grolla *et al*, 2012). They are caused by RNA viruses (Mackenzie *et al*, 2004). Yellow fever virus is found in the tropical and sub-tropical areas of South America and sub-Saharan Africa. The global travel may result in the spread of the virus to Asia and Europe including North America (Johansson *et al*, 2010; Hammarlund *et al*, 2012; Wang and Zhou, 2016). Dengue virus is much more widespread than YFV which has never been documented in Asia (Gould and Solomon, 2008). The WNV has a wide geographical distribution that includes Europe, Asia, Africa and America (Filette *et al*, 2012). Both yellow fever and dengue fever have a common transmission vector and can co-circulate in the same area (Appawu *et al*, 2006; Patel *et al*, 2013).

Generally, viral haemorrhagic fevers (VHFs) typically manifest as rapidly progressing acute febrile syndromes with profound haemorrhagic manifestation and very high case fatality rates (Warfield *et al*, 2003; Zhai *et al*, 2006). The clinical course of flaviviruses in humans may include; asymptomatic infection, mild illness with flu like symptoms whilst the severe disease includes fever, haemorrhage and death (Garske *et al*, 2014). The diseases may not easily be distinguished from other febrile diseases such as viral hepatitis and malaria due to

the non-specific manifestations of the VHFs (Sutherland *et al*, 2011; Domingo *et al*, 2012). The burden of viral hepatitis in Africa is not well known but it is believed to be significantly high (Patel *et al*, 2014).

The outbreaks of flaviviral diseases are increasingly being reported in Africa and South America in the recent years due to high number of international trips. The growth of international travel with people travelling from Asia, Europe and North America to endemic areas has increased the risk of introduction of flaviviruses into areas where competent vectors are present (Johansson *et al*, 2010). The ecological disruption may increase the possibility of introduction of these viruses into new areas or increase incidence in endemic countries (Patel *et al*, 2013; Escadafal *et al*, 2014). The unplanned urbanization, population movement, poor sanitation, climate change, deforestation and human encroachment in wildlife sanctuaries may increase the possibility of human-vector contact (Gubler and Clark, 1995; Guzman *et al*, 2009; Murray *et al*, 2013).

Following the reclassification of Zambia as a low risk YF Zone, the Ministry of Health (MOH) in collaboration with World Health Organization (WHO) instituted a risk assessment in 2013 followed by YF case based surveillance in January, 2014. This program was designed to confirm all suspected YF cases within Zambia at University Teaching Hospital Virology Laboratory (UTHVL). According to WHO clinical case definition, a YF suspect is defined as "Any case presenting with acute onset of fever, with jaundice appearing within 2 weeks after onset of symptoms" (Gould *et al*, 2005; WHO, 2005; Kwallah *et al*, 2015). The samples collected from suspected YF suspects are primarily screened using IgM-capture ELISA. The program is conducted in North-Western, Western, Muchinga, Copperbelt and Lusaka Provinces. None of the presumptive positive samples has been confirmed as a case of YF at

the Institute de Pasteur, Dakar, Senegal. This study aimed to determine the serological and molecular epidemiology of flaviviruses in selected provinces of Zambia.

1.2 Statement of the Problem

Flaviviral infections are capable of causing high morbidity and mortality. Globally, the incidence of flaviviral outbreaks has been on an increase in the recent years (Patel, 2013; Sessions *et al*, 2013). Yellow fever and DF outbreaks have been documented in the Democratic Republic of Congo (DRC) and Angola in the recent past (Dash *et al*, 2012; Meltzer *et al*, 2014; WHO, 2014, Barret, 2016). In Zambia, no outbreak has been documented for at least 60 years although recent data suggest low levels of flaviviruses in circulation (Robinson, 1950; Liwewe *et al*, 2014, Babaniyi *et al*, 2015). Since Zambia shares a common border with the DRC and Angola, there is a risk that flaviviruses from these endemic countries could be imported into the country. Several importations of VHFs to countries where these diseases are non-endemic have been documented, and this has been attributed to international travel, porous border and trade (Bannister, 2010; CDC, 2010, Barret, 2016). In 2010, the WHO reclassified part of Zambia as an area with potential for YF transmission mainly North-Western and Western regions of the country which are contiguous with DRC and Angola. The recent data suggest the presence of suitable vectors of flaviviruses (Kuniholm *et al*, 2006; Masaninga *et al*, 2014; Verma *et al*, 2014; WHO, 2014). The risk of dengue infection on Zambian population is attributed by the activity of *Aedes aegyptis* mosquito (Gibbons and Vaughn, 2002; Liwewe *et al*, 2014). Additionally, the disease is more likely to be misdiagnosed because of its low awareness by health workers (Amarasinghe *et al*, 2011; Sessions *et al*, 2013).

The symptoms of flaviviral disease are usually non-specific. It is possible these viruses or other non flaviviruses that present with YF like illness could be circulating unnoticed with potential to cause a large outbreak unexpectedly. This could pose a challenge to our untested public health rapid response systems. Therefore, we need to be certain if these flaviviruses or other viruses are circulating in our environment so that appropriate preventive measures can be undertaken.

1.3 Justification of the Study

Zambia has recognized the importance for an early warning system for these flaviviral outbreaks. The Ministry of Health introduced an enhanced YF case based surveillance in January, 2014. This system has been capturing cases fitting the YF case definition. A case of YF is defined as "any case presenting with acute onset of fever, with jaundice appearing within 2 weeks after the onset of symptoms" (Gould *et al*, 2005; WHO, 2005; Kwallah *et al*, 2015). Samples are collected from YF suspects in all health facilities in Western, North-Western, Muchinga, Copperbelt and Lusaka Provinces. These samples are tested for YF IgM antibodies using an ELISA assay at the UTHVL. To date, none of the presumptive YF IgM positive cases detected have been confirmed as YF by Plaque reduction neutralization test (PNRT) at the WHO AFRO Regional Reference Laboratory (Institut Pasteur, Dakar, Senegal). There is therefore a need to establish the aetiology of this YF-like illness. Yellow fever infection produces similar symptoms to infections with other flaviviruses. It is important to ascertain whether these other viruses could be causing the illnesses currently seen.

A YF risk assessment was conducted by the WHO in collaboration with the MOH in 2013 in North-Western and Western Provinces of Zambia to confirm whether the classification of

these areas as being low risk for transmission is justified. The assessment showed some evidence of past and recent infection with YF in both provinces with an overall sero prevalence of 0.3% in these two provinces (Babaniyi *et al*, 2015). However, serological data has its limitations. Assays used tend to be non-specific due to cross reactivity of flavivirus antibodies (Mansfield *et al*, 2011). The study did attempt to check for cross reactions by measuring antibody titres using PNRT and considering only cases above certain threshold titres as confirmed YF cases. However, interpretation of such results is very difficult. This study employed the more specific molecular methods to confirm these cases.

Appropriate prevention and control measures are dependent on a thorough knowledge of the distribution and frequency of other non-YF flaviviruses in Zambia. As long as we remain unaware about their presence, we may be directing efforts in the wrong direction. There may be need to modify our surveillance methodology to capture relevant information. The study has provided baseline data on viruses circulating in our environment and will enhance efforts to prevent and control further disease transmission in Zambia and worldwide.

1.4 Literature Review

1.4.1 General Characteristics and Classification of Flaviviruses

The genus *Flavivirus* of the *Flaviviridae* contains approximately 70 viruses including YFV, DENV and WNV that are of major human public health concern (Mackenzie *et al*, 2004; Patel *et al*, 2013; Kwallah *et al*, 2015). Yellow fever is thought to have originated from West Africa (Staples and Monath, 2008; Lima *et al*, 2012). The DENV has four distinct serologic sub types: DEN-1, DEN-2, DEN-3 and DEN-4. There is extensive cross reactivity among these viruses, but there is no cross protective immunity in humans and all four serotypes have been reported in Asia, Africa and America (Gubler and Clark, 1995; Mackenzie *et al*, 2004; Sood, 2013). West Nile virus is also a member of the Japanese encephalitis (JE) serocomplex which include; St. Louis encephalitis (SLE) virus and Murray Valley encephalitis (Riabi *et al*, 2014).

1.4.2 Epidemiology of Flaviviruses and Hepatitis viruses

Globally, YF remains an important public health problem for populations in 45 African and Latin American countries, and almost 900 million individuals are at risk of being exposed to YF (Thomas *et al*, 2012; Escadafal *et al*, 2014). Brazil is among other South American countries where the YF is endemic and the disease principally occurs in the Amazon region and contagious grasslands of South America (Romano *et al*, 2014). Yellow fever mainly affects men engaged in lumbering or clearing land for agriculture (Manoth, 2001). However, YF outbreaks have never been documented in Asia despite the conditions being conducive for the introduction of the virus following the presence of a large susceptible human population and suitable urban vector (Hanley *et al*, 2013).

In Africa, the distribution of YF is largely restricted to tropical rain forests, moist savannah and contagious dry savannah areas (Weir and Haider, 2004). The revised global YF risk map of 2011 showed that 27 out of 32 countries have been classified as endemic countries in Africa and the other 5 countries have been designated as a low potential for exposure to YF (Agampodi and Wickramage, 2013). In West Africa, YF is actually re-emerging following the discontinuation of mass vaccination approximately 20-30 years ago following a noted association with a high incidence of encephalitis in children below 10 years old and most of which has remained unknown due to poor disease surveillance (Wiysonge *et al,* 2008; Briand *et al*, 2009). The largest recorded outbreak in the post-YF vaccine era occurred in Ethiopia during 1960-1962 with more than 100,000 people acquiring YF that led to 30,000 deaths (Agampodi and Wickramage, 2013).

Yellow fever disease remains an important public health problem because of its case fatality of 20-50% (Bourgaral *et al,* 2010). The true incidence of YF is unknown worldwide but it has been estimated at 200,000 cases and 30,000 deaths that occur each year, and 90% of these cases occur in sub-Saharan Africa (Talwani *et al,* 2011; Markoff, 2013). However, these figures are under reported, annually approximately 5000 cases are reported to the WHO from Africa and 300 cases from South America (Almeida *et al*, 2014). The continued presence and epidemic potential of YF virus have made it a global health threat following an increased number of travelers who are potentially exposed to the virus and pose a risk of introducing YF in areas where competent vectors are present (Johnsson *et al*, 2010; 2012).

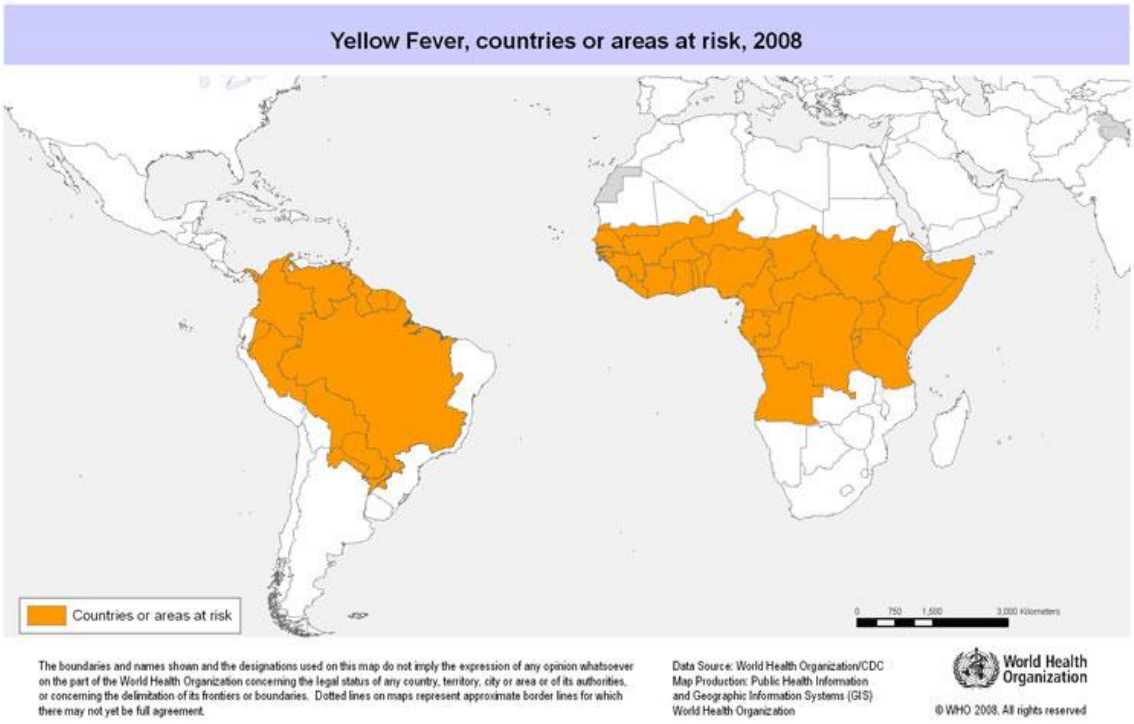

Figure 1.4.2.1: Global epidemiology of Yellow fever (Reference: WHO, 2008)

Recently, Zambia was reclassified as an area with low potential for exposure because the Western and North-Western regions of the country are contiguous with endemic countries such as the DRC and Angola, where the disease is prevalent (WHO, 2014). The first reported suspected case was described from the North-Western Province in 1943, and antibodies were also detected in the Western part of Zambia with a seroprevalence rate up to 18% in 1951-1953 (Jentes *et al*, 2010). Following the recent YF risk assessment, there is evidence of past and recent infection of YF in both provinces with seroprevalence of 0.3% (0.2% in North-Western and 0.4% in Western Provinces) (Babaniyi *et al*, 2015).

Dengue is endemic in more than 100 countries found in most tropical areas of Asia, America and some parts of sub-Saharan Africa (Guzman and Kouri, 2004; Mansfield *et al*, 2011;

Wichmann *et al*, 2011). Over 2.5 billion people or 40% of world population are at risk of dengue infection, and about 390 million people are infected annually (Guzman and Isturiz, 2010). The annual average number of dengue fever/dengue haemorrhagic fever (DF/DHF) cases reported to the WHO has increased dramatically in recent years, although there has never been official report to the WHO from countries in Africa and the Eastern Mediterranean region (Guzman *et al*, 2010; Were, 2012). Yet, outbreaks of suspected dengue have been documented in Pakistan, Saudi Arabia, Yemen, Sudan, Madagascar and Cape Verde Island (Guzman *et al*, 2010). The unplanned urbanization, population movement, lack of proper sanitation facilities and also viral evolution are the important factors that have resulted in an increased disease burden (Gubler and Clark, 1995; Guzman *et al*, 2009; Murray *et al*, 2013). The classical dengue fever is not lethal but the fatality rates in untreated cases may exceed 20% (Singhi *et al*, 2007; Sherin, 2011). Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS) forms of the disease lead to death, and are often associated with infections by a different b serotype (Sood, 2013; Lizarranga and Nayar, 2014). However, these conditions are rarely seen in Africa (Kuniholm *et al*, 2006; Gardener and Sarkar, 2013). The WHO estimates that dengue virus alone produce 50-100 million clinical cases each year, but the true disease burden is unknown in most endemic countries (WHO, 2009; Gregory *et al*, 2010; Vong *et al*, 2010; Sherin, 2011; Rao *et al*, 2013).

Although Japan has a temperate climate, the first dengue outbreak in over 70 years occurred prompting the Japanese Red Cross Society (JRCS) to advise people who recently visited the Tokyo parks to refrain from donating blood (Kyodo, 2014). As of September 2014, the cumulative number of people infected with the disease stood at over 70 cases since the first case was reported in August, 2014 (Carney, 2014). In Africa, the disease is endemic in 34 countries (Were, 2012). According to a study conducted in the Western and North-Western

Provinces of Zambia, the seroprevalence rate of Dengue specific IgG antibodies was 4.1% (Liwewe *et al*, 2014). However, the disease is more likely to be misdiagnosed or underreported in Africa because of its low awareness by health workers (Amarasinghe *et al*, 2011; Sessions *et al*, 2013).

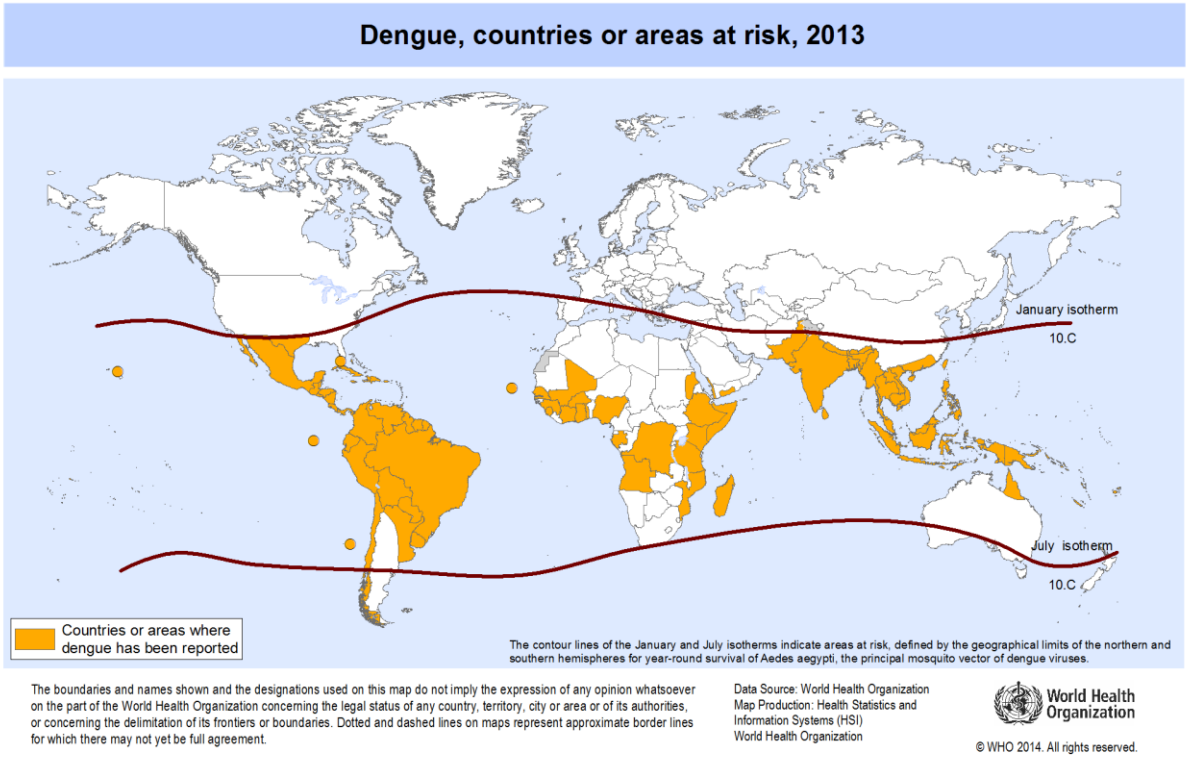

Figure: 1.4.2.2: Global epidemiology of Dengue haemorrhagic fever (Reference: WHO, 2013)

West Nile virus is the the most widespread flavivirus in the world, with the geographical distribution covering Amerca, Eurasia and Africa (Tran *et al*, 2014). Migratory birds are thought to be primarily reponsible for viral dispersal, including the reintroduction of WNV from endemic areas into regions that experience sporadic outbreaks (Yeh *et al*, 2011; De filette *et al*, 2012). Febrile illness occurs in approximately 20% of WNV infected individuals;

neurologic complications (meningitis, encephalitis, flaccid paralysis) occurs in less than 1% and 80% remain asymptomatic (Zayman and Venter, 2012; Paz, 2015).

Geograghically, the virus has been in circulation in Africa since 1937 (Riabi *et al*, 2003). Several human outbreaks have been reported in DRC, Uganda, Sudan, Tunisia, South Africa and Kenya mainly associated with mild febrile illnesses (Nur, *et al*, 1999; Raibi *et al*, 2014). In Zambia, no study has been done on human population to determine the presence this flavivirus.

Zika virus (ZIKV) has been reported in sub-Saharan Africa, Asia, Australia, Oceania, south and central America. Zika is not endemic in Poland because the *Aedes* mosquitoes that transmit that virus is absent. However, there is potential risk of importing the disease due to increased global travel and trade from endemic countries (Korzeniewski *et al*, 2016). Typically, ZIKV causes a mild self limited illness and 80% of ZIKV infections remain asymptomatic (Boef *et al*, 2016). The spectrum of the disease mimic other arboviral infections.

In Africa, the virus has been in circulation in Africa since 1947 when it was first described in rhesus monkey in the Zika forest of Uganda and has emerged as a global threat with an accelarated geographic spread noted in the last 5 years (Gyawali *et al*, 2016; Sikka *et al*, 2016). The human illness caused by ZIKV was first recognised in Nigeria in 1953 (Peterson *et al*, 2016). However, it was not until 2007 that virus was recognised as clinically important pathogen capable of causing disease outbreaks (Posen *et al*, 2016; Wikan and Smith, 2016).

In Zambia, findings from the previous seroepidemiological survey suggested Zika virus Ig G positivity of 6.1% in children less than 5 years old in North-western and Western province (Babaniyi *et al,* 2014; 2016).

Japanese encephalitis (JE) a mosquito borne disease is a principal viral encephalitis in Asia, the Western Pacific countries and Northern Australia (Wang and Liang, 2015). About 3 billion people live in JE endemic countries and foreingn travellers are at risk of acquiring the disease. China accounts for 50% of these cases (Zheng *et al,* 2012). About 1% of human JEV infections result in JE with a case fatality of 20-30% and 30-50% of the survivors suffer significant neurologic or psychiatric sequelae (Malhotra *et al,* 2015). Although several vaccines are currently available, approximate 67,000 cases and 20,400 deaths occur annually (Ishikawa and Konishi, 2014). The true disease burden is not known (Batchelor and Peterson, 2015). Majority of these cases occur among children below the age of 15 years and the risk is highest among the rural and agricultural areas (CDC, 2013; Lopes *et al,* 2015).

Although, reports of presence of JEV have neven been documented in Africa, where other flaviruses circulate they should also be included in the differential diagnosis especially that only with appropriate diagnostic tests dengue viruses and West Nile virus can distinguished from JEV (Solomon *et al*, 2000).

Saint Louis encephalitis virus is the major representative of the Japanese encephalitis serocomplex in America and Caribbean Islands where it is transmitted by the mosquitoes of the genus *Culex* (Hoyos-Lopes *et al,* 2015). It is usually maintained in cycles between birds and mosquitoes; humans and horses are the dead end hosts (Diaz *et al*, 2011). The majority of

the cases remain asymptomatic. However, the mortality rates of symptomatic patients vary from 5% to 20% with a disproportionate impact on the elderly and young patients (Kopp *et al*, 2013).

Hepatitis A is an acute self-limiting infection of the liver caused by HAV with an annual estimate of 1.4 million cases of new infection worldwide (Verma and Khanna, 2012; Pereira *et al*, 2014). More than 80% of the adult patients with hepatitis A have symptoms but the majority of children do not have symptoms (Suiijkerbuijk *et al*, 2009). In a study conducted in Thailand, the mean age of symptomatic HAV has shifted from childhood towards early adulthood in rural areas and transitioning from early adulthood to middle adulthood in urban areas (Effelterre *et al*, 2016). Despite the low mortality, it is a significant cause of morbidity and socio economic losses in many part of the world (Pereira *et al*, 2014). Africa has generally been considered a region of high endemicity of hepatitis A infection with South Africa as the only exception (Poovovawan *et al*, 2002; Kanyenda *et al*, 2015). In Zambia, there is a scarcity of data on HAV infection. This suggests that HAV infection is thus still considered a neglected disease.

Hepatitis B is a serious global public health problem. Of the approximately 2 billion people who have been infected in world, more than 350 million are chronic carriers of Hepatitis B (Nkhrumah *et al*, 2011; Bekondi *et al*, 2015; Papastergious *et al*, 2015). Approximately 15-45 % of the infected patients will develop cirrhosis, liver failure or hepatocellular carcinoma (HCC) with a case fatality of 500,000-1.2 million deaths worldwide (Hou *et al*, 2005; Musa *et al*, 2014).

The WHO has stated that the prevalence of hepatitis B is highest in sub Saharan Africa and East Asia and 5-10 % of the patients are chronically infected (Howell *et al*, 2014; Muller *et al*, 2015). However, this figure is an underestimate of true disease burden due to poor surveillance in many poor countries where the disease is endemic (Howell *et al*, 2014). However, the true burden of disease is not well known in Zambia as data is only limited to blood donors who are routinely screened for hepatitis B virus. Therefore, no epidemiological study has ever been conducted on human population.

Hepatitis C virus is a leading cause of chronic liver disease in the world. The WHO estimates 185 million people with HCV substantially higher than Human immunodeficiency virus (HIV) rates (Layden *et al*, 2014). About 3-4 million of hepatitis C infections occur yearly making it one of the leading public health in the world (Blankson *et al*, 2005). Globally, the geographical distribution of the infection appears to vary from one region to another region. The sub Saharan Africa has the highest disease burden of 5.3% prevalence (Chaabna *et al*, 2014). In Japan, up to 90% of all reported cases of HCC are caused by HCV infection (Averhoff *et al*, 2012). Egypt has the highest prevalence of 14.7 % of HCV in the world (Karoney and Siika, 2013; Chaabna *et al*, 2014). This is a result of Egypt's government not agreeing to use somewhat expensive diagnostic tests to determine if donor blood is infected with HCV (Miller *et al*, 2015). Zambia is one of the countries in Africa with the lowest prevalence (0.2 %) of HCV (Karoney and Siika, 2013).

Hepatitis E is an acute self-limiting disease which does not result in chronic infection and has a broad worldwide distribution (Pelosi and Clarke, 2008). About 20 million infections and 3.4 million cases occur annually worldwide with an estimated 70,000 deaths (Khurooo *et al*,

2016). HEV infection is particularly severe during pregnancy; pregnant women are at risk to develop liver and obstetric complication, with mortality rate as high as 10-25% (Gerbi *et al*, 2015). Outbreaks of hepatitis E have been reported in African countries and between 5 % and 70 % of sporadic cases of acute hepatitis present with laboratory evidence of acute of HEV infection (Shimakawa *et al*, 2016). However, the disease burden of acute hepatitis E on Zambian population is unknown due to lack of capacity for acute viral hepatitis surveillance.

1.4.2 Transmission of Flaviviruses and Hepatitis viruses

The principal vector of DENV is an infective female mosquito, *Aedes aegyptis*, which is found in tropical and sub-tropical areas and a secondary vector, *Aedes albopiticus,* which has been implicated in Asia (Moncayo *et al*, 2004). The *Aedes* species is a day time feeder, an efficient vector and restless mosquito as the slightest movement interrupts feeding, thus several people may be bitten in short period during one blood meal (Gibbons and Vaughn, 2002).

Yellow fever is transmitted by a bite of *Aedes* species mosquitoes in Africa or *Haemagogus* species mosquitoes in South America (Farnon *et al*, 2010). Yellow fever infection occurs in three cycles known as jungle fever, intermediate (Savannah) and urban fever. In the first transmission cycle, the virus circulates in the forest mainly between non-human primates and various mosquito species. In the urban cycle, the virus spreads and solely circulates among humans (Lima *et al*, 2012). In Africa, an intermediate cycle exists that involve transmission of YF virus from tree hole-bleeding *Aedes* species to humans living or working in jungle border areas (CDC, 2010).

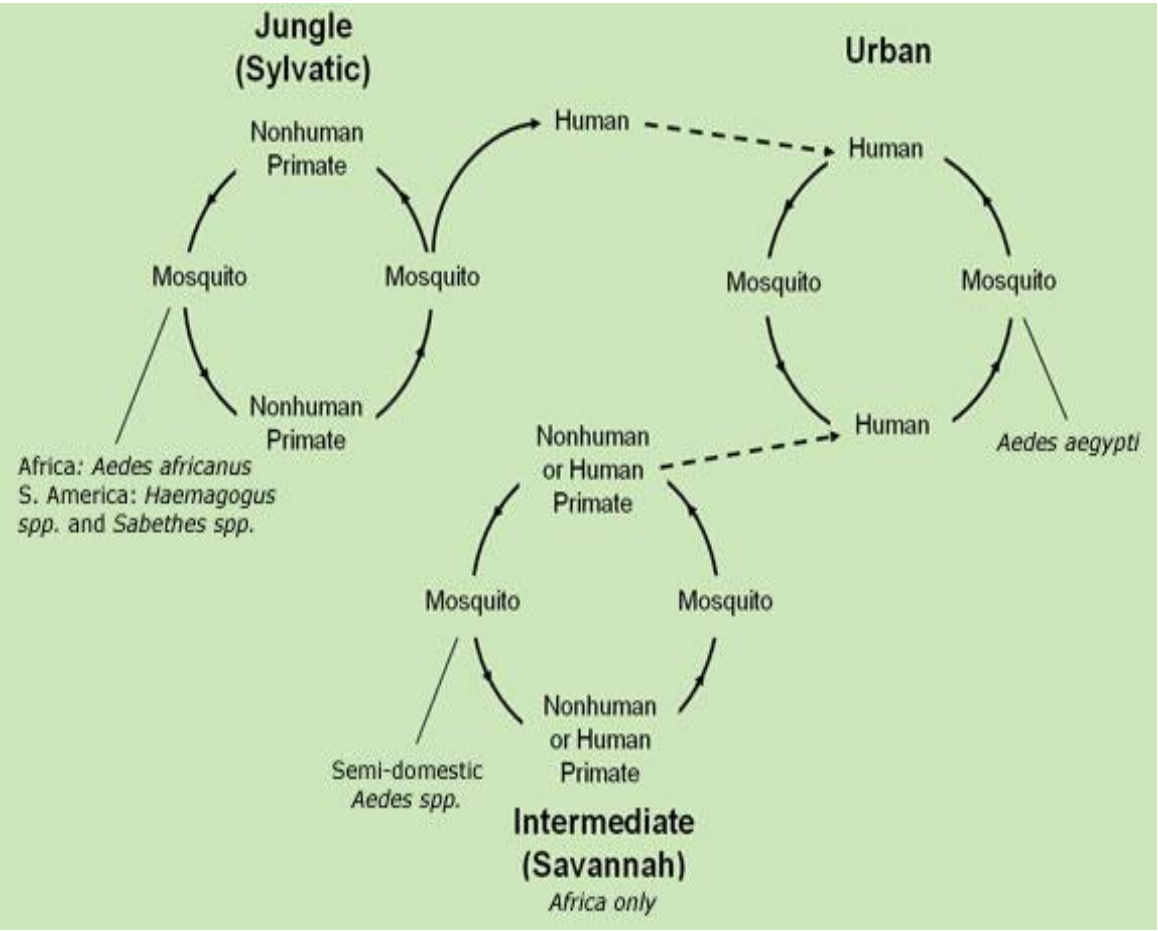

Figure 1.4.2.3: Transmission events of Yellow fever (Reference: CDC, 2010)

West Nile virus is maintained in a cycle between birds and mosquitoes. Although many different species of mosquito are capable of maintaining this cycle, the *Culex* species play the largest role in natural transmission (Rossi *et al*, 2011). Human and Horses are accidental or "dead end" hosts in this cycle since the concentration of the virus within blood is insufficient to infect feeding mosquitoes. Its transmission can also occur between mother and new born via the intra uterine route or possibly breast feeding (Alpert *et al*, 2003; Hinckley *et al*, 2007).

Zika virus is transmitted by the day active *Aedes* mosquitoes and potentially by sexual contacts, blood transfusion and from mother to fetus causing microcephaly in a child (Korzeniewski *et al*, 2016). In Africa, ZIKV exists in a sylvatic transmission cycle involving

non-human primates and forest dwelling species of *Aedes* mosquitoes. In Asia, a sylvatic transmission cycles has not yet been identified. In urban and sub urban environment, ZIKV is transmitted in a human-mosquito-human transmission (Peterson *et al*, 2016).

Japanese encephalitis is transmitted in zoonotic cycle among mosquitoes and vertebrates amplifying hosts, chiefly pigs and wading birds (Hassain *et al*, 2004). Humans are the dead end host and as such there is no human to human transmission. This *Culex* mosquito species preferentially breed in the rice paddies and is a duck and evening feeder (Batcher and Peterson, 2015).

HAV and HEV are generally acquired by the faecal oral either through ingestion of contaminated food and water (Jacob *et al*, 2014). Person to person transmission is common and generally limited to close contacts (Franco *et al*, 2012). HBV and HCV are spread through contact with blood and bodily fluids including saliva and sexual transmission (Karoney and Siika, 2013). Perinatal transmission has also been implicated (Hou *et al*, 2005).

1.4.4 Clinical Presentation

The onset of YF and DF is usually sudden and the duration of illness may vary from 2-6 days. Yellow fever is characterized by non-specific fever and headache while the severe form of the disease is manifested by high fever, jaundice and death (Domingo *et al*, 2012). Dengue infection in human causes a spectrum of clinical systems ranging from acute self- limited DF to DHF and DSS (Chen and Wilson, 2004; Das *et al*, 2008; Murray *et al*, 2013). Dengue haemorrhagic fever is characterized by abnormalities in haemostasis and increased vascular permeability. It may progress to the life threatening DSS which is a result of hypovalaemic shock associated with haemo concentration (Umareddy *et al*, 2008). West Nile virus in

human presents with non-symptomatic disease or mild influenza-like illness that goes undetected and generally characterized by acute fever, headache, myalgia malaise, nausea and vomiting (Raibi *et al*, 2003). Symptoms of ZIKV disease includes fever, skin, rashes, conjunctivitis, muscle and headache which normally last for 2-7 days (Hayes, 2009). Furthermore, the symptoms of flaviviral diseases apart being clinical in presentation could be mimicking malaria which has been known to be endemic in Zambia.

The incubation period of viral hepatitis varies depending on the type (Pereira *et al*, 2014). The symptoms of acute viral hepatitis include fatigue, flu-like symptoms, dark urine, fever and jaundice; however viral hepatitis may occur with minimal symptoms that resolve spontaneously (Nagu *et al*, 2008; Grossner *et al*, 2015). Rarely, acute viral hepatitis (HAV and HEV) causes fulminant hepatic failure (Verma and Khanna, 2012). However, the development of chronic hepatitis linked to HEV has been described in immunosuppressed patients (Jacob *et al*, 2014). The symptoms of chronic viral hepatitis often are mild and non-specific. HBV- and HCV- related chronic hepatitis is considered the main cause of cirrhosis, hepatocellular carcinoma (HCC) and liver transplantation (Kamal *et al*, 2010).

1.4.5 Diagnosis

The diagnosis of VHFs is based initially on clinical judgement with laboratory testing to confirm or exclude clinical suspicion. Most diagnostic laboratories use serological assays for *Flavivirus* testing. These tests are commonly based on the Enzyme–Linked Immunosorbent Assays (ELISA), and detect virus-specific IgM and IgG antibodies. An IgM-specific test is suitable for detecting recent infection, but the relevance of IgM testing for acute VHFs depends on the virus and duration of illness. An IgG-specific ELISA is efficacious, not only in the diagnosis of a large number of VHFs cases but also for epidemiological studies in

endemic regions (Fukushi *et al*, 2012). The antibody titres tend to be low at least in the early phase of the disease, and this renders serological assays not to be the primary option (Srikiathachorn and Spiropoulou, 2014). The IgM antibodies are not detectable prior to 5-7 days after the onset of infection, and detectable levels of IgG antibodies appear at about 10-12days (Ksiazek*et al*, 1999; Patel *et al*, 2013). Dengue IgM has been shown to appear from the seventh day of illness onward with 70% positivity, and by the tenth day the patients are expected to achieve 100% positivity of antibodies. A major drawback of serological assays is that there is no assay that can provide definite diagnosis because they all remain presumptive (Rathakrishanan and Sekaran, 2012). The IgM antibodies against YFV and other flaviviruses have a strong cross-reactivity which may generate false results in serological results (Crill and Chang, 2004; Mansfield *et al*, 2011; Tigoi *et al*, 2015). The possibility of cross-reaction occurring as a result of recent or passive exposure to other flaviviruses can be ruled out by additional test such as Polymerase chain reaction (PCR).

Viral identification in cell culture is the gold standard of viral detection (Leland and Ginocchio, 2007). However, testing requires a Biosafety Level 3 (BSL-3) facility because of the risks associated with handling clinical samples (Vanhomwegen *et al*, 2012). Electron microscopy can be an important adjunct to other methods for virus identification but possibly cannot be relied on its own (Samuel and Tyagi, 2006). However, the Electron microscopy can also be employed to confirm cases of VHFs within a few days of the onset of symptoms (Hezelton and Gelderblom, 2003; Adegboro and Adeola, 2011; Ahmed and Broor, 2014).

The PCR has now become a standard diagnostic and research tool due to its high sensitivity and specificity, allow a rapid, efficient method of detecting, identifying and quantifying the

pathogens (Das *et al*, 2008; Faye *et al*, 2013; Maheaswari *et al*, 2016). The RT-PCR can rapidly detect and identify the selected gene target of the RNA viruses and pathogens that are difficult or impossible to culture and detect by traditional methods (Speer, 2006; Trombley *et al*, 2010). This molecular method can also be used to diagnose the unknown virus by a high throughout sequencing which uses random amplified DNA product sequences and compare the obtained product sequences with available extensive banks of sequences for final viral identification and confirmation (Marher-sturgess *et al*, 2008; Goldsmith *et al*, 2013). Despite its widely application in diagnosis, the method requires primers with perfect or close sequence to the pathogen genome. Although it is not difficult to design primers specific to an individual strain of a pathogen, genetic drift and selection produce a variety of sequence variants that can be difficult to target effectively (Gijavanekar *et al*, 2011). A panel of comprehensive one step real time reverse transcriptase Polymerase chain reaction (RT-PCR) assays covering all the important pathogens suitable for multiplex screening with fast turn-around time and identical cycling parameter, is a necessity so that the unknown samples can be analyzed simultaneously and effectively (Pang *et al*, 2014).

1.5 Research Questions

1.5.1 Could we be missing YF cases when using serological methods in our surveillance?

1.5.2 Could cases presenting with YF like symptoms that are not YF be due to other viruses?

1.6 General Objective

1.6.1 Objective

To determine the extent to which flaviviruses are causing YF-like illnesses in selected Provinces of Zambia.

1.6.2 Specific Objectives

(a) To determine the proportion of YF-like illness that is due to flaviviruses.

(b) To analyze the genotype of flaviviruses detected in Zambia.

(c) To ascertain whether there could be other viruses responsible for the YF-like cases identified in Zambia.

Chapter 2

Materials and Methods

2.1 Study Design

This was a cross-sectional laboratory based study.

2.2 Study Sites

The MOH established a YF case based surveillance in 5 Provinces of Zambia including North-Western, Western, Copperbelt, Muchinga and Lusaka Provinces. North-Western and Western Provinces were selected because they share a contiguous with endemic countries and also to supplement the previous YF risk assessment's findings (Babaniyi *et al*, 2015). Copperbelt Province was selected because a case of Chikungunya fever and presence of the suitable vector have been previously documented (Roger, 1961; Kumar and Gopal, 2010). The increased global movement of people across endemic countries leaves the human populations in Lusaka province vulnerable to emerging infection (Bannister, 2010). Whilst Muchinga was included due to its new provincial status. These provinces are already familiar with Integrated Disease Surveillance Response (IDSR) procedures and trainings were conducted to equip them with appropriate skills to roll out the YF surveillance to all health facilities in the provinces such as identify patients that meet YF case definition, collect samples and ship samples to the national laboratory. In this study, all available serum samples were drawn from these provinces.

Figure 2.2: Map of Zambia Showing the Study Sites (circled Provinces) (Source: Google maps, 2015)

2.3 Sample Size

A convenient sample of 93 bio-banked serum samples from UTHVL collected from January, 2014 to July, 2015 was used in this study. These samples were drawn from North-Western, Western, Copperbelt, Muchinga and Lusaka Provinces.

2.3.1 Sampling Frame

A convenient sample of 93 available archived serum samples from cases suspected of having YF-like illnesses stored in the Virology Laboratory was used in this study. These samples were collected for YF case-based surveillance in Zambia from January, 2014 to July, 2015.

2.3.2 Inclusion Criteria

Archived serum samples from cases suspected of having YF-like illnesses from both children and adults regardless of sex were included. Yellow fever-like illnesses was defined as "any case presenting with acute onset of fever, with jaundice appearing within 2 weeks of onset." Only samples that initially tested malaria negative at health facility by thick blood smear or RDT were enrolled in the study.

2.3.3 Exclusion Criteria

Serum specimens from cases with fever of known bacterial or parasitic origin and present with jaundice were excluded.

2.4 Determination of Serological Epidemiology of YF

2.4.1 Specimen Storage and Processing

Serum samples were prepared from acute-phase specimens and stored on -40 ^{o}C at UTHVL before they were evaluated for the flaviviruses and hepatitis viruses by RT-PCR and nPCR assays.

2.4.2 Enzyme Linked Immunosorbent Assay of YF Specific IgM Antibodies

The IgM-Capture ELISA was performed as previously described (Duermeyer *et al*, 1979). Briefly, 96-well round bottom polyvinyl chloride microplates were coated with 75µl diluted goat anti-human IgM antibody (1:500) for the assay and the plates were incubated overnight at 4^{o}C and plates were washed four times with Phosphate buffered saline/Tween. Two hundred microlitres of 10% horse serum in PBS/Tween/Milk (Sigma Aldrich, Missouri,

USA) were loaded in the plates to block the anti-human IgM antibody and incubated for 1 hr at 37ºC and plates were four times washed with PBS/Tween. Ten microlitres of patient samples, positive and negative control sera (Institut Pasteur, Dakar, Senegal) diluted in PBS/Tween/Milk (1:100dilutions) were loaded in the plates and incubated for 1 hr at 37ºC. After another four times washing with PBS/Tween, 75 µl YF antigen and negative control antigen were then dispensed to appropriate wells. These samples were tested in triplicate against YF virus. Seventy five microliters of horseradish peroxidase anti-Flavivirus monoclonal antibody was dispensed to each well and incubated at 1 hr for 37ºC. The plates underwent a new cycle of five times washing with PBS/Tween. One hundred microlitres of ABS (2, 2'-azino-di [3 ethyl benziazoline-6-sulphonate]) substrate (Sigma Aldrich, Missouri, USA) was added to each well and the plates incubated for 30 min at room temperature in the dark, the reaction was stopped by adding 1% sodium dodecyl sulphate (Sigma Aldrich, Missouri, USA) to the mixture. The optical density (OD) was measured at 405nm with 620nm reference in an automated ELISA reader (Thermo Scientific, USA). An adjusted OD for each sample was determined by subtracting the average antigen OD from the average positive antigen OD. The cut-off of each assay was the mean OD plus 3 SDs rounded up to nearest tenth. This OD was typically 0.2. A sample was considered positive if the OD was greater than or equal to this cut off.

Reference: Institute Pasteur de Dakar, Senegal (WHO-AFRO Regional Reference Laboratory)

2.4.2 Detection of DF specific IgM antibodies using chromatographic immunoassay

The test device was labeled with specimen' identification number. The capillary tube was filled with serum not to exceed the specimen line. Holding the capillary tube vertically, 5µl of serum was dispensed into the centre of the sample well (S well) making sure that there was no air bubbles. Immediately, 3 drops (about 90-120µl) of sample diluent was dispensed into

the buffer well with the bottle positioned vertically. The results was read at 20 minutes. To ensure assay validity, a procedural control was incorporated in the device and labelled "Control". If the control bar did not turn red by assay completion, the test was invalid and the sample was repeated. If red bar appeared in both control window (labelled "Control) and the patient window (labelled "Patient") of the strip, the patient sample was interpreted as positive. If one bar appeared in the Control window of the test strip and no red bar in the patient window, the patient sample was interpreted as negative.

Reference: (CTK BIOTCH, San Diego, CA, USA)

2.4.3 Determination of Molecular Epidemiology and genotypes of Flaviviruses

2.4.3.1 RNA Preparation

Viral RNA was prepared from 140 µl of the serum using the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five hundred and sixty µl of prepared Buffer AVL containing carrier RNA was pipetted in each 1.5 ml microcentrifuge tube. After this pipetting, 140 µl of serum was added to the Buffer AVL-carrier RNA in each microcentrifuge. These were mixed by pulse vortexing for 15 seconds and incubated at room temperature (15-25^{o}C) for 10 min. The tubes were briefly centrifuged to remove drops from the inside of the lids. After the centrifugation, 560 µl of ethanol (96-100%) was added to the samples, and mixed by pulse vortexing for 15 seconds. The tubes were again briefly centrifuged to remove drops from inside the lids. Six hundred and thirty microlitres of the solution from the previous step was carefully applied to each QIAamp Mini column (in a 2 ml collection tube) without wetting the rims. The caps were closed and centrifuged at 6000 x *g* for 1 min. The QIAamp Mini columns were placed into the clean 2 ml collection tubes and the tubes containing the filtrate were discarded. The QIAamp Mini columns were carefully opened and the previous step was repeated. The QIAamp Mini columns were carefully

opened and 500 µl of Buffer AW1was added to each tube. After the addition of the buffer, the caps were closed and centrifuged at 6000 x *g* in the clean 2 ml collection tubes and the tubes containing the filtrate were discarded. The QIAamp Mini columns were carefully opened and 500 µl of Buffer AW2 was added each tube. The caps were closed and centrifuged at full speed (20,000 x *g*) for 3 minutes. To eliminate any chance of possible Buffer AW2 carryover, the QIAamp Mini columns were placed in the new 2 ml collection tubes and the old collection tubes with the filtrate were discarded. After centrifugation at full speed for 1 minute, the QIAamp Mini columns were placed in the clean 1.5 ml microcentrifuge tubes and the old collection tube containing the filtrate were discarded. The QIAamp Mini columns were carefully opened and 60 µl of Buffer AVE equilibrated to room temperature was added to each tube. The caps were closed and incubated at room temperature for 1 minute. After incubation, the tubes were centrifuged at 6000 x *g* for 1 min. Finally, RNAs were eluted in 60 µl volumes and stored at -80^{o}C until use.

Reference: QIAamp Protocol

2.4.3.2 Molecular Determination of YF virus using One-step RT-PCR

RT-PCR assay for YF detection was performed as previously described (Ogawa *et al*, 2011). The reaction mixtures were prepared with QIAGEN one step RT-PCR as follows: 5.6 µl of RNase, 2 µl of 5x buffer, 0.4µl of dNP, 0.6 µl of each 10 µM primer (YFV-29: AATCGAGTTGCTAGGCAATAAACAC; YFV141RTCCCTGAGCTTTACGACCAG), 0.4 µl of the template and 0.4 µl of Polymerase added in the final volume of 10 µl (Qiagen, Hilden, Germany). Samples underwent an initial cycle of 50^{o}C for 30 min and 95^{o}C for 15 min, followed by further 40 cycles at 94^{o}C for 30 s (denaturation), 57^{o}C for 30 s (annealing), 72^{o}C for 30 s (extension). Final elongation step was carried out at 72^{o}C for 10 min. The reactions were cooled down to 4^{o}C. The reactions were carried out using a Veriti 96 well

Thermal Cycler (Applied Biosystem, USA). Each assay contained a negative control which consisted of distilled water. A 5 µl of each sample was electrophoresed on 1.5% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were photographed on agarose gel using a BioDoc.It^{TM} Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.3 Molecular Determination of DF viruses using RT-PCR

RT-PCR assay for DF detection was performed as described previously (Ogawa *et al*, 2011). Briefly, each initial reaction mixture contained 2.5 µl of sample RNA and 1 µl of 10 µm of each D1: (TCAATATGCTGAAACGCGCGAGAAACCG) and D2 TTGCACCAACAGTCAATGTCTTCAGGTTC and was subjected to a temperature of 65^{o}C for 5 min and cooled down to 4^{o}C. Furthermore, 2.5 µl of Nuclease free water, 2 µl of 5 x buffer, 0.5 µl of dNTP, 1 µl of 0.1M DTT, 0.25 µl each of RNase inhibitor and M-MLV RT were added in a 10 µl final reaction volume. The one-step RT-PCR program for reverse transcription was done at 42^{o}C for 1 hour followed by initial inactivation at 98^{o}C for 5 min. The second PCR reaction utilized a 10 µl total volume of 2µl of RT reactive product of the previous mixture, 5.75µl of DW, 1 µl of 10 x buffer, 0.2 µl of each 10 µM D1: (TCAATATGCTGAAACGCGCGAGAAACCG) and D2: (TTGCACCAACAGTCAATGTCTTCAGGTTC), 0.8 µl of dNTP and 0.05 µl of Ex Taq HS in mixture reaction tubes. The samples were amplified as follows: incubation at 94^{o}C for 2 min followed directly by 35 cycles of amplification consisting of 94^{o}C for 30s, 55^{o}C for 1min and 72^{o}C for 2min, ending with a final extension of 72^{o}C for 7 min. The reactions were cooled down to 4^{o}C. The amplification step was performed in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA) and distilled water was used as negative control. Five microlitres of each PCR product was analyzed by electrophoresis in 1.5% agarose gel

containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were visualized on agarose gel in a BioDoc.It™ Imaging System (Upland, CA, USA).

2.4.3.3.1 Molecular Typing of DF viruses using RT-PCR

The first PCR DNA product diluted x 100 with the buffer was added to the reaction tube that included 1 µl of x 10 buffer, 0.2 µl of each 10 µM D1 and Typing primer were also added. D1 was a combination with TS1: (TCAATATGCTGAAACGCGCGAGAAACCG + CGTCTCAGTGATCCGGGGG), D1 was again in combination with TS2 (TCAATATGCTGAAACGCGCGAGAAACCG + CGCCACAAGGGCCATGAACAG, D1 was again in combination with TS3 (TCAATATGCTGAAACGCGCGAGAAACCG + TAACATCATCATGAGACAGAGC) and D1 was again combined with TS4 (TCAATATGCTGAAACGCGCGAGAAACCG + CTCTGTTGTCTTAAACAAGAGA). 0.8 µl of dNTP and 0.05 µl of Ex Taq HS were also added to the reaction tube. The thermal cycling conditions used were as follows: incubation at 94°C for 2 min, followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 2 min. A final extension step was conducted at 72°C for 7 min and the reactions were cooled down to 4°C. The amplification was allowed to proceed in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA) and every assay was accompanied by negative control which consisted of distilled water. After completion of the amplification, 5 µl portion of each reaction mixture was electrophoresed through 4% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were photographed on agarose gel using a BioDoc.It™ Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.4 Molecular Determination of pan-Flaviviruses using one-step RT-PCR

Briefly, the protocol consisted of one-step RT-PCR with the following: 5 µl of RNase, 2 µl of 5 x buffer, 0.4 µl of dNTP, 0.6 µl of 10 µM forward primers Flavi all S (PanFV: TACAACATgATggggAARAgAgARAA) and DEN4 F (PanFV: TACAACATgATgggRAAACgTgAGAA), 0.6 µl of reverse primer of 10 µM Flavi AS 2 (PanFV: gTgTCCCAgCCNgCKgTgTCATCWgC), 0.4 µl of each template and DNA polymerase were used in the final volume of 10 µl according to the manufacturer's instruction (Qiagen, Hilden, Germany). The mixture was incubated at 50^{o}C for 30 min and initial PCR inactivation at 95^{o}C for 15 min. This was later subjected to 45 cycles of denaturation at 94^{o}C for 30 s, annealing at 51^{o}C for 30 s, extension at 72^{o}C for 30 s and final extension of 72^{o}C for 7 min. The reactions were cooled down to 4^{o}C. The amplification step was allowed to proceed in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA). Distilled water was used as negative control in every run. After completion of the amplification, 5 µl of reaction products were identified by their molecular weights analyzed by electrophoresis through 1.5% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were visualized on agarose gel in a BioDoc.It^{TM} Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.5 Molecular Determination of WNV using one-step RT-PCR

The RT-PCR reaction mixture was carried out in a 10 µl reaction mixture containing 5 µl of RNase free water, 2 µl of 5 x buffer, 0.4 µl of dNTP, 0.6 µl of 10 µM of each forward primer Fla-U5004: ggAACDTCMggHTCNCCHAT; Fla-5457: gTgAARTgDgCYTCRTCCAT, 0.4 µl of each temperate and DNA Polymerase (Qiagen, Hilden, Germany). The RT-PCR was performed under the following thermal cycling parameters: incubation at 50^{o}C for 30 min and

initial PCR activation at 95ºC for 15 min followed by 45 cycles of denaturation at 94ºC for 1 min, annealing at 53ºC for 1 min, extension at 72ºC for 1 min with a final extension at 72ºC for 10 min and cooled down to 4ºC. The reactions were carried out in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA). Each amplification incuded a negative control which consisted of distilled water. The PCR products in the volumes of 5 µl were analyzed by electrophoresis through 1.5% agarose gel stained with 0.5 µg/L of ethidium bromide in TBE buffer gel. Following the electrophoresis, the amplified DNA fragments were visualized on agarose gel in a BioDoc.It™ Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.6 Molecular Determination of Hepatitis A virus using one-step RT-PCR

The One step RT-PCR was used with a 10 µl reaction mixture including 5µl of RNase, 2µl of 5 x buffer, 0.4 µl of dNTP, 0.6 µl of 10 µM each forward primers: +300; GCTGTAGGAGTCTAAATTGGGGAC and 0.6 µl of reverse primer of 10 µM: -516; ACTCAATGCATCCACTGGATGAG, 1 µl DNA polymerase according to the manufacturer's instruction (Qiagen, Hilden, Germany). The thermal profile of this assay included: incubation at 50ºC for 30 min and initial PCR inactivation at 95ºC for 15 min followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 30 s, extension at 72ºC for 30 s. The RT-PCR cycle ended with a final extension of 72ºC for 10 min followed by 4ºC hold. This assay was allowed to proceed in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA) and distilled water was ran as negative control. A 5 µl of each sample was analyzed by electrophoresis through 1.5% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were observed on agarose gel under ultravoilet light in a BioDoc.It™ Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.7 Molecular Determination of Hepatitis B virus using one-step RT-PCR

The one step RT-PCR (Qiagen, Hilden, Germany) was used with a final volume of 10 µl reaction mixture including 1 µl of sample DNA, 1 µl of 10 x buffer, 0.8 µl of dNTP, 0.2 µl of 10 µM each forward primer: TSB1; TCACCATATTCTTGGGAACA and 0.2 µl of reverse primer of 10 µM:TSB2; TTCCTGAACTGGAGCCACCA, 0.05 µl Ex taq HS and 6.75 µl Distilled water were added. The reaction was accomplished in one-step RT-PCR with the following optimized thermal programme: 94^{o}C for 3 min and followed by 40 cycles of denaturation at 94^{o}C for 45 s, annealing at 53^{o}C for 1 min, extension at 72^{o}C for 90 s and final extension of 72^{o}C for 10 min. The reactions were cooled down to 4^{o}C and the thermal cycling was allowed to proceed in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA) and distilled water was used as negative control. After completion of the amplification, 5µl of each reaction mixture was resolved on 1.2% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were visualized on agarose gel by BioDoc.It^{TM} Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.8 Molecular Determination of Hepatitis C virus using Nested RT-PCR

The reaction mixture contained 5 µl of RNase, 2 µl of 5 x buffer, 0.4 µl of dNTP, 0.6 µl of 10 µM forward primer: #32S; CTGTGAGGAACTACTGTCTT and 0.6 µl of reverse primer of 10 µM: #36AS; AACACTACTCGGCTAGCAG, 1 µl temperate and 0.4 µl DNA polymerase to complete a 10 µl volume according to the manufacturer's instruction (Qiagen, Hilden, Germany). The following thermal profile was used for reverse transcription at 50^{o}C for 30

min and initial PCR inactivation at 95ºC for 15 min followed by 40 cycles of denaturation at 94ºC for 30 s, annealing at 58ºC for 30 s, extension at 72ºC for 30 s and final extension of 72ºC for 10 min. The reactions were cooled down to 4ºC. The Nested PCR (nPCR) reactions were carried out at total volume of 10 µl containing 2 µl of first PCR product, 1 µl of 10 x buffer, 0.8 µl of dNTP, 0.2 µl of 10 µM each forward primers:
#33S; TTCACGCAGAAAGCGTCTAG and 0.2 µl of reverse primer of 10 µM: # 48 AS; GTTGATCCAAGAAAGGACCC, 0.05 µl ExTaq HS and 5.75 µl Distilled water. The reaction mixture was then incubated at 94ºC for 1 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s. The final extension was conducted at 72ºC for 5 min followed by a 4ºC hold. The reaction was carried out in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA) and distilled water was used as negative control. Five microlitres of each reaction product was loaded on 1.5% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer and separated by electrophoresis. The amplified DNA fragments were photographed on agarose gel using a BioDoc.It™ Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.9 Molecular Determination of Hepatitis E virus using Nested RT-PCR

The reaction was performed in final volume of 10 µl containing 5 µl of RNase, 2 µl of 5 x buffer, 0.4 µl of dNTP, 0.6 µl of 10 µM forward primer: HEV-F1;TAYCGHAAYCAAGGHTGGCG and 0.6 µl of reverse primer of 10 µM: HEV-R2; TGYTGGTTRTCRTARTCCTG 1 µl temperate and 0.4 µl DNA polymerase according to the manufacturer's instruction (Qiagen, Hilden, Germany). The cycling conditions were in consistent with those described previously: incubation at 50ºC for 30 min and initial PCR inactivation at 95ºC for 15 min followed by 40 cycles of denaturation at 94ºC for 30 s,

annealing at 55ºC for 30 s, extension at 72ºC for 30 s and final extension of 72ºC for 5 min. The reactions were cooled down to 4ºC. The nPCR reaction mixture was carried out in final volume of 10 µl and contained 2 µl of first PCR product, 1 µl of 10x buffer, 0.8 µl of dNTP, 0.2 µl of 10 µM each forward primer: HEV-F2; GGBGTBGCNGAGGAGGAGGC and 0.2 µl of reverse primer of 10 µM: HEV-R1; CGACGAAATYAATTCTGTCG, 0.05 µl ExTaq HS and 5.75 µl Distilled water. The amplification step was carried out in a Veriti 96 well Thermal Cycler (Applied Biosystem, USA) using the following procedure: incubation at 94ºC for 1 min followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s and extension at 72ºC for 30 s. The RT-PCR cycle ended with a final extension of 72ºC for 5 min followed by a 4ºC hold. Distilled water was used as negative control. Upon completion of the amplification, 5µl of the nested PCR mixture was subjected to electrophoresis through 1.5% agarose gel containing 0.5 µg/L of ethidium bromide in TBE buffer. The amplified DNA fragments were observed on agarose gel under UV light using a BioDoc.It™ Imaging System (Upland, CA, USA).

Reference: Manual of National Institute of Infectious Diseases in Japan

2.4.3.10 DNA Sequencing

The Forward and reverse linear amplification was performed in 10 µl final volume including 0.75 µl of the purified PCR product, 0.5 µl BigDye, 1.75 µl of 5 x Buffer, 0.5 µl of 3.2 µM primer and 6.5 µl of template. The profile of sequencing profile consisted of 25 cycles of initial denaturation at 96ºC for 1 s followed by denaturation at 96ºC for 10 s, annealing at 50ºC for 50 s and elongation at 60ºC for 4 min. The reaction was cooled down to 4ºC. The DNA product was purified using the ethanol precipitation method. Briefly, the entire extension products was carried out in 28 µl of freshly prepared precipitation solution (2 µl of 125mM EDTA and 2 µl of NaAcetate), 90 µl of 100% Ethanol, incubated at room

temperature for 10 min and then centrifuged at 15 000 rpm for 20 min. After carefully removing the supernatant, 200 µl of 70% of Ethanol was added and centrifuged at 13 000 x *g* for 5 min at 4 ^{o}C. The supernatant was aspirated and discarded. Another 200 µl of 70% of Ethanol was added and centrifuged at 15 000 rpm for 5 min at 4^{o}C. The samples was then covered with aluminum foil and dried up in a vacuum machine for 5 min. After additional of 20µl of Formaldehyde, the samples underwent denaturing programme in AB Thermo cycler and analyzed in 3130 Genetic Analyzer (AB Bio Applied System, HITACHI, Japan) for 10 hours. The DNA sequence reads were edited using Ridom TraceEdit Software (Ridom Bioinformatics GmbH, Wurzburg, Germany) and use to search the National Center for Biotechnology Information (NCBI) RefSeq database using BLASTN Software htt:// www,ncbi,nlm.nih.gov/BLAST.

2.5 Statistical Analysis

Data were entered into Excel spread sheet and analyzed by using STATA Version 12 (StataCorp, College Station, TX, USA). Relevant clinical and demographic data were retrieved from the laboratory registers and request forms that accompanied the samples. Associations between the outcome variable (yes or no for presence of Flaviviruses) and categorical variables; sex, age, vaccination status, travel history, sample collection sample time from onset of symptoms and provinces were assessed using the Chi- square test. Multivariate logistic regressions, two-sample test of proportion statistical methods were used for data analysis. Two-simple test of proportion was used to ascertain the statistical significance of two proportions. Univariate and multivariate analysis was used to assess the factors associated with the outcome. Confidence interval (CI) of 95% and 5% level of significance was set to assess statistical significance.

2.6 Ethics Consideration

Ethics approval was reviewed and approved by the Institutional Review Board of Excellence in Research Ethnics and Science Converge (ERES Converge IRB) Ref. No. 2015-Apr-003 and National Health Research Authority Ref MH/101/23/10/1. Permission to conduct research and use of the archived samples was obtained from management of University Teaching Hospital and Ministry of Health. Study numbers were used to identify the archived samples to maintain confidentiality and the data base was restricted to other staff apart from the investigators. Results will be shared with the relevant health authorities.

Chapter 3

Results

3.1 Demographic characteristics of YF suspects

From January 2014, through July 2015, a total of 93 patients' samples that met the case definition of YF suspect were collected from health facilities in 5 provinces of Zambia and shipped to UTHVL. Serological tests and RT-PCR assays were tested on these serum samples which were collected from YF suspects in the following provinces; Western 44/93 (47.3%), North Western 29/93 (31.2%), Muchinga 11/93 (11.8%), Lusaka 4/93 (4.3%) and Copperbelt 5/93 (5.4%) as shown in figure 3.1. The majority 73/93 (78.5%) of the YF cases were reported from Western and North Western provinces of Zambia where the YF risk assessment was initially instituted by Ministry of Health in collaboration with World Health Organization.

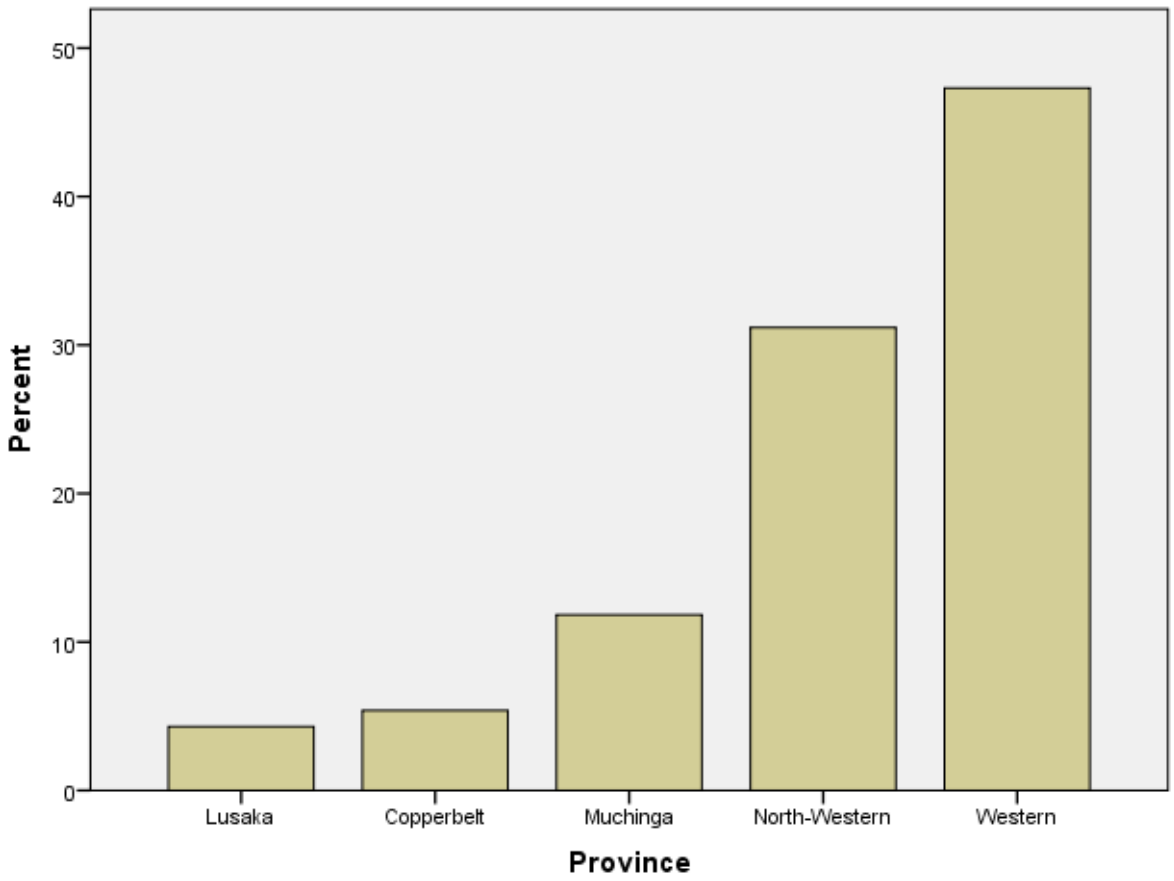

Figure 3.1: Frequency (%) of serum samples by Province

The study sample consisted of 48/93 (51.6%) male and 45/93 (48.5%) female. The median age for YF suspect was 19 years and the range was 1 to 83years. The ages were further classified into six age groups 0-5 years 19 (20.4%), 6-15 years 22 (23.7%), 16-25years 19 (20.4%), 26-35years 11 (11.8%), 36-45 years 12 (12.9%), >45 years 10 (10.8 %). None of the patients reported having been vaccinated against YF vaccine and 1.1% (1/93) of the total patients had history of travel to other country in the proceeding 14 days. (Tables 3.1)

Table 3.1: Demographic characteristic of YF suspects

Characteristic	no. of Patients	Percentage (%)
Total Tests	93	100
Sex		
Male	48	51.6
Female	45	48.4
Travel History		
Yes	1	1.1
No	92	98.9
Vaccination Status		
Yes	0	0
No	93	100
Sample collection time		
≤ 5 days	38	40.9
> 5 days	55	59.1
Age		
0-5yrs	19	20.4
6-15yrs	22	23.7
16-25yrs	19	20.4
26-35yrs	11	11.8
36-45yrs	12	12.9
>45yrs	10	10.8
Residence (provinces)		
Copperbelt	5	5.4
Lusaka	4	4.3
Muchinga	11	11.8
North-Western	29	31.2
Western	44	47.3

no: number

3.2 Clinical Characteristics of Yellow fever suspects

As shown in table 3.2 below, 49/93 (52.7%) had a body temperature of ≥38^{o}C, the rest were the unknown, not done and missing data. However, the most common reported symptoms at presentation were jaundice 52/93 (55.9%), reduced urine output 23/93 (24.7%) and

proteinuria 15/93 (16.1%). A total percentage of 3/93 (3.2%) patients had unexplained bleeding manifestations.

Table 3.2: Clinical manifestations of YF suspects

Clinical manifestations	**Number (%)***
Fever	49 (52.7)
Jaundice	52 (55.9)
Slow pulse	11 (11.8)
Bleeding	3 (3.2)
Reduced urine	23 (24.7)
Proteinuria	15 (16.1)

*__%__: Percentage

3.3 Serological Prevalence of YF Immunoglobulin M cases

The YF IgM was detected in 13/93 (14%) of the YF suspects. The median age of patients with positive presumptive results was 14 years old (range: 3-53 years) and those without evidence of HAV infection was 19 years old (range: 1-83). The medians were statistically significant ($p < 0.001$). Among all acute febrile patients, 5/93 (5.4%) male and 8/93 (8.6%) female were YF IgM presumptive positive respectively. The YF suspects with presumptive positive results against YF IgM antibodies were reported from Copperbelt 3/13 (23.1%), Muchinga 1/13 (7.7%), North-Western 3/13 (23.1%) and Western Provinces 6/13 (46.2%). None of the serum samples tested positive in Lusaka province as shown in Figure: 3.3.1. The age group distribution were shown as follows; 0-5 years 3 (23.1%), 6-14 years 4 (40.6%), 16-25 years 2 (15.4%), 26-35 years 2 (15.2%), 36-45 years 1 (7.7%), >45 years 1 (7.7%).

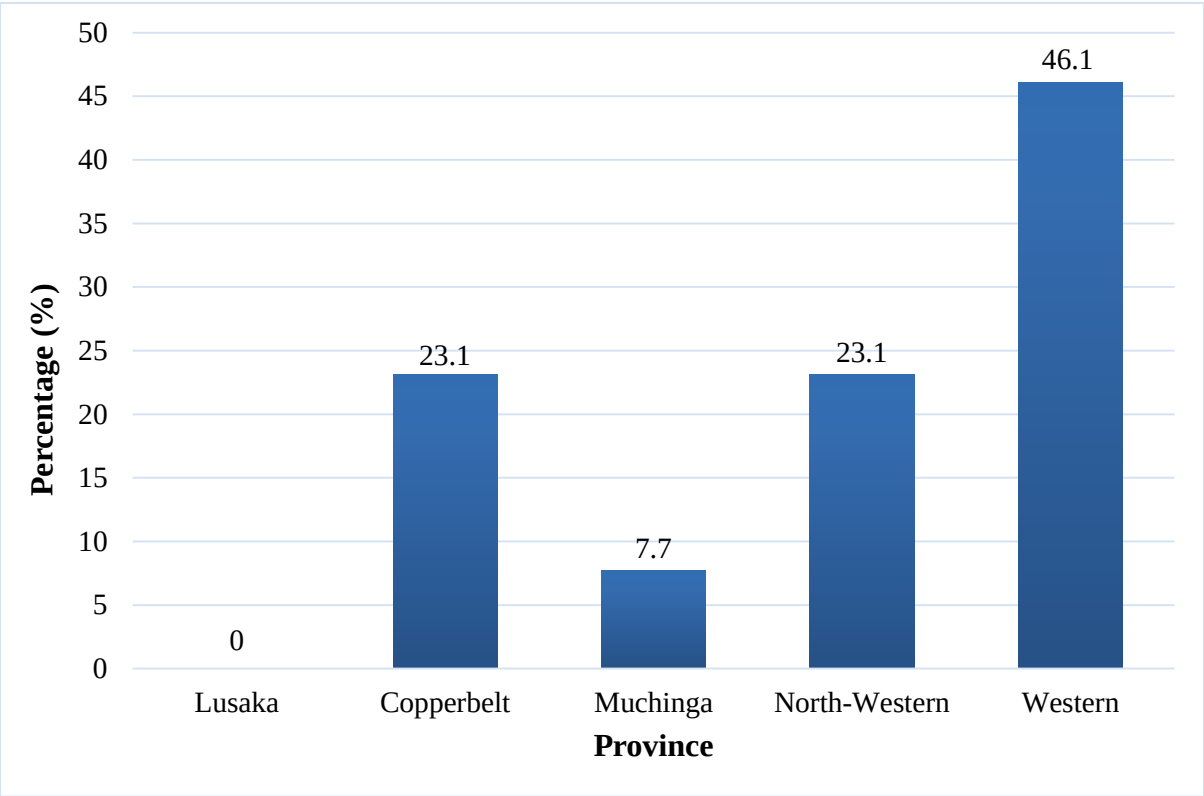

Figure 3.3.1: YF IgM presumptive positivity rate by Province

Table 3.3.1: Comparison of YF IgM cases with independent variables

	number (%)		
Characteristics	**YF IgM+**	**YF IgM-**	***p*-value[c]**
Number	13 (14)	80 (86)	
Sex			
Male	5 (38.5)	43 (53.8)	0.306
Female	8 (61.5)	37 (46.2)	
Total	13 (100)	80 (100)	
Age category(in years)			
0-5	3 (23.1)	16 (20.0)	0.947
6-15	4 (30.8)	18 (22.5)	
16-25	2 (15.4)	17 (21.2)	
26-35	2 (15.4)	9 (11.2)	
36-45	1 (7.7)	11 (13.8)	
>45	1 (7.7)	9 (11.2)	
Total	13 (100)	80 (100)	
Provinces			
Lusaka	0 (0)	4 (5.0)	0.04
Copperbelt	3 (23.1)	2 (2.5)	
Muchinga	1 (7.7)	10 (12.5)	
North western	3 (23.1)	26 (32.5)	
Western	6 (46.2)	38 (47.5)	
Total	13 (100)	80 (100)	

YF IgM: Yellow fever Immunoglobulin M, +: positive, -: Negative, [c:] chi-square

As shown in table above, sex and age category did not show an association except the provinces (p= 0.04).

Table 3:3.2: Predictors of YF IgM cases in the study population using univariate and multivariate logistic regression

Variable	Odds Ratio[u] (95% CI)	*p*-value	adjusted Odds[m] Ratio OR(95%CI)	p- value
Sex				
Male	1.00	0.344	1.00	0.177
Female	1.67 (0.58, 4.86)		0.44 (0.13, 1.45)	
Age category (in years)				
0-5	1.00		1.00	
6-15	1.75 (0.42, 7.25)	0.440	1.63 (0.36, 7.39)	0.523
16-25	0.20 (0.20, 2.06)	0.181	0.12 (0.01, 1.50)	0.100
26-35	0.83 (0.12, 5.50)	0.850	0.76 (0.16, 5.64)	0.784
36-45	0.34 (0.04, 3.49)	0.364	0.38 (0.04, 4.00)	0.424
>45	0.94 (0.14, 6.28)	0.947	1.07 (0.15, 7.91)	0.944
Provinces				
Copper belt	1.00		1.00	
Muchinga	0.20 (0.02, 1.81)	0.153	0.16 (0.01, 1.85)	0.141
North western	0.25 (0.04, 1.56)	0.138	0.21 (0.02, 1.88)	0.163
Western	0.15 (1.03, 0.94)	0.043	1.11 (0.14, 0.94)	0.043

CI: Confidence Interval, **OR**: Odds Ratio, **[u]**: Univariate, **[m]**: Multivariate

As shown in the table above, patients from the Western province were less likely to have presumptive YF IgM case than those from Copperbelt province. This association was statistically significant (p=0.04). Lusaka province had no YF IgM case; therefore it was not included in the univariate and multivariate logistic regression.

All of the 93 (100%) serum samples tested negative for YFV, DENV, WNV and pan-Flaviviruses by PCR assays. We also confirmed HBV DNA in 2/20 pooled sera of YF suspects' samples whilst 5/20 were suspected for hepatitis B. In addition, none of the YF

suspects tested positive for HCV RNA and HEV RNA by PCR assays. Among the 90 samples (100%) tested, none of the patients tested positive for DENV IgM antibodies. The aetiologies of 85/93 (91.4%) of cases presenting with acute febrile jaundice remained unknown.

Table 3.3.3: Serological and Molecular Prevalence of Flaviviruses

Assays	**Positivity (%)**
Serological Assays	
YF IgM ELISA	13 (14)
DF IgM ELISA	0 (0)
Molecular Assays	
YFV RT-PCR	0 (0)
DFV RT-PCR	0 (0)
WNV RT-PCR	0 (0)
Pan -Flavi RT-PCR	0 (0)

YFV: Yellow Fever Virus, **DFV:** Dengue fever virus, **WNV**: West Nile Virus, **Pan-Flavi**: Pan-Flaviviruses, **RT-PCR**: Reverse transcriptase, **IgM**: Immunoglobulin M, **ELISA**: Enzyme-linked immunosorbent assay.

3.4 Prevalence of Hepatitis A

The design was adjusted to incorporate a wider range of tests for viral hepatitis. However, 8/93 (8.6 %) of the patients had evidence of acute hepatitis A, consisting of 3 (37.5%) males and 5 (62.5%) females. The median age of patients with Hepatitis A was 9.5 years old (range: 1-33 years) and those without it was 19 years old (range: 1-83). The medians were not statistically different ($p > 0.05$). These cases were detected from the following provinces; Lusaka 1/8 (12.5%), Copperbelt 2/8 (25%), Muchinga 1/8 (12.5%), North-Western 2/8 (25%) and Western 2/8 (25%) as shown in figure 3.4.1.

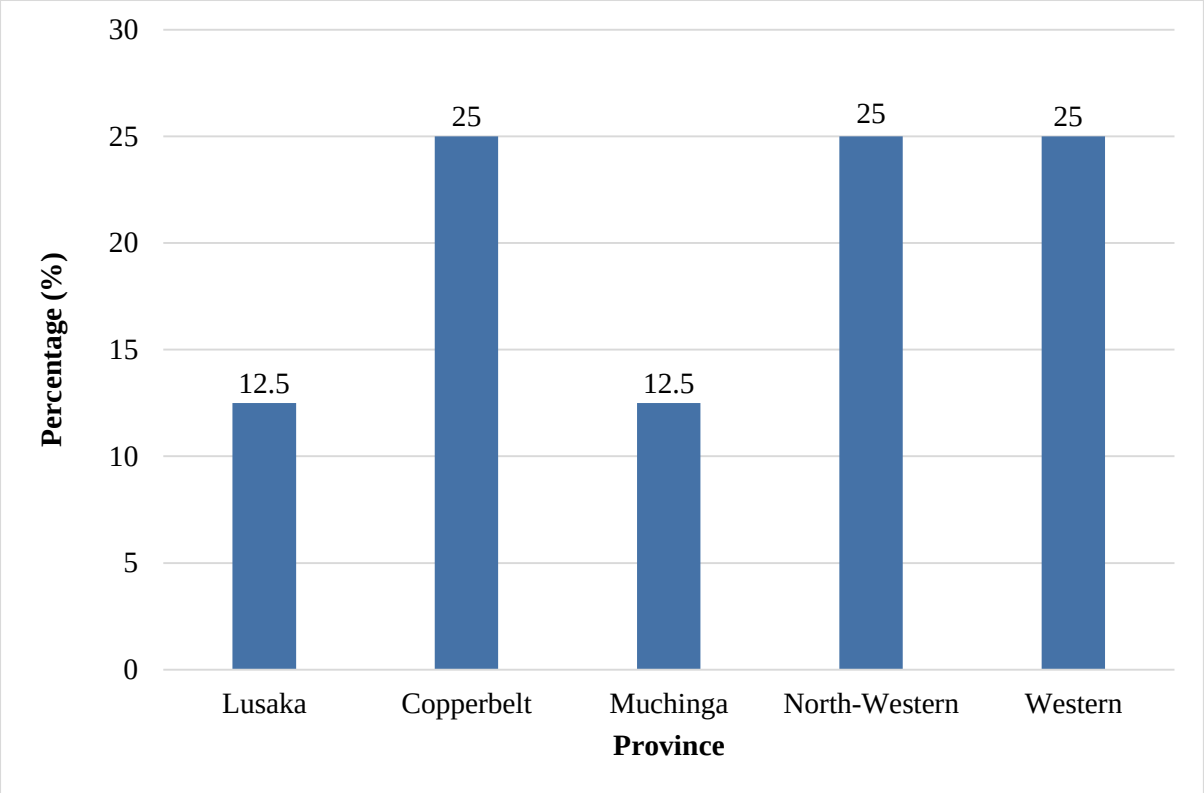

Figure 3.4.1: Hepatitis A positivity rate by Province

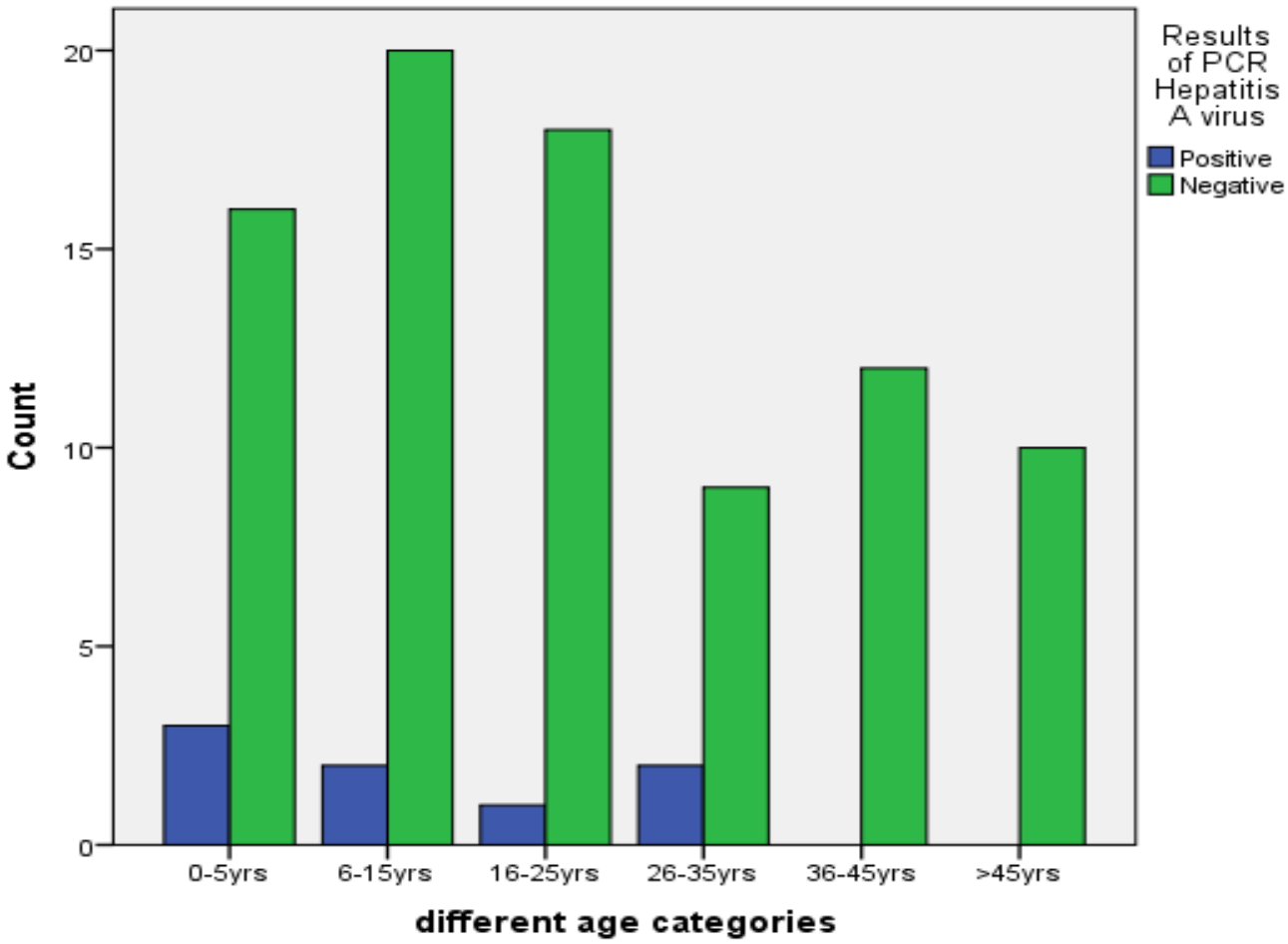

Figure 3.4.2: Results of PCR Hepatitis A virus

The age group distribution of hepatitis A patients in this study were as follows; 0-5 years 3/8 (37.5%), 6-15 years 2/8 (25%), 16-25 years 1/8 (12.5%), 26-35 years 2/8 (25%), 36-45 years 0/8 (0%), >45 years 0/8 (0 %) as shown in figure 3.4.2. The result showed that most of the hepatitis A patients were in the age group between (0-5years), (6-15 years) and (26-35).

Table 3.4.1: Comparison of Hepatitis A with independent variables

Characteristics	HAV DNA+ (%)	HAV DNA- (%)	p-value[c]
N	8 (8.6)	85 (91.4)	
Sex			
Male	3 (37.5)	45 (52.9)	0.403
Female	5 (62.5)	40 (47.1)	
Total	8 (100)	85 (100)	
Age category (in years)			
0-5	3 (37.5)	16 (18.8)	0.431
6-15	2 (25)	20 (23.5)	
16-25	1 (12.5)	18 (21.2)	
26-35	2 (25)	9 (10.9)	
36-45	0 (0)	12 (14.1)	
>45	0 (0)	10 (11.8)	
Total	8 (100)	85 (100)	
Provinces			
Lusaka	1 (12.5)	3 (3.5)	0.070
Copperbelt	2 (25.5)	3 (3.5)	
Muchinga	1 (12.5)	10 (11.8)	
North western	2 (25)	27 (31.8)	
Western	2 (25)	42 (49.4)	
Total	8 (100)	85 (100)	

HAV DNA: Polymerase chain reaction hepatitis A virus, **+**: positive, **-**: negative, **n**: number of tests

As shown in the table 3.4.1, the total percentage of hepatitis A cases was 8/93 (8.6%) consisting of 3 males and 5 females. However, sex, age category and provinces were not statistically associated with hepatitis A infection.

Chapter 4

Discussion

4.0 Discussion

Although the current evidence from other sub-Saharan countries close to Zambia strongly suggests that flaviviruses would be among the main causes of acute febrile jaundice (Dash *et al*, 2012; Meltzer *et al*, 2014; WHO, 2014). These viruses have not been thoroughly investigated in Zambia. Therefore, this study was aimed to determine the serological and molecular epidemiology of flaviviruses in selected provinces of Zambia.

The main finding from our data is that the flaviviruses were not contributing to hospital morbidity and mortality in the study areas. However, this does not prove that flaviviruses are absent due to the limited sample size. Moreover, the previous studies suggest inconclusive low circulation of flaviviruses in North-Western and Western provinces of Zambia (Babaniyi *et al*, 2015; Liwewe *et al*, 2015). However, the existing evidence of their circulation in Zambia has been based on serology which has its own limitations due to flavivirus cross reactivity (Crill and Chang, 2004; Mansfield *et al*, 2011). Instead of the flaviviruses, a high incidence of viral hepatitis was found.

The case definition of YF suspect used to screen for potential flaviviruses was intentionally broad to promote a "high clinical index of suspicion" which allows for urgent referral to diagnostic facilities for case confirmation. Subsequently, this will trigger timely and effective control measures (WHO, 2010). The clinical complications of flaviviruses are frequently observed in other febrile illnesses in Africa such as bacterial and viral diseases. For example,

fever with jaundice is a frequent complication of viral hepatitis. In the process, the broad case definition facilitated the sampling of patients with other acute febrile undifferentiated illnesses. The WHO clinical case definition of YF suspect seems to be more sensitive but not sufficiently specific since symptoms of flaviviruses can mimic other febrile illnesses (WHO, 2010; Baba *et al*, 2013; Monath and Vasconeles, 2015).

Because the samples of YF suspects shipped to UTHVL were collected from patients with an acute febrile illness, YF IgM-capture ELISA and RT-PCR assays were used to screen and confirm the flaviviral infections and for which malaria was initially excluded at the site of sample collection. There is however, no single test that can guarantee reliable results for different febrile illnesses, hence the need to perform serological and molecular methods simultaneously to encourage proper diagnosis and treatment (Tadesse and Tadesse, 2013). Nevertheless, serological tests are broadly used and do have value in the diagnosis of flaviviruses and their negative predictive value gives the high confidence that negative results correctly identifies patients without disease (Dayan *et al*, 2015). In this study, the absence of IgM antibodies against YF virus was found to range from 86% to 100%.

The PCR methodologies used in this study would be more sensitive for samples collected during the first days of illness while serological testing would be more sensitive for samples taken after the onset of fever (Khongwichit *et al*, 2015). However, PCR may show false negativity as the viraemia in YF and WNF is usually of short duration (Thomas *et al*, 1995; WHO, 2004; Gray and Webb, 2014). In this study, 40.9% (38/93) of the serum samples were collected within 5 days after onset of symptoms but none of the samples tested positive. This is when the PCR is considered more sensitive than serology because the flaviviruses are often detected in the acute phase serum samples whilst antibodies are undetectable prior to 5 days

after the onset of infection (WHO, 2010; Patel *et al*, 2013; Susilawati and McBride, 2014; Dayan *et al*, 2015).

Although, 14% (13/93) of the YF suspects tested had results suggesting a possibility of recent infection, the PCR testing failed to identify flaviviruses in all samples (100%) confirming the absence of flaviviruses among the febrile jaundice cases because by the time overt symptoms are recognized the viral RNA is usually undetectable (WHO, 2010). However, because of the decline in the level of viraemia overtime and possible inaccuracy in reporting of dates of illness onset, a negative RT-PCR result does not exclude flaviviral infections (Rabe *et al*, 2016). Since we had only acute-phase samples, therefore our serological results can only be considered presumptive. The possibility of cross-reaction occurring as a result of recent exposure to other non flaviviruses should be ruled out by an additional test. Therefore, we also used the pan-Flavi PCR assay to absolutely confirm the absence of flaviviruses and complement species specific PCR flavivirus assays (Patel *et al*, 2013). However, the sensitivity and specificity of the in-house ELISA used in this study was not 100% (Institute Pasteur de Dakar, Senegal). IgM ELISA reported in previous study suggests that few of the cases are misclassified (Vaughn *et al*, 2000). Furthermore other studies have shown that using IgM ELISA alone may give false positive results underestimating the proportion of undiagnosed cases (Susilawati and McBride, 2014). Thus, it is possible that some if not all results were false positive cases. Moreover, no antibodies to DFV another flavivirus were detected in this study. The serology results may probably be due to a cross-reaction from a similar yet previously unidentified non-flaviviruses including *Alphavirus* genus (Gudo *et al*, 2015; Kam *et al*, 2015; Tigoi *et al*, 2015). Therefore, the possibility of cross-reactivity with other pathogens cannot be excluded. Despite this limitations, YF IgM seropositivity was found to be associated with Copperbelt, Muchinga, North-Western and Western provinces

(p= 0.04). Probably, this could have been attributed by the close proximity to Angola and DRC which are high risk YF zones or false positive due to cross reactions with other pathogens including *Alphavius* (Susilwati *et al,* 2014). The patients' samples that came from Western province were less likely have YF presumptive infection than those from Copperbelt province. However, this cross-reactivity was not a limitation in this study since the absence of evaluated flaviviruses was largely confirmed by the molecular methods (Milosevic *et al*, 2014).

The data from our study which were collected over a period of one and half years confirmed the absence of flaviviruses as the cause of acute febrile jaundice in selected provinces of Zambia. This finding is consistent with other published studies in Nigeria and Ghana. In other previous studies, 1-2% of YF suspects had laboratory evidence of infection (WHO, 2012; Monath and Vasconeles, 2015). Similarly in a previous study conducted from 2009 to 2011 on 258 patients with VHF symptoms in Ghana, none of the patients tested positive for VHFs (Bonney *et al*, 2013). Available data strongly suggest that none of the presumptive YF IgM positive cases detected at UTHVL for YF case based surveillance have been confirmed as a case of YF by PNRT at the WHO AFRO Regional Reference Laboratory (Institut Pasteur, Dakar, Senegal) (UTHVL, Unpublished data).

Despite all samples meeting the case definition of yellow fever suspicion, only 55.9% (52/93) cases were documented as having jaundice. However, the diseases may not easily be distinguished from other febrile diseases such as viral hepatitis due to the non-specific clinical manifestations of the VHFs (Ahmed and Ahmed, 2010; Sutherland *et al*, 2011; Domingo *et al*, 2012).

Therefore, the design of the main study was adjusted to include the methodologies of viral hepatitis. The hepatitis A and E viruses are both enterically transmitted and individuals living in conditions with poor sanitation and hygiene are at risk of acquiring the disease. The acute disease in young children is often asymptomatic or mild; adults may occasionally experience increased risk of morbidity and mortality (WHO, 2012; Kanyenda *et al*, 2015). Our findings have shown a moderate proportion of HAV in selected provinces of Zambia. This is lower than the HAV prevalence obtained in Ghana (Bonney *et al*, 2013). However, there were no differences in the proportion of Hepatitis A virus in all the study sites. Although, Lusaka and Copperbelt provinces had the highest proportion. This had a similar findings with a previous study conducted in Zambia and Tunisia where Hepatitis A was more common in children below 15 years (UTHVL, Unpublished data; Letaief *et al*, 2005; Franco *et al*, 2012). However, our study does not agree with findings in the previous study done in Egypt where the mean age of symptomatic hepatitis A has shifted to adult age due to better sanitation (Zakaria *et al*, 2007). The proportion of female febrile jaundiced patients who were positive for HAV (62.5 %) was not similar to that of the male febrile jaundiced patients (37.5%) and the difference was not statistically significant ($p= 0.403$). Additionally, age category and provinces were also not statistically associated with HAV infection in this study ($p > 0.05$).

Though HAV has been implicated as a causative agent of acute febrile jaundice in this study, further investigation is needed to determine the risk factors (social economic indicators) associated with the disease and expand our standing of the epidemiology in Zambia.

Regardless of much publication in Africa about acute HAV infection, no published data are available on the epidemiology of HAV in Zambia. Previous studies conducted at UTH in early 1990s' showed seroprevalence of 97.8 % in primary school children and 4% in patients with liver diseases in Lusaka respectively (UTHVL, unpublished data). In places, where HAV is not documented as a health problem, it may be neglected as a cause of acute hepatitis delaying implementation of preventive measures and leading to increased morbidity and mortality.

We also found a high incidence of Hepatitis B cases although we were unable to determine whether these were acute or chronic infection due to lack of IgM anti-HBc test during our study. Hence, the exact incidence of acute hepatitis B was very difficult to assess. Furthermore, hepatitis B can also persist in a chronic asymptomatic infection; therefore the jaundice observed in our cases may possibly be due to other unknown causes.

However, available evidence strongly suggests that other pathogens not tested in our study but causing acute febrile jaundice may be circulating in our environment. Other aetiologies that may cause jaundice but were not evaluated in this study include Salmonella *typhi*, Salmonella *paratyphi*, Leptospira *interrogans*, Brucella species, Entamoeba *histolytica* and drug toxicity (Isa *et al*, 2014). On the basis of one case (1.0%) with a history of travel to Tanzania (an area with low potential for YF exposure) before an illness we may speculate that most if not all infections were locally acquired. In Africa where there is a risk of flaviviruses, the areas are typically endemic with other pathogens that cause acute undifferentiated febrile illness in patients and their specific aetiologies are often unknown to make accurate diagnosis, effective treatment and targeted public health measures difficult

(Manock *et al*, 2009; WHO, 2010; Kasper *et al*, 2012; Mueller *et al*, 2014; Susilawati and McBride, 2014). Hence, it is not surprisingly to note that large proportion of febrile jaundice cases still remain largely unknown with aetiological agents and need further investigations.

4.1 Limitations of study

This study was conducted at UTHVL on serum samples of YF suspects collected from five provinces of Zambia over one and half years. Therefore, we cannot be confident that these findings can be generalized to the whole country. The small size was another study limitation. We were not able to exclude the possibility of drug toxicity or bacterial infections as cause of acute febrile jaundice. The other limitation was the non-inclusion of the positive controls in the PCR assays due to non-availability of the positive controls. However, virus identification was done using the BLAST and sequences were deposited in GenBank.

4.2 Conclusion

Viral hepatitis was the cause of morbidity and mortality in some of the patients that presented with acute febrile jaundice in selected health facilities of Zambia. The proportions of hepatitis A and hepatitis B were found. Hence, hepatitis viruses need to be screened when flaviviruses are suspected. In addition, a large proportion of acute febrile cases remained undiagnosed with unknown aetiologies.

4.3 Recommendations

1. The availability of rapid serological diagnostic test for Hepatitis A which are quick and require minimal user expertise should be considered.

2. Further research to understand other causes of febrile jaundice in Zambia should be promoted in the context of on-going surveillance.
3. There is need to perform both serological and molecular surveillance systems in patients presenting with YF like illnesses which may provide timely and comprehensive information regarding virus introduction and circulation.

5.0 Reference

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Appendices

Appendix A: Letter from National Health Research Authority

THE NATIONAL HEALTH RESEARCH AUTHORITY
C/O Ministry of Health
Ndeke House
P.O. Box 30205
LUSAKA

MH/101/23/10/1
In reply please quote:

No.....

December 7th, 2015

Boniface Kabungo
University of Zambia
School of Medicine
P. O. Box 50110
Lusaka

Dear Mr. Kabungo,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Serological and Molecular Epidemiology of Flaviviruses in Selected Provinces of Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been approved to carry out the above mentioned exercise on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, and all key respondents.

Yours sincerely,

Dr. P. Chanda-Kapata
For/Director
National Health Research Authority

Appendix B: Ethical approval letter

ERES CONVERGE

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16th November, 2015

Ref. No. 2015-Apr-003

The Principal Investigator
Mr. Boniface Kabungo
The University of Zambia
School of Medicine
Dept. of Biomedical Sciences
P.O. Box 50110,
LUSAKA.

Dear Mr. Kabungo,

RE: SEROLOGICAL AND MOLECULAR EPIDEMIOLOGY OF FLAVIVIRUSES IN SELECTED PROVINCES OF ZAMBIA.

Reference is made to your resubmission dated 9th November, 2015. The IRB resolved to approve this study and your participation as principal investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2015-Apr-003**
Approval and Expiry Date	Approval Date: 16th November, 2015	Expiry Date: 15th November, 2016
Protocol Version and Date	Version-Nil	15th November, 2016
Information Sheet, Consent Forms and Dates	• English.	15th November, 2016
Consent form ID and Date	Version-Nil	15th November, 2016
Recruitment Materials	Nil	15th November, 2016
Other Study Documents	Data Extraction Tool.	15th November, 2016
Number of participants approved for study	-	15th November, 2016