NOVEL APPROACHES IN THE DETECTION OF YELLOW FEVER VIRUS IN FEBRILE PATIENTS VISITING SELECTED HEALTH FACILITIES IN WESTERN KENYA

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Novel approaches in the detection of Yellow Fever Virus in febrile patients visiting selected health facilities in Western Kenya

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2015

DECLARATION

This thesis is my original work and has not been presented for a degree at any other university.

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DEDICATION

to my loving wife Noreen Njeri and daughters Shantal Timpiyian and Colleen Nashipai

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TABLE OF CONTENTS

DECLARATION ii

DEDICATION iii

ACKNOWLEDGEMENTS iv

TABLE OF CONTENTS vi

LIST OF FIGURES xiv

LIST OF TABLES xiv

LIST OF APPENDICES xvii

LIST OF ABBREVIATIONS AND ACRONYMS xviii

ABSTRACT xx

CHAPTER ONE 1

1.0 INTRODUCTION 1

1.1 Background 1

1.2 Problem statement 3

1.3 Justification 4

1.4 Research Questions 4

1.5 Hypothesis 5

1.6 General objective 5

1.7 Specific objectives 5

CHAPTER TWO 7

2.0 LITERATURE REVIEW 7

2.1 Yellow fever 7

2.2 Yellow fever virus 8

2.2.1 Structure and genomics 8

2.2.2 YFV transmission cycles 9

2.2.3 YFV signs, symptoms and pathogenesis 12

2.2.4 Populations at risk of YFV 13

2.2.5 YFV vaccination 15

2.2.6 Diagnostics of yellow fever 16

2.2.6.1 Serological tests 18

2.2.6.1.1 Heamagglutination inhibition (HI) test 18

2.2.6.1.2 IgM-capture enzyme-linked immunosorbent assay (IgM-capture ELISA) 18

2.2.6.1.3 IgG indirect ELISA 19

2.2.6.1.4 Plaque reduction neutralization test (PRNT) 20

2.2.6.1.5 Focus reduction neutralization test (FRNT) 21

2.2.6.1.6 Indirect Immunofluorescence antibody assay (IFA) 22

2.2.6.2 Virological assays 22

2.2.6.2.1 Heamagglutination (HA) test 22

2.2.6.2.2 Antigen detection ELISA 23

2.2.6.2.3 Plaque Assay 24

2.2.6.2.4 Focus Assay 25

2.2.6.2.5 Direct immunofluorescence antibody assay (IFA) 25

2.2.6.3 Viral genome detection 26

2.2.6.3.1 Reverse transcriptase - polymerase chain reaction (RT-PCR) 26

2.2.6.3.2 Real time RT-PCR 27

2.2.6.3.3 Real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP) 28

2.2.6.3.4 DNA sequencing 29

2.2.7 Recombinant antigen YFV Non Structural protein 1 (NS1) 30

CHAPTER THREE 33

3.0 MATERIALS AND METHODS 33

3.1 Sampling sites 33

3.1.1 Sites in Trans Nzoia County 33

3.1.2. Alupe site 33

3.2 Study population 33

3.3 Recruitment into the study 34

3.4 Inclusion criteria.. 34

3.5 Exclusion criteria 35

3.6 Sample size.. 35

3.7 Sample collection, storage, transportation and processing 36

3.8 Virus strains and cell cultures 36

3.9 Virus titration 37

3.10 Preparation of purified virus 38

3.11 Harvest of ICF and concentration 38

3.12 Sucrose Density Gradient Ultracentrifugation 39

3.13 Development and optimization of an in-house indirect IgG ELISA, antigen detection ELISA and IgM-capture ELISA system using anti-YFV monoclonal antibody (2D12). 40

3.13.1 Patients and panel of control sera 40

3.13.2 IgG indirect ELISA 41

3.13.3 Antigen detection ELISA 42

3.13.4 IgM capture ELISA 43

3.14 Monoclonal antibody 2D12 propagation, purification and conjugation 44

3.15 Focus Reduction Virus Neutralization Test 47

3.16 Design and optimization of a Reverse Transcription Loop-Mediated Isothermal Amplification Assay (RT-LAMP) for yellow fever virus 50

3.16.1 RT-LAMP primer design 50

3.16.2 RNA extraction 50

3.16.3 RT-PCR 51

3.16.4 RT-LAMP 51

3.16.5 Real-time RT-PCR 52

3.16.6 Feasibility of RT-LAMP 52

3.17 Development of non structural (NS1) recombinant antigen for yellow fever virus (YFV) 53

3.17.1 NS1 primer design 53

3.17.2 RNA extraction and NS1 RT-PCR 54

3.17.3 DNA ligation and transformation 55

3.17.4 Western Blot of the YFV NS1 recombinant antigen 58

3.18 Data management 59

CHAPTER FOUR 61

4.0 RESULTS 61

4.1 Development and optimization of an in-house indirect IgG ELISA, antigen detection ELISA and IgM-capture ELISA system using anti-YFV monoclonal antibody (2D12) 61

4.1.1Production of purified virus 61

4.1.2 Monoclonal antibody 2D12 propagation, purification and conjugation 62

4.1.3 Sociodemographic charactersistics 63

4.1.4 IgM capture ELISA and Indirect IgG ELISA 66

4.1.5 Focus Reduction Neutralization Test (FRNT) 68

4.1.6 Flavivirus cross reactivity 71

4.2 The design, optimization and testing of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid diagnosis of YFV. 72

4.2.1 YFV RT-LAMP primer design 72

4.2.2 YFV RT-LAMP limit of detection 74

4.2.3 YFV RT-LAMP specificity 77

4.2.4 YFV RT-LAMP sensitivity 78

4.3 Development of YFV NS1 recombinant antigen using an *E. coli* expression system. 80

4.3.1 YFV NS1 recombinant antigen RT-PCR 80

4.3.2 YFV NS1 recombinant antigen cloning and protein expression 80

4.3.3 Western blot and CBB staining of the recombinant YFV NS1 82

CHAPTER FIVE 83

5.0 DISCUSSION 83

5.1 Seroprevalence of YFV in febrile patients visiting selected health facilities in Western Kenya 83

5.2 A real-time reverse transcription loop-mediated isothermal amplification assay for the rapid detection of Yellow Fever virus ... 85

5.3 Development of YFV NS1 recombinant antigen ... 87

CHAPTER SIX ... 90

6.0 CONCLUSION AND RECOMMENDATION ... 90

6.1 CONCLUSION ... 90

6.2 RECOMMENDATION ... 91

REFERENCES ... 93

APPENDICES ... 106

LIST OF TABLES

Table 2.1: WHO revised case definitions for public health surveillance of YF, 2010. 17

Table 3.1: Different concentrations of sucrose in STE buffer 33

Table 3.2: RT-PCR primers used for the amplification of YFV 52

Table 4.1: Sociodemographic characteristics of study participants by facility 65

Table 4.2: Neutralization test against six different flaviviruses using $FRNT_{50}$ of all the 28 YFV IgG ELISA positive samples 69

Table 4.3: Details of the RT-LAMP primers used for the amplification of the YFV NS1 region 73

Table 4.4: Detection limit of three assays; RT-LAMP, Real-time RT-PCR and RT-PCR against yellow fever virus strains: 17D and two Kenya strains (1992-1993). 79

LIST OF FIGURES

Figure 1.1: Endemic regions of Yellow Fever in Africa... 2

Figure 2.1: Schematic representation of a flavivirus particle 9

Figure 2.2: Yellow fever transmission cycles... 11

Figure 2.3: Major events in the pathogenesis of YF in humans and animal models. 13

Figure 2.4: Areas with risk of YFV transmission in Africa, 2010. 14

Figure 2.5: Areas with risk of YFV transmission in South America, 2010. 15

Figure 4.1: Production of purified virus.. 62

Figure 4.2: Monoclonal antibody 2D12 anti-YFV culture fluid fraction purification using MAbTrap kit 63

Figure 4.3: Map showing seropositivity in the hospital based sampling sites. 676

Figure 4.4: Microscopic photos of YFV17D infected Vero cells after FRNT immunostaining using DAB... 68

Figure 4.5: Flavivirus IgG indirect ELISA using JEV antigen versus YFV IgG indirect ELISA using YFV antigen for 28 Western Kenya samples. 71

Figure 4.6: The RT-LAMP turbidimeter and EIKEN's reaction tubes showing turbidity. 75

Figure 4.7: Analytical sensitivity of YFV RT-LAMP on YFV 17D ICF. 75

Figure 4.8: Detection limit of YFV using real-time RT-PCR... 75

Figure 4.9: Detection limit of YFV using RT-PCR. 75

Figure 4.10: Detection specificity of the YFV RT-LAMP. 77

Figure 4.11: Analytical sensitivity of the YFV RT-LAMP on human serum spiked using YFV 17D. 78

Figure 4.12: YFV NS1 RT-PCR agarose gel. 76

Figure 4.13: YFV NS1 cloning and recombinant antigen expression. 77

Figure 4.14: YFV NS1 recombinant antigen Western blotting and CBB staining 78

LIST OF APPENDICES

Appendix I: SSC approval for SSC# 1829 90

Appendix II: ERC approval for SSC# 1829 91

Appendix III: Continued ERC approval for SSC# 1829 92

Appendix IV: YFV NS1 recombinant antigen sequence 93

Appendix V: Questionnaire 94

Appendix VI: Informed consent forms for adults 97

Appendix VII: Assent forms for minors 101

Appendix VIII: pET 28a showing BamHI and SalI sites 105

Appendix IX: Reprints of published papers 106

Appendix X: Publications and presentations 136

LIST OF ABBREVIATIONS AND ACRONYMS

CHIKV	Chikungunya virus
CPE	Cytopathic Effect
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DENV	Dengue virus
DNA	Deoxyribonucleic acid
ELISA	Enzyme Linked Immunoassay
EMEM	Eagles Minimum Essential Medium
FCS	Fetal calf serum
FFU	Focus forming unit
IgG	Immunoglobulin G
IgM	Immunoglobulin M

JEV	Japanese encephalitis virus
NUITM	Nagasaki University Institute of Tropical Medicine
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
RNA	Ribonucleic Acid
RPM	Revolutions per minute
RT-LAMP	Reverse Transcriptase Loop Amplification Test
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
RVFV	Rift Valley fever virus
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow Fever virus

ABSTRACT

Globally, the threat of emerging and re-emerging arboviral infections continues to be a major public health concern. The detection and diagnosis of many of these diseases remains a challenge, and it is clear that novel approaches to their identification and control must be continually developed. Clearly there is an overarching need to accurately detect priority febrile illnesses and their causative agents in Kenya. This study designed and optimized a Reverse-Transcription Isothermal Loop Mediated Assay (RT-LAMP) for yellow fever virus (YFV) assay, developed and optimized ELISA systems using anti-yellow fever virus monoclonal antibody (2D12) as well as used the developed assays to determine the seroprevalence of YFV in selected health facilities in Western Kenya during the period 2010-2012. The study design was experimental. RT-LAMP was done isothermally at 62 °C using a real-time turbidimeter which allowed detection within 1 h. RT-PCR and real time RT-PCR using YFV primers was also performed. Specificity of RT-PCR, real-time RT-PCR and RT-LAMP assays was determined using RNA from other related flaviviruses (WNV, DENV1-4, JEV) as well as RVFV and CHIKV where only YFV RNA was detected. In addition, equal sensitivity was also observed when the RT-LAMP assay and the quantitative real-time RT-PCR were compared using YFV spiked human serum samples with a detection limit of 0.29 PFU. The RT-LAMP assay further showed equal detection limit sensitivity of 0.29 PFU while using two local YFV wild isolates and the vaccine strain YFV 17D. A total of 469 serum samples from febrile patients were tested for YFV antibodies using in-house IgM-

capture ELISA, in-house IgG indirect ELISA and focus reduction neutralization test ($FRNT_{50}$). The present study did not yield any IgM ELISA positive cases an indication of absence of recent YFV infection in the area. Twenty eight samples (6%) tested positive for YFV IgG. These 28 cases detected during the study were due to either YFV vaccination or past exposure to various flaviviruses including YFV. There were five cases confirmed by $FRNT_{50}$, of these, four cases were either vaccination or natural infection during YF outbreak (1992-93) or another period and one case was confirmed as a West Nile virus infection. The YFV NS1 recombinant antigen was successfully cloned and expressed in a pET 28a system. Sequencing data showed 99% homogeneity with the consensus YFV 17D strain. Confirmation of the expressed YFV NS1 was performed using a penta-his tag in a Western blot.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

In Kenya a plethora of infectious diseases such as yellow fever, malaria, typhoid, dengue fever and influenza, whose cardinal sign is fever occur. There is anecdotal evidence that arbovirus infection is an important cause of morbidity and mortality in Kenya and the tropics (Morrill *et al*., 1991; Reiter *et al*., 1998; Sang *et al*., 2001; Woods *et al*., 2002). However, the true extent of the problem has never been accurately described. In the last twenty years there have been outbreaks associated with significant morbidity and mortality (Johnson *et al*., 1983; Sang *et al*., 2001; Woods *et al*., 2002; LaBeaud *et al*., 2007). On each occasion, the outbreaks have spread rapidly.

Yellow fever (YF), is an important viral hemorrhagic fever endemic in equatorial Africa and South America (Gould *et al*., 1989; Monath, 2001). The epidemiology of the disease differs between the continents, even though the disease is caused by the same virus. In South America it mainly affects forest workers, whereas in Africa there have been serious epidemics in unimmunized populations in both rural and urban areas. The potential exists in African and South American cities for epidemics if *Aedes aegypti*, the mosquito that carries the YF virus, occurs in densely populated areas (WHO, 2005). Yellow fever virus (YFV) is the prototype of the family *Flaviviridae* and the genus

Flavivirus (Monath *et al*., 1984). The genus *Flavivirus* contains approximately 70 viruses including YFV, dengue viruses (DENVs), Japanese encephalitis virus (JEV) and West Nile virus (WNV) that are of major human public health concern (Ellis, 2008). An estimated annual incidence of 200,000 cases of YF causing 30,000 deaths is reported each year worldwide (WHO, 2004). Case fatality rates of YF can be as high as 50% in severely affected persons without treatment (Ellis, 2008; WHO, 2010). In the Eastern African region YF outbreaks have been reported in Kenya, 1992-93 (Reiter *et al*., 1998; Sanders *et al*., 1998), South Sudan 2003; 2005 (Onyango *et al*., 2004; Gould *et al*., 2005), Uganda, 2010-11 (McMullan *et al*., 2012), Sudan, 2012 (Markoff, 2013) and Ethiopia, 2013 (WHO, 2013) (**Figure 1.1**).

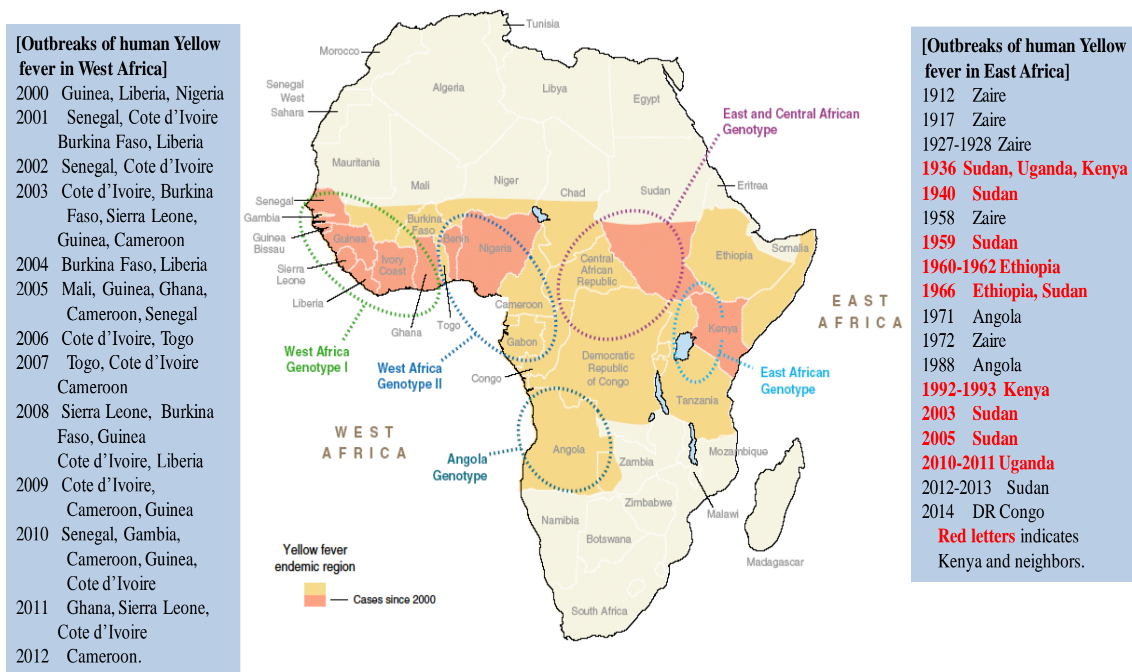


Figure 1.1: Endemic regions of Yellow Fever in Africa. Barrett and Monath, 2003

1.2 Problem statement

On each occasion, YF outbreaks have spread rapidly as many unvaccinated people become infected leading to a public health concern. Diagnostic tests are not routinely available for YF, not just in Kenya but also in East Africa for this reason, assays to deal with YF outbreaks are inadequate. Where available, test assays for several key infectious agents are highly specialised and can only be offered in research institutions. Yet, these assays are needed where outbreaks are more likely to occur and where the priority diseases are prevalent. At the diagnosing laboratory various challenges arise such as; the availability of reagents, throughput time of assays, biosafety level of the laboratory, technical capabilities and competencies of the laboratory staff, differential diagnostic capabilities and communication of assay results. The difficulty faced by many research teams has been the lack of laboratory assays specific to YFV. In addition, the occurence of several flaviviruses in one geographic region offers diagnostic challenges due to cross-reactions between the closely related flaviviruses. Kenya continues to be reliant on donor-driven research projects to diagnose most arboviruses which makes her vulnerable should the projects come to a halt or if research priorities shift. There is hence need for in country development of reliable, replenishable and cost effecient diagnostic platforms to address this need.

1.3 Justification

There exists a huge risk of expansive YF outbreaks in unimmunized populations even when the confirmed cases are few. This is further confounded in children who are especially vulnerable. Hence proactive surveillance offers the best tool to detect and prompt response to YF outbreaks in populations whose vaccine coverage remains low (WHO, 2005). Kenya continues to import ELISA reagents for routine surveillance and outbreak response of YFV. In order, to address the lack of specific ELISA systems, the use of a YFV specific monoclonal antibody 2D12 in an in-house antigen detection and IgM capture ELISA's will ensure a reliable and constant supply of reagents not just for Kenya but the larger Eastern Africa region where a burden of YF exists. In the present study, an RT-LAMP assay, taking only 1 hour and testing up to 32 samples at a time which will offer increased sensitivity over the already established conventional RT-PCR will be designed, optimised and developed. This rapid detection assay method will assist in timely reporting of YFV outbreaks once domesticated in-country. There will also be the development of a YFV NS1 recombinant antigen using *E. coli* expression system. The recombinant antigen may be used to develop an easy to use rapid strip that could be used as point of care tool or openly in the field even where resources are limited as is mostly the case in Sub Saharan Africa.

1.4 Research Questions

1. What proportion of seropositive YFV patients would be detected using inhouse ELISA assays?

2. How sensitive and specific would an RT-LAMP assay be in the diagnosis of yellow fever virus?
3. Can an NS1 recombinant antigen for yellow fever virus using an *E. coli* expression system be developed for use in resource limited settings?

1.5 Hypothesis

Null Hypothesis

YFV infection is not detectable in febrile patients visiting selected health facilities in Western Kenya.

1.6 General objective

To design and develop assays for the detection of YFV infection in febrile patients visiting selected health facilities in Western Kenya.

1.7 Specific objectives

1. To develop and optimize an in-house indirect IgG ELISA, antigen detection ELISA and IgM-capture ELISA system using anti-YFV monoclonal antibody (2D12).
2. To design, optimise and test a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid diagnosis of YFV.
3. To develop NS1 recombinant antigen of YFV using an *E. coli* expression system.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Yellow fever

Yellow fever (YF), a mosquito-borne disease, is an important viral hemorrhagic fever in Africa and South America where it is endemic (Gould *et al*., 1989). In a report by, Sternberg 1890, on the etiology and prevention of YF, the American Bacterilogist, describes it as a feared perstilence. Notable was a requirement to fly a '*Yellow jack*' on ships due to quarantine of the disease with unknown cause and effective treatment (Freirson, 2010). According to WHO fact sheet, '*yellow*' is due to the jaundice sympomized by some patients (WHO, 2010). An estimated annual incidence of 200,000 cases of YF causing 30,000 deaths is reported each year worldwide (WHO, 2004). Case fatality rates of YF can be as high as 50% in severely affected persons without treatment (WHO, 2010; Ellis & Barret, 2008).

Between 1990 and 2004, 14,281 human cases were reported to the WHO, of which 11,763 (82%) occured in Africa, but there is significant underreporting (Monath, 2007). Africa continues to be faced with the risk of an YF outbreak as evidenced by past events. Reported YF outbreaks include Southern Africa in Angola 1977, 1988 (WHO, 2005). In Western Africa, outbreaks have been reported in Guinea 2010 (WHO, 2011), Senegal 2010 (WHO, 2010), Cameroon 2010 (WHO, 2011), Côte d'Ivoire 2011 (WHO, 2011)

and Sierra Leone 2011 (WHO, 2011). In the Eastern African region outbreaks have been reported in Kenya 1992-93 (Sanders *et al*., 1998), South Sudan 2003, 2005 (Onyango *et al*., 2004; Gould *et al*., 2008), the Democratic Republic of Congo 2010 (WHO, 2011), Uganda 2010-11 (McMullan *et al*., 2012), Sudan 2013 and Ethiopia 2013. The resurgence of YF in the Eastern African region raises public health concerns, creating a need to improve on surveillance, early warning systems and expanded immunization programs (Ellis & Barrett, 2008).

2.2 Yellow fever virus

2.2.1 Structure and genomics

Yellow fever virus (YFV) is the prototype of the family *Flaviviridae* and the genus *Flavivirus* (Monath & Nystrom, 1984). The genus flavivirus contains approximately 70 viruses including YF, dengue viruses (DENVs), Japanese encephalitis virus (JEV) and West Nile virus (WNV) that are of major human public health concern (Ellis & Barrett, 2008). YFV is a non-segmented, single-stranded, positive-sense RNA of approximately 11 kb nucleotides in length encoding three structural (capsid, pre-membrane/membrane, and envelope) proteins as shown in **Figure 2.1** and seven non structural; NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 proteins (Vratskik *et al*., 2013; Rice *et al*., 1985). On the basis of genetic homology YFV is closely related to nine other flaviviruses; Banzi, Bouboui, Edge Hill, Jugra, Saboya, Potiskum, Sepik, Uganda S and Wesselsbron viruses. However, New Guinea's Sepik virus, is genetically the most closely related to YFV rather than the African flaviviruses (Gaunt *et al*., 2001).

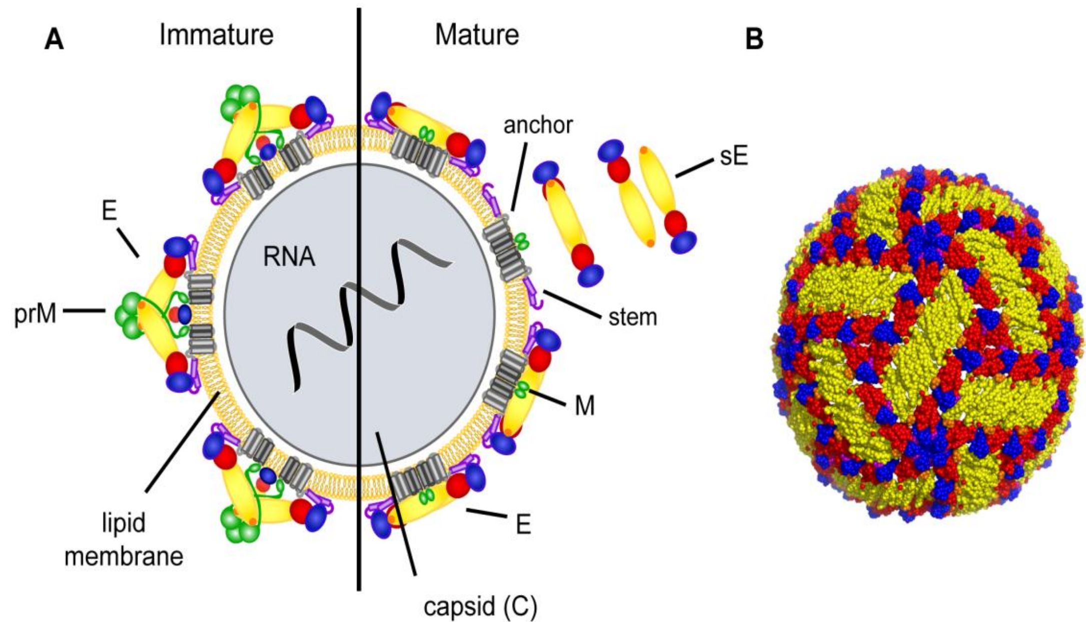


Figure 2.1: Schematic representation of a flavivirus particle

(A) Immature virion and (B) mature virion. Vratskik *et al*., 2013

2.2.2 YFV transmission cycles

There are three well described YFV transmission cycles: sylvatic, urban and savannah as shown in **Figure 2.2**. In the sylvatic transmission cycle also known as the "Jungle cycle" the virus is transmitted between non-human primates by *Aedes africanus* in Africa and canopy-dwelling mosquitoes such as *Hemagogus janthinomys* in Southern America (Barrett & Monath, 2003; Weaver *et al*., 2010). Other mosquito species involved in the transmission include *Ae. africanus, Ae. furcifer, Ae. vittatus, Ae. luteocephalus, Ae. opok, Ae. metallicus* and *Ae. simpsoni* in Africa and *Sabethes chloropterus* in South America (Barrett & Monath, 2003). The urban cycle is

characterised by transmission between humans and the anthrophilic *Ae aegypti* in Africa and South America (Weaver, 2010). *Ae. aegypti's* high densities in urban areas enables it to be a vector for urban YF (Barrett & Monath, 2003). The savannah cycle also known as the intermediate cycle between humans and various *Aedes* mosquitoes is only reported in Africa (Barrett & Monath, 2003). When humans in the moist savannah regions come into contact with the jungle cycle a "Zone of Emergence" arises (Barrett & Monath, 2003). Arthropods including *Amblyomma variegatum* ticks may act as vectors and are involved in the biologic transmission of the virus to vertebrate hosts (Barrett & Monath, 2003).

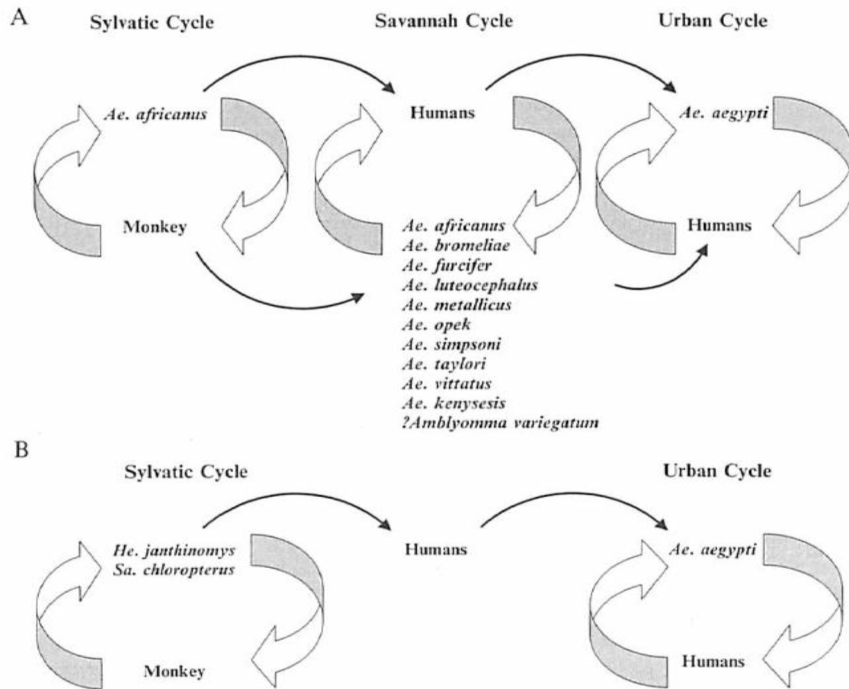


Figure 2.2: Yellow fever transmission cylces. A. YF transmission cycles in Africa. B. YF transmission cycles in South America. Barrett & Monath, 2003

2.2.3 YFV signs, symptoms and pathogenesis

These three transmission cycles lead to the same clinical disease (Vainio & Cutts, 1998). In the acute phase of YF, after an incubation period of three to six days, there is a sudden onset of fever, chills, severe headache, anorexia and viremia as shown in **Figure 2.3** (Monath, 2008). Laboratory examination findings include leukopenia, neutropenia, elevated liver enzyme levels and proteinuria (Markoff, 2013). Over several days to a week, most pateints recover, but 15% to 25% of them enter a more toxic second phase within 24 hours after an initial remission (WHO, 2010). The disease progresses with the appearence of severe asthenia, acute fulminant hepatitis develops leading to jaundice, hematemeis and hematochezia or melena, renal failure, cardiovascular instability and shock. Half the patients who enter the toxic phase die within 10 to 14 days with case fatality rates of more than 50% (Monath *et al*., 2008; WHO 2010). The rest recover without clinically significant organ damage. Naturally acquired infection confers lifelong protection against YF (Markoff, 2013). Increasing evidence shows that the systemic inflammatory response syndrome (SIRS) also known as cytokine storm, contributes to terminal events and death. Early signs of infection are probably due to the innate immune response to infection, including interferon-α, Tumour necrosis factor (TNF-α) and other acute-phase reactants (APRs) (Monath 2008).

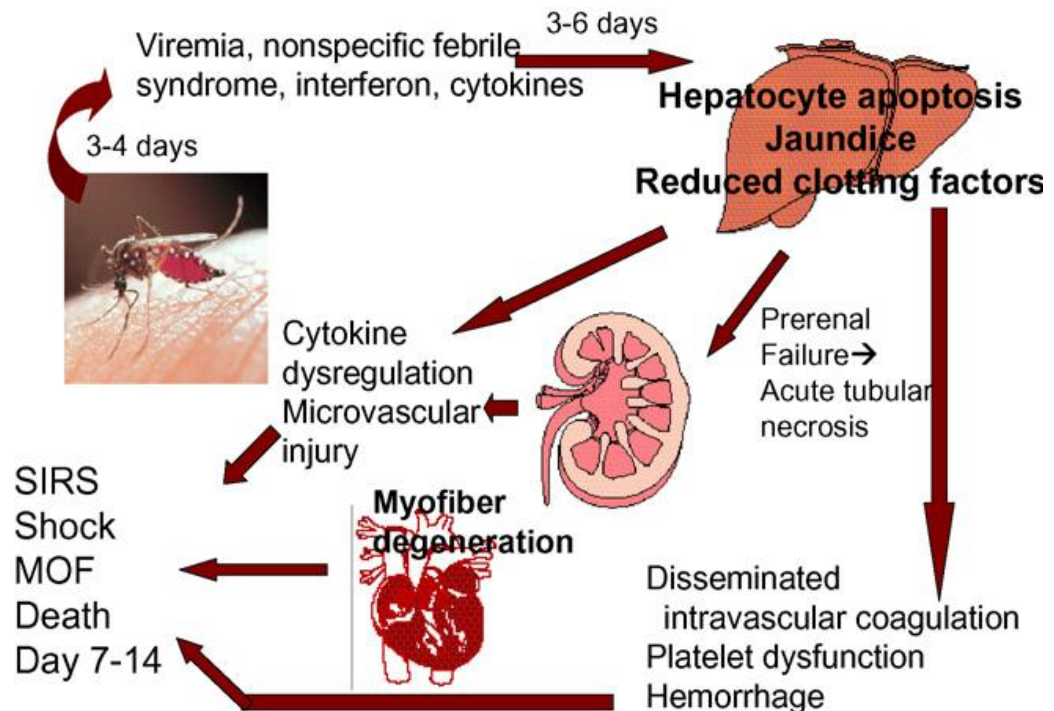


Figure 2.3: Major events in the pathogenesis of YF in humans and animal models.

Monath, 2008

2.2.4 Populations at risk of YFV

YFV is only found in sub-Saharan Africa, **Figure 2.4** and tropical South America, **Figure 2.5**, including 34 countries in Africa and 14 countries in South America; the virus is not found in Asia (Barrett & Monath, 2003). Countries where the vector and non-human primate hosts exist are vulnerable to the introduction of YFV, even if the disease is not endemic (Jentes *et al*., 2011). A combined population of >900 million are at risk in the 45 endemic countries in Africa and South America (WHO, 2010). Since

the mid-20th century, the global YF risk map has depicted the best estimate of the distribution of the virus (Jentes *et al*., 2011).

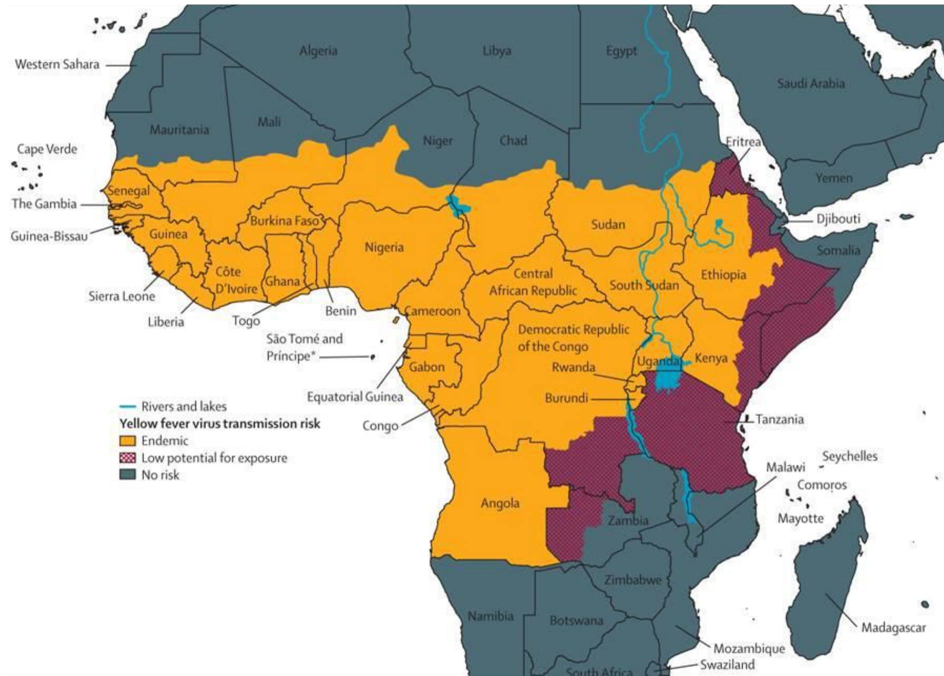


Figure 2.4: Areas with risk of YFV transmission in Africa, 2010.

Figure 2.5: Areas with risk of YFV transmission in South America, 2010. Jentes *et al*., 2011

2.2.5 YFV vaccination

Vaccination offers an important measure in the prevention of yellow fever. A live-attenuated yellow fever vaccine based on 17D virus strain was developed in 1936 by Max Theiler (Theiler & Smith, 1937). Since then several hundred million doses have been administered and its effectiveness in protecting from disease has been reported to be at least 90% (Vratskikh, 2013). A single dose provides protection for 10-35 years or more and probably for life (WHO, 2010). Recent studies, analyzing innate, cellular and humoral immune responses after YF vaccination indicate that all arms of the immune system are activated leading to a polyfunctional response that is most likely essential for

the long lasting immunity induced by the vaccine (Vratskikh, 2013). Strong evidence suggests that humoral immunity mediated by virus-neutralizating antibodies is the primary effector mechanism of protection (Barrett, 2009). Although the effective vaccine has been available for more than 70 years the disease still remains endemic in tropical South America and sub-Saharan Africa (Markoff, 2013). Failure to eradicate the disease in these regions can be attributed to several factors, including the sylvatic life of the virus and intermitent or absent vaccination campaigns, especially in politically unstable and economically disadvantaged African countries (Markoff, 2013). In high-risk areas where vaccination coverage is low, prompt recognition and control of outbreaks through immunization is critical to prevent epidemics (WHO 2010). In rare cases some adverse effects of the YFV 17D vaccine strain may occur by invasion of the central nervous system and cause meningoencephalitis after immunization, which is usually self-limited; young age (<6 months), advanced age and immune deficiency are risk factors for yellow ferver vaccine-associated neurotropic disease (YEL-AND) and yellow ferver vaccine-associated viscerotropic disease (YEL-AVD) (Monath *et al.*, 2008). The risk of death from yellow fever is much bigger than the risks related to the vaccine (WHO 2010). Although an attempt was made to treat YF using ribavirin, research results showed that the drug was ineffective.

2.2.6 Diagnostics of yellow fever

YF cases are difficult to diagnose from clinical symptoms in the early stages of an illness due to "influenza-like symptoms" which are similar to other febrile illnesses,

many of them highly prevalent in Africa and South America. Differential diagnosis may include malaria, viral hepatitis, dengue, leptospirosis or other haemorrhagic fevers (WHO 2008). WHO-recommends case definition for suspect YF as shown in **Table 2.1** as; "Any case presenting with acute onset of fever, with jaundice appearing within 14 days after the onset of the first symptoms (WHO 2010)."

Table 2.1: WHO revised case definitions for public health surveillance of YF, 2010.

Type of case	Definition
Suspect	Any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms
Probable	A suspected case **AND** 1 of the following: • presence of yellow fever IgM in the absence of yellow fever immunization within 30 days before onset of illness; • positive postmortem liver histopathology; • epidemiological link to a confirmed case or an outbreak
Confirmed	A probable case **AND** 1 of the following: • detection of yellow fever-specific IgM; • detection of fourfold increase in yellow-fever IgG titres between acute and convalescent serum samples, or both; • detection of yellow fever-specific neutralizing antibodies **AND** • absence of yellow fever immunization within 30 days before onset of illness **OR** 1 of the following: • detection of yellow fever virus genome in blood or other organs by PCR; • detection of yellow fever antigen in blood, liver or other organs by immunoassay; • isolation of yellow-fever virus **AND** • absence of yellow fever immunization within 14 days before onset of illness

Weekly epidemiological record, No. 47, Nov 2010

2.2.6.1 Serological tests

2.2.6.1.1 Heamagglutination inhibition (HI) test

The HI test is widely used as a quantitative test based on the ability of virus to agglutinate goose red blood cells (GRBC) for diagnosis of various viruses, especially influenza virus and dengue virus (WHO, 2009). Although influenza virus's heamagglutination, is temperature dependent, flaviviruses and Toga-alpha viruses are pH dependent. Anti-flavivirus antibodies in sera can inhibit this agglutination and the potency of this inhibition is measured in the HI test. Serum is heat treated at 56 °C for 30 min to inactivate hemolysin, then treated with acetone or kaolin to remove non-specific inhibitors of heamagglutination and adsorbed with GRBC to remove natural agglutinins. The assay requires the use of pair sera i.e. acute and convalescent phase of infection, or sera with an interval of more than seven days. It does not discriminate between infections of closely related falviviruses (YFV, DENV, JEV or WNV) or between immunoglobulin isotypes (WHO, 2009). While proposing the concept of arboviral groups, HI test showed the least resolution of antigenic difference, the neutralization test was the most specific, and the complement-fixation (CF) test was intermediate between these two (Casal, 1957).

2.2.6.1.2 IgM-capture enzyme-linked immunosorbent assay (IgM-capture ELISA)

The IgM-capture ELISA relies on the capture of total IgM in patient's sera by anti-µ chain specific antibodies coated onto a microplate (WHO, 2009). YFV antigens are

bound to the captured anti-YFV IgM and are detected by monoclonal or polyclonal YF antibodies directly or indirectly conjugated with an enzyme that will transform a non-coloured substrate into coloured products (WHO, 2009). Serum can be used for the detection of IgM if the samples are taken five days or more after the onset of fever. The YFV antigens used for this assay are derived from the infected culture fluid (ICF) or suckling mouse brain preparations (WHO, 2009). The IgM-capture ELISA has good sensitivity but it is however, plagued by, cross-reactivity with other circulating flaviviruses such as DENV and WNV. Patients with a history of other flavivirus infections may interfere with the test in a phenomenon known as "antigenic sin" (Zompi & Harris, 2013). These limitations have to be taken into account when using the tests in regions where these pathogens co-circulate (WHO, 2009).

2.2.6.1.3 IgG indirect ELISA

The IgG indirect ELISA is used for the detection of recent or past YFV infections since IgG production is life-long. A microplate is coated with YFV sucrose-purified antigen, where YFV antibodies in patient sera bind. Anti-YFV IgG antibodies are then detected by a polyclonal anti-human IgG conjugated with an enzyme that will transform a non-coloured substrate into coloured products (WHO, 2009). The test acquires it reliability in the presence of paired sera as a fourfold or greater increase in IgG antibodies in acute and convalescent paired sera can be used to document recent infection. In addition, test results correlate well with the HI test. However, the IgG indirect ELISA test lacks

specificity within the flavivirus serocomplex groups serving as a limitation when using this test.

2.2.6.1.4 Plaque reduction neutralization test (PRNT)

The PRNT is a sensitive method for the detection and quantification of specific virus neutralizing antibodies in serum and is carried out in three steps (Russel *et al.*, 1967). The first, neutralizing step, is performed by incubating serum dilutions against a standard dose of virus. In the second, infection step, the virus/serum mixture is inoculated to Vero cell monolayers in 6-well plate and incubated to allow any remaining live virus to infect the cells. The cells are overlaid with methylcellulose and further incubated. The third, staining step, the cell monolayers are then fixed and the methylcellulose overlay removed, then stained with crystal violet to allow visualization and counting of plaques formed. The reciprocal of the serum dilution that reduces the number of plaques by 50% as compared to the virus control is considered positive for neutralizing (Russel *et al.*, 1967). Since PRNT only measures neutralizing antibody titres, thus, it is more specific than other antibody test such as ELISA, CF test and immunofluorescent antibody assay (IFA). The PRNT test has proved to be confirmatory method for assaying YFV neutralizing antibody, however, technical difficulty is experienced depending on laboratory settings.

2.2.6.1.5 Focus reduction neutralization test (FRNT)

The FRNT is also a sensitive method for the detection and quantification of specific virus neutralizing antibodies in serum and is carried out in four steps (Hashimoto *et al*., 1971). The first, neutralizing step, is performed by incubating serum dilutions against a standard dose of virus. The second, infection step, the virus/serum mixture is inoculated to Vero cell monolayers in a microplate and incubated to allow any remaining live virus to infect the cells. The cells are overlaid with methylcellulose and further incubated. The third, fixation step, the methylcellulose overlay is removed, fixed with formaldehyde and permeabilized with NP-40. The fourth, immunostaining step, is the additional step in FRNT, hence a deviation from PRNT. The cells are then blocked using a blocking solution, then reacted with monoclonal or polyclonal YFV antibodies directly or indirectly conjugated with an enzyme that will transform a non-coloured substrate into coloured products. The reciprocal of the serum dilution that reduces the number of plaques by 50% as compared to the virus control is considered positive for neutralizing (Hashimoto *et al*., 1971). Similar to PRNT, FRNT only measures neutralizing antibody titres, thus, are more specific than other antibody test such as ELISA, CF and IFA. In addition, FRNT can obtain results within two to three days while PRNT requires seven days or more. However, FRNT can be performed only if YFV antibody is available.

2.2.6.1.6 Indirect Immunofluorescence antibody assay (IFA)

The indirect IFA is a four step procedure, intended for the qualitative detection of human IgM to YFV in a single sample or to identify sero-conversion in acute and convalescent specimens and as an aid in the diagnosis of current or past infection (Henchal *et al.*, 1982). In the first step, YFV infected cells are attached and fixed with cold acetone. In the second step, the patient serum is diluted in PBS, added to appropriate slide wells in contact with the virus infected cells, and incubated. Following incubation, the slide is washed in PBS which removes unbound serum antibodies. In the third step, antigen-antibody complexes react with the fluorescein isothiocyanate-labeled anti-IgM. In the fourth step, the slide is washed, dried and examined using a fluorescence microscope. Positive reactions appear as cells exhibiting bright cytoplasmic fluorescence against a background of negative control cells. Semi-quantitative endpoint titer is obtained by testing serial dilutions of sample serum (Henchal *et al.*, 1982). Serologic cross-reactivity among flaviviruses such as DENV infection may complicate the interpretation of a positive YF IFA hence a limitation of the assay.

2.2.6.2 Virological assays

2.2.6.2.1 Heamagglutination (HA) test

The HA test is a simple method for titrating viruses based on their ability to agglutinate GRBC. YFV ICF can agglutinate GRBC, thus serial dilution of ICF in a microplate with a consistent amount of GRBC can indicate virus titre as HA units. The test though not

serological, is used as a basis for the HI test. The limitation of HA test is that it is not suitable to identify the virus (WHO, 2009).

2.2.6.2.2 Antigen detection ELISA

The antigen-detection ELISA is a simple and quantitative method that uses anti-YFV IgG (polyconal Ab: human high titre IgG, hyperimmune mouse ascitic fluid; monoclonal Ab: 2D12, 5E3, 5E5, 8A3 and 6B6C-1), immobilized on a solid phase, to capture YFV virus in ICF (Monath and Nystrom, 1984). A limiting factor of laboratories attempting to establish the assay is the difficulty to obtain suitable high titre IgG antibody-containing human or animal sera. However, YFV monoclonal antibodies have the advantage of availability in standardized and with virtually unlimited supply (Schlesinger *et al*., 1985; Monath & Nystrom, 1984). The broadly reactive flavivirus monoclonal antibody (6B6C-1) prepared against St. Louis encephalitis virus and conjugated to enzyme proved to be the most useful for detection of YFV in the ELISA (Monath & Nystrom, 1984). The antigen detection ELISA is convenient for screening large number of ICF samples to detect and quantify virus and also less expensive than RT-PCR. The limitation of this ELISA is that it is less sensitive in detecting low concentrations of virus, compared to RT-PCR, thus, amplification of virus by growth in cell culture may be necessary.

2.2.6.2.3 Plaque Assay

The plaque assay is the most commonly used virological assay to determine viral titer as plaque-forming units per ml (pfu/ml) (Russell *et al.*, 1967). In this assay, cell monolayers are infected with a low ratio of virus, such that sporadic cells become infected. An overlay medium, methylcellulose, is added to semi solidify the culture fluid so as to limit the spread of virus. When each infected cell produces virus and eventually lyses, only the immediately adjacent cells become infected forming a plaque. The methylcellulose overlay is removed, the cell monolayer fixed and stained with crystal violet to allow visualization and counting of plaques formed. The plaque assay is also used to purify a clonal population of virus in virus cloning experiments. Each plaque represents a single virus. Therefore, clonal virus populations may be purified by picking individual plaques. The advantages of the plaque assay include; its ability to quantify and titrate viruses, cloning capability especially in co-infecting strains of virus, offers better adherence to less CPE type viruses unlike HA, the plaque sizes generated can be used to morphologically characterise virus and are bigger in size as opposed to those of focus assay and there is no need for anti-virus antibody as no immunostaining step occurs in the plaque assay. However, the time taken to perform the plaque assay to completion is longer than focus assay or HA test, there also needs to be a good combination between the virus-cell line for its success and the slow growth viruses with small plaque sizes remain a challenge as they do not increase in size even when the incubation time is increased.

2.2.6.2.4 Focus Assay

The focus assay offers two functions; virus titration and identification of virus by immunostaining (Igarashi & Mantani, 1974). The assay only quantifies, the infectious virus particles, thus, making it a very accurate technique for virus titration with similarity to plaque assay titration. The YFV is inoculated to Vero cell monolayers in a microplate and incubated to allow the virus to infect the cells. After incubation, the cells are overlaid with methylcellulose and further incubated. The methylcellulose overlay is removed, the cell monolayer fixed, permeabilized and blocked. An immunostaining step where detection is performed by using monoclonal or polyclonal antibodies against YFV directly or indirectly conjugated with an enzyme that will stain YFV in the infected cells (Igarashi & Mantani, 1974). The advantages of this assay are the shortened incubation time from seven days in plaque assay to three days in focus assay and increased number of samples that can be screened due to the microplate format. However, the assay is limited by its inability to morphologically characterize viruses and also it cannot be applied for viral cloning unlike plaque assay as visualization is made after virus and cell fixation.

2.2.6.2.5 Direct immunofluorescence antibody assay (IFA)

This technique is used to detect viral protein in ICF using specific fluorescein isothiocyanate-labeled antibody (Henchal *et al*., 1982). The steps involved are: Fixation of smear on the slide, treating with labeled antibody, incubation, washing to remove

unbound excess labeled antibody and visualization under fluorescence microscope. When viewed under fluorescence microscope, the field is dark and areas with bound antibody fluoresce green. This technique can be used to detect viral antigens from monolayer of cells and also identification of anatomic distribution of an antigen within compartments of a cell (Henchal *et al*., 1982). The advantages of the direct IFA include: it's very sensitive, even single positive cell can be visualized over a non-fluorescing negative background and due to its rapidity it offers early inferences of a disease especially in an outbreak situation. A standardized anti-viral antibody is supplied for most flaviviruses detection. The direct IFA is limited due to the need of a fluorescence microscope, it's subjective leading to intra-person variations, normally not a good assay method for screening large numbers of samples unlike the Antigen detection ELISA and its less quantitative compared to plaque/focus assay or Antigen detection ELISA.

2.2.6.3 Viral genome detection

2.2.6.3.1 Reverse transcriptase - polymerase chain reaction (RT-PCR)

Genome based molecular assays, antigen detection ELISA and virus isolation augment each other in the detection of YFV in the acute phase of infection before the appearance of antibody (Dash *et al*., 2011). Since the advent of RT-PCR, several of these assays have been developed. They all involve three basic steps: (1) nucleic acid extraction and purification, (2) reverse transcription and DNA amplification and (3) detection and characterization of the amplified product. The quantification of viral load has

traditionally been performed using plaque assay, but quantitative RT-PCR is now preferred, because of its simplicity, speed, amenable to high-throughput screening and sensitivity. Compared to plaque assay, the sensitivity of RT-PCR methods varies from 80% to 100% and depends on the region of the genome targeted by the primers, the approach used to amplify or detect the PCR product either one-step RT-PCR or two-step RT-PCR. It is imperative to target specific regions of the YFV genome, to avoid false positive results due to non-specific amplification and not those conserved among flavi- or other related viruses. False positive may also occur as a result of contamination by amplicons from previous amplifications (WHO, 2009). In addition, RT-PCR is faced by the challenge of detecting even non infectious virus particles unlike plaque assay or focus assay that target only infecting virus.

2.2.6.3.2 Real time RT-PCR

The real-time RT-PCR is a one step assay system used to quantify viral RNA using fluorescent probes. Many real-time RT-PCR assays have been developed employing TaqMan or SYBR green technologies. The TaqMan real-time RT-PCR is highly specific due to sequence-specific hybridization of the probe. The TaqMan probe chemistry assays include assays developed by: Drosten *et al*., 2002, Bae *et al*., 2003, Chao *et al*., 2007 and Mantel *et al*., 2008. The SYBR green real-time RT-PCR has the advantage of simplicity in primer design and uses universal RT-PCR protocols but is theoretically less specific. Dash *et al*. (2012) proposed a SYBR green chemistry test for YFV detection that offers a simpler and less expensive alternative whilst maintaining the

advantages of real-time RT-PCR assays. However, a limitation of the real-time RT-PCR is its inability to detect low amounts of viral RNA as the amplification curves diminish due to the delayed start of amplification unlike in real-time RT-LAMP.

2.2.6.3.3 Real-time reverse transcription loop-mediated isothermal amplification (RT-LAMP)

The real-time RT-LAMP is an RNA-specific amplification assay that does not require thermal cycling instrumentation. In the initial step, reverse transcription, single-stranded RNA target is reverse transcribed using a native Avian Myeloblastosis Virus (AMV) reverse transcriptase to a single stranded cDNA molecule (WHO, 2009). Subsequently, the assay relies on autocycling strand displacement DNA synthesis by a recombinant *Bacillus stearothermophilus* (*Bst*) DNA polymerase and a set of four or six primers. Two inner primers and two outer primers define the target region, and an additional set of primers termed loop primers can be added to increase the sensitivity of the assay. The final products of the RT-LAMP are DNA molecules with a cauliflower-like structure of multiple loops consisting of repeats of the target sequence. The products can be analyzed by real-time monitoring of the turbidity resulting from the production of magnesium pyrophosphate precipitate during the DNA amplification reaction (Notomi *et al*., 2000; Roux *et al*., 2009). Advantages of RT-LAMP include: highly sensitive and specific method for virus titration, isothermal nature allows for less infrastructural investment unlike RT-PCR, amplification peaks are not viral-RNA copy dependent. However, primer design is complicated and there is need to use a set of four/six primers

in RT-LAMP. Several RT-LAMP diagnostic approaches are in place for the identification of different infectious diseases SARS (Hong *et al*., 2004; Fuxun *et al*., 2005), Dengue (Parida *et al*., 2005) and West Nile (Parida *et al*., 2004).

2.2.6.3.4 DNA sequencing

DNA sequencing using Sanger method requires that the DNA fragment be located close to a known sequence and has to be available in single strands. DNA synthesis is initiated from a nucleotide primer binding to a known sequence and then continued along the unknown sequence by DNA polymerase (Wink, 2011). Dideoxyribonuleoside triphosphates (ddNTPs) that bind to the single-stranded DNA same as fluorescence-labeled nucleoside triphosphates are introduced as specific competitive inhibitors. However, lacking the essential hydroxyl group in the 3'-position the ddNTPs are unable to bind to the next nucleotide to be incorporated (Wink, 2011). Hence four separate reactions are run for each of the nucleotides (ddATP, ddGTP, ddCTP or ddTTP) that compete with their normal counterparts. After the DNA synthesis and base-specific fluorescent marking, all four reaction products can be simultaneously separated by capillary electrophoresis using a fluorescence detector to read the nucleotide sequences (Wink, 2011). However, DNA sequencing remains considerably expensive and highly specialized laboratory and personnel are key for its success.

Several diagnostic approaches are hence in place for the identification of YFV. The tests include ELISA, RT-PCR, PRNT and virus isolation. Although the use of combinatory test diagnosis is recommended in case of outbreaks, there arise huge obstacles where resources are limited especially in Sub-Saharan Africa. Moreover, epidemics of the past happened in marginalized areas and most ended up unreported or under-reported. Hence, the present study will strive to improve the detection of YFV in Kenya. An YFV RT-LAMP, YFV Ag detection and IgM-capture ELISA systems will be established as well as the development a recombinant Ag against YFV NS1 which may be used in future for the development of an immunochromatography test. The tests developed will be cost effective, readily available and replenishable hence enabling better response to outbreaks that continue to befall the Eastern African region.

2.2.7 Recombinant antigen YFV Non Structural protein 1 (NS1)

Conventionally, whole infected culture fluid (ICF) or purified virion have been the medium of choice as diagnostic tools for most arboviruses including YFV (Chanyasanha *et al*., 1999). However, the production of ICF and purified virion are still quite an economic challenge in most developing countries, notwithstanding the potential risk that researchers expose themselves to while working with live virus. Moreover, the use of whole antigen exposes the test to cross-reactivity with closely related flaviviruses such as dengue virus (DENV) and West Nile virus (WNV). NS1 was first identified in flavivirus infected suckling mouse brain extracts and was originally referred to as the soluble complement fixing (SCF) antigen (Russell *et al*., 1970). Since then it has been

recognized as being important for its immunogenicity. NS1 is located in various regions in infected cells: in association with membranes in the cytoplasm (Stohlman *et al*., 1975), at the cell surface in association with the plasma membrane (Cardiff & Lund 1976; Gould *et al*., 1985; Westaway & Goodman, 1987) and in infected culture supernatants as a secreted species (Winkler *et al*., 1989; Mason 1989; Despres *et al*., 1991). NS1 exists as both a monomer (46 kDa) and a dimer (86 kDa) in flavivirus infected mammalian cells (Winkler *et al*., 1988; Chambers *et al*., 1989; Mason, 1989). Dimerization of NS1 occurs in the ER prior to transport to the Golgi where processing of some of the N-linked glycans occurs (Winkler *et al*., 1989, Despres *et al*., 1991). The conversion of monomers to dimers has been related to the membrane-association of NS1. It appears that the NS1 protein exists temporarily in a hydrophilic form before becoming associated with the membrane.

In addition to the envelope (E) protein and prM, NS1 has been associated with antibody-mediated protective immunity. NS1 exists in cell associated (Westaway, 1987), cell surface (Schlesinger *et al,* 1985) and extracellular forms (non-virion associated) (Mason, 1989; Smith & Wright, 1985; Winkler *et al*., 1988) as mentioned earlier and does not form part of the virion. Consequently, antibodies raised against NS1 have no neutralizing or haemagglutination inhibition activity, although some are able to fix complement (Schlesinger *et al*., 1985; Smith & Wright, 1985; Gibson *et al*., 1988; Falconar & Young, 1991). Schlesinger *et al*., (1985) demonstrated that anti-NSI MAbs

afforded passive protection to adult mice against lethal intra-cerebral (IC) challenge with the YFV 17D-204 strain. Protection was most likely mediated by antibody binding to cell surface expressed NS1 and complement mediated lysis of infected cells. It was also shown that active immunization with NS1 led to solid protection against lethal IC challenge with YFV 17D-204 strain (Schlesinger *et al*., 1985). NS1 immunization was also demonstrated to protect rhesus monkeys (the natural host of YF) from challenge.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sampling sites

3.1.1 Sites in Trans Nzoia County

Human blood samples were collected from Kitale District Hospital, Andersen Medical Centre and Endebess Sub District Hospital. These facilities serve a large part of Trans Nzoia county. The surrounding areas are heavily forested. The slopes of the mountain nearby have several caves, infested with bats, monkeys and rodents which form hosts.

3.1.2. Alupe site

Human blood samples were also collected from Alupe Sub District Hospital, located in Busia county, 8 Km north of the border town of Busia. This facility is located in the same compound as the Centre for Infectious and Parasitic Diseases Control and Research, (CIPDCR) of the Kenya Medical Research Institute, (KEMRI). The surrounding countryside is bushy woodland and infested with mosquitoes and tsetse flies and to the south of Alupe is the Budalangi Flood Plain.

3.2 Study population

The population served in the Trans Nzoia sites include Sabaot, Luhya, Pokot and Kalenjin ethnic groups, while that at Aulpe site is mainly Luhya and Teso ethnic groups,

as well as migrants from Uganda. It is important to note that in both study areas human migration, which predisposes to disease transmission is rampant.

3.3 Recruitment into the study

During the present study period, patients presenting with fever at the study sites were seen by the resident clinicians in the study sites. Upon establishment of high fever and of short duration the clinician explained in detail to the patient about the YFV study. Consent was obtained from the patient by the clinician and as part of the laboratory request the clinician indicated that 5 ml of blood be drawn from the patient for the YFV study. Since the clinician has medical records to the patient, follow up and immediate response to avert an outbreak would have been undertaken by the hospital administration or relevant authorities such as Ministry of Health.

3.4 Inclusion criteria

1. Blood samples were taken from children aged 5 years and above and adults: Assent was obtained from the parents/guardians of children aged 5-17 years and consent from adults aged 18 years and above.

2. Fever including haemorrhagic fever and of short duration with temperatures greater than 38.5 °C.

3. Those whose laboratory request form seeks a Malaria or typhoid test were included in the study too.

3.5 Exclusion criteria

1. Unable or unwilling to give consent

2. Aged below 5 years

3. Not febrile

Outpatients presenting to the laboratory (with fever and either headache, rash, jaundice or haemorrhage) for tests would receive an explanation of the study. They were requested to give informed consent by the phlebotomist, and if they so agreed, they got the benefit of testing for arboviruses in addition to the specific tests that their clinician had requested. All study subjects were free to leave the project at will without any form of victimisation.

3.6 Sample size

The study design being experimental in a hospital-based setting, the sample size was calculated based on the formula (Cochran, 1977).

$$N = \frac{Z^2P(1-P)}{d^2}$$

Where,

N- Minimum sample size required

Z- 1.96 (Standard error)

P- Expected prevalence (assuming a 50% prevalence)

d- 0.05 (The inverse of 95% confidence limit - the allowable error)

The minimum sample size required to determine prevalence of arbovirus exposure is:

$1.96^2 \times 0.5(1-0.5)/ 0.05^2 = 384$ blood samples, this is rounded to 400 samples.

3.7 Sample collection, storage, transportation and processing

Five millilitres of human whole blood was collected by venipuncture in vacutainer (BD, New Jersey, USA). The tubes were transported on ice to the laboratory where they were processed as soon as possible after collection. The total time taken to transport samples from the field to the lab did not exceed 48 h. Once in the lab, the whole blood samples were centrifuged at 600 × *g* for 10 min to clarify the serum formed. A total of 2 ml of serum (1 ml for virus isolation and detection; 1 ml for serology) was aliquoted into two separate 1.5 ml screw-cap cryotubes for storage at -80 ^{o}C.

3.8 Virus strains and cell cultures

The live attenuated vaccine strain 17D, which is a Bio-Safety Level 2 containment pathogen in Kenya, was propagated in Vero cells (African green monkey kidney cell line) maintained at 37 $^{\circ}$C in Eagle's minimum essential medium (EMEM) (Life Technologies, New York, USA) supplemented with 2% fetal calf serum (FCS) (Life Technologies, New York, USA) and 0.2 mM nonessential amino acids (NEAA) (Life Technologies, New York, USA) and harvested after 7 days. In addition, YFV (strain:

Baringo 1), YFV (strain: Baringo 2), dengue virus serotype 1 (DENV1) (strain: Hawaii), dengue virus serotype 2 (DENV2) (strain: 00St-22A), dengue virus serotype 3 (DENV3) (strain: SLMC50), dengue virus serotype 4 (DENV4) (strain: SLMC318), Japanese encephalitis virus (JEV) (strain: ML-17), chikungunya virus (CHIKV) (strain: S27), Rift Valley fever virus (RVFV) (strain: Smithburn), and West Nile virus (WNV) (strain: Eg101) viruses were propagated by regular sub-culturing in either Vero cells or *Ae. albopiticus* clone C6/36 cells (Igarashi, 1978). A confluent monolayer of Vero or C6/36 cells was inoculated with the respective virus and then incubated at 37 °C or 28 °C, respectively. Cells were observed daily for cytopathic effect (CPE); at 80% or more CPE, the infected culture fluid (ICF) was harvested by centrifugation at 600 × *g* for 10 min at room temperature.

3.9 Virus titration

The propagated viruses were titrated using a modified plaque assay to establish the virus titer (De Madrid & Porterfield, 1969). Briefly, Vero cells were seeded in a 6-well plate (IWAKI, Tokyo, Japan) and cultured until a monolayer of cells was observed. Tenfold serial dilutions of respective virus (1×10^1 to 1×10^7) were prepared in EMEM containing 2% FCS and inoculated to each corresponding well in duplicate. After 1.5-2.0 h incubation for virus absorption, overlay medium, EMEM containing 2% FCS and 1.25% (w/v) methylcellulose 4000 (Wako, Osaka, Japan) was added. The plates were placed in a 5% CO_2 incubator at 37 °C for three to six days. Cells were then fixed with 5% formaldehyde solution for 2 h to overnight. The cells were stained using 0.5% (w/v)

Crystal violet (Wako, Osaka, Japan) and the plaque forming units per ml (PFU/ml) derived.

3.10 Preparation of purified virus

Purified virus was prepared by sucrose density gradient ultracentrifugation (described below) with some modifications to suit the situation on the ground at KEMRI laboratories. The YFV17D vaccine strain and other viruses were propagated in mass culture of Vero cells, either using Cytodex beads (GE Healthcare, Uppsala, Sweden) in spinner flasks (Corning, New York, USA) or in tissue culture flasks (NUNC, Roskilde, Denmark). Every second day the spinner flasks were inspected for fungal and bacterial infections.

3.11 Harvest of ICF and concentration

After culturing the cells on a magnetic stirrer (As one, Osaka, Japan) (speed: 20 rpm) at 37 $^{\circ}$C for 7-10 days, the stirrer was stopped to allow the beads to settle down in the spinner flasks. While working in a level II Biosafety cabinet, the ICF was filtered using a large funnel containing a Whatman 1 filter paper (Whatman, Maidstone, England) into a 500 ml Beckman centrifuge tube (Beckman, California, USA) to remove dead cells and the cytodex beads from the ICF. The tubes were then centrifuged at 7,741 $\times$ *g* for 30 min at 4 $^{\circ}$C in an Avanti J-26 XP high-speed centrifuge (Beckman, California, USA). The supernatant was pooled into a 3 litre erlenmeyer flask with a magnetic stirrer bar. Into the flask, 22.2 g of NaCl (Wako, Osaka, Japan) and 60g of Polyethelyene Glycol

(PEG) 6000 (Wako, Osaka, Japan) per 1L of ICF were added and the ICF stirred very slowly at 35 × *g* at 4 °C for one overnight. The next morning, the ICF was aliquoted into 6 × 500 ml Beckman centrifuge tubes and centrifuged at 7,741 × g for 30 min at 4 °C in an Avanti J-26 XP high-speed centrifuge. The supernatant was discarded and 18ml of Sodium Chloride-Tris-EDTA (STE) buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH7.4) was used to re-suspend the faint precipitate on the surface of the centrifuge tubes. The pooled resuspended virus precipitate was then centrifuged at 12,096 × *g* for 20 min at 4 °C in an Avanti J-26 XP high-speed centrifuge. The supernatant was harvested for the sucrose density gradient ultracentrifucation step while the precipitate was resuspended with another 18 ml of STE buffer.

3.12 Sucrose Density Gradient Ultracentrifugation

Sucrose of different concentrations in STE buffer was autoclaved and a step gradient was prepared in a centrifuge tube (UltraClear tubes, Beckman No. 344058) **(Table 3.2)**:

Table 3.2 Different concentrations of sucrose in STE buffer

[Sucrose]	50%	45%	40%	35%	30%	15%	Sample	Total
volume	4ml	3ml	3ml	3ml	3ml	4ml	18ml	38ml

The tube was centrifuged at 48,384 × g for 14 hrs at 4 °C in a Beckman Coulter Optima 80 XP (Beckman, California, USA) ultracentrifuge using a SW32 Ti rotor. After 14 h of ultracentrifugation, centrifuge tubes containing the sucrose gradient were retrieved.

Most of the virus was contained in the visible bands and was retrieved using a density gradient fractionater (Instrument Specialties Co. Nebraska, USA). All the fractions taken from the start of the peak absorbance to the end were pooled together. The concentration of the virus was measured using an ultraviolet spectrophotometer at 280 nm and 260 nm using the below formula.

$$\textit{Protein concentration (mg/ml)} = (1.45\ OD_{280} - 0.74\ OD_{260}) \times \textit{Dilution factor}$$

The purified virus was aliquoted and frozen at -80 ºC in cryovials until use. When necessary, the purified virus was thawed then diluted with $PBS^{(-)}$ to the desired level of concentration for use.

3.13 Development and optimization of an in-house indirect IgG ELISA, antigen detection ELISA and IgM-capture ELISA system using anti-YFV monoclonal antibody (2D12).

In a bid to better equip Kenya to respond to the burden of YFV, the present study established in-house indirect IgG ELISA, antigen capture ELISA and IgM-capture ELISA with slight modifications from Chanyasanha *et al*., 1999.

3.13.1 Patients and panel of control sera

A total of 469 blood samples were collected from febrile patients visiting the selected Western Kenya hospitals and who either consented or assented to participate in the study. Sample collection was only done after approval, SSC protocol # 1829, was

received from the KEMRI Scientific Steering Committee (SSC) (**Appendix I**) and Ethics Review Committee (ERC) (**Appendix II & III**). The KEMRI-Arbovirus laboratory, National reference laboratory for YF diagnosis, kindly provided six IgM positive sera for YFV that were used as positive controls for the in-house IgM ELISA establishment (*courtesy of Dr. Rosemary Sang*). Nagasaki University's Institute of Tropical Medicine kindly availed a strong positive serum sample that was used to generate the standard curves for this work and served as the flavivirus IgG positive control. The negative controls used in the present study were generated from healthy volunteers who had not received YFV vaccine or pre-exposed to YFV field infection.

3.13.2 IgG indirect ELISA

Ninety-six well flat-bottomed microtitre plates (Maxisorp, Nalge Nunc International, Roskilde, Denmark) were coated with purified antigen (250 ng/100 µl/ well) diluted using coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) at 37 °C for 1 h or at 4 °C overnight. A hundred microlitres of Blockace (Yukijirushi, Sapporo, Japan) were added to each well except the blank wells and the plate incubated at room temperature for 1 h. The microtitre plates were then washed three times using PBS containing 0.05% Tween 20 (PBS-T). Control and patient sample serum, 100µl of 1:1,000 dilution in 10 % Blockace in PBS-T, was added to each well in duplicate and incubated at 37 °C for 1 h. This allowed the antibody to react with the antigen-coated wells. The plates were washed as described above.

A hundred microlitre of 1:5,000 diluted horseradish peroxidise (HRPO)-conjugated goat anti-human IgG (American Qualex, California, USA) was added to each well and the plates incubated at 37 °C for 1 h. The plates were then washed as described above. A hundred µl of substrate (5 mg *o*-phenylenediamine dihydrochloride (OPD) (Sigma Chemical, St. Louis, USA) and 0.03 % hydrogen peroxide in 10 ml of 0.05 M citrate-phosphate buffer at pH 5.0) was added to each well and subsequent incubation in the dark at room temperature for 20-30 min. The reaction was stopped by adding 100 µl of 1N H_2SO_4 to each well. The OD at 492 nm for each well was measured using Multiscan EX reader (Thermo Scientific, Massachusetts, USA) using Ascent Software Version 2.6 (Thermo Scientific). Each sample was tested in duplicate, the mean OD for each sample measured and IgG titres calculated using a standard curve.

3.13.3 Antigen detection ELISA

A simple *in vitro* YFV titration protocol was established with slight modification from Monath and Nystrom's 1984 protocol using the YFV specific monoclonal antibody 2D12. Ninety six well microtitre plate was coated with 100 µl (2.0 µg/ml) of anti-flavivirus human IgG diluted in ELISA coating buffer in all wells except for the blank, and the coated plate incubated at 37 °C for 1 h or at 4 °C overnight. All wells except for the blank wells were blocked with 100 µl of the original concentration of Blockace and incubated at room temperature for 1 h. Plate was washed three times with phosphate buffered saline containing 0.05% Tween 20 (PBS-T). Duplicates of 100 µl, positive standard (6.25, 12.5, 25, 50, 100, and 400 ELISA units and unknown sample infected

culture fluids were added to the microtitre plate, then incubated at 37 °C for 1 h and the plate was washed as described above. One hundred microliters of 200 × diluted anti-YFV monoclonal antibody (2D12)(American Type Culture Collection (ATCC) CRL-1689) (ATCC, Virginia, USA) in-house conjugated to horseradish peroxidase-conjugated (HRPO) was added to each well except from the blank wells and the plate incubated at 37 °C for 1 h. Plate was washed as described above. Colour was developed after washing the plate and adding 100 µl of substrate: 5 mg *o*-phenylenediamine dihydrochloride (OPD) and 0.03 % hydrogen peroxide in 10 ml of 0.05M citrate-phosphate buffer at pH 5.0, to each well and the subsequent incubation in the dark at room temperature for 20-30 min. The reaction was stopped by adding 100 µl of 1N H_2SO_4 to each well. OD at 492 nm for each well was measured using Multiscan EX reader using Ascent Software Version 2.6. The generated OD_{492} values were used to draw a semi log standard curve and the ELISA units of each sample calculated from this.

3.13.4 IgM capture ELISA

Ninety six well microtitre plate was coated with 100 µl (5.5 µg/100 µl) of goat anti-human IgM (µ-chain specific), (Cappel ICN pharmaceuticals, Aurora, USA) diluted in ELISA coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6, containing 0.02% sodium azide) in all wells except for the blank, and the coated plates incubated at 37 °C for 1 h or at 4 °C overnight. All wells except for the blank wells were blocked with 100 µl of original concentration Blockace and incubated at room temperature (r.t.) for 1 h. Plate was washed three times with phosphate buffered saline containing 0.05% Tween

20 (PBS-T). Duplicates of 100 μl, positive control, negative control and patient serum sample diluted 1:100 using PBS-T were added to the microtitre plate, incubated at 37 °C for 1 h and the plate washed as described above. The purified YFV17D antigen (100-ELISA units), diluted in PBS-T, was then added to each well except for the blank well as an assay antigen and the plate incubated at 37 °C for 1 h. Plate was washed as described above. One hundred microliters of 1:400 dilution of anti-YFV monoclonal antibody (2D12) were added as a primary antibody and the plate incubated at 37 °C for 1 h. Plate was washed as described above.

As a conjugate, 100 μl of 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (American Qualex, California, USA) were added and the plate incubated at 37 °C for 1 h. Colour was developed after washing the plate and adding 100 μl of substrate: 5 mg *o*-phenylenediamine dihydrochloride (OPD) and 0.03 % hydrogen peroxide in 10 ml of 0.05M citrate-phosphate buffer at pH 5.0, to each well and the subsequent incubation in the dark at r.t. for 20-30 min. The reaction was stopped by adding 100 μl of 1N H_2SO_4 to each well. OD at 492 nm for each well was measured using Multiscan EX reader using Ascent Software Version 2.6. A P/N (positive control (or sample) OD_{492}/negative control OD_{492}) ratio ≥2.0 was considered positive.

3.14 Monoclonal antibody 2D12 propagation, purification and conjugation

The anti YFV MAb 2D12 (ATCC CRL 1689, Virginia, USA) producing hybridoma was donated by Nagasaki University Institute of Tropical Medicine (NEKKEN), Nagasaki,

Japan. The hybridoma 2D12 was propagated initially in complete RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 10% FCS, 0.02 mM NEAA, 0.29 mM L-glutamine, 100 U penicillin, 0.01 mM streptomycin (PS) and sodium bicarbonate. Thereafter, the hybridoma cells were transferred into Hybridoma SFM media (Life Technologies, New York, USA) supplemented with 0.02 mM NEAA, L-glutamine, PS and 7.5% sodium bicarbonate to allow for monoclonal antibody secretion. Stationary cultures using 75 cm^2 tissue culture flasks (Nunc International, Roskilde, Denmark) were performed until 400 ml of culture fluid was attained. A protein G affinity chromatography purification of the 2D12 monoclonal antibody was done using MAbTrap kit (GE Healthcare, Uppsala, Sweden) from the cell culture supernatant. Briefly, culture fluid was thawed, centrifuged at 1,673 × *g* for 5 min at 4 ºC using an Avanti J-26 XP high-speed centrifuge and filtered using a 0.45µm filter (Whatman, Mildestone, England). Double distilled water was then added to the provided column to remove the 20% ethanol used as a storage buffer. A TRIS™ peristaltic pump (Teledyne ISCO, St. Lincoln, USA) was used to apply, (1) the provided 5ml of binding buffer solution, (2) ≈ 400ml of filtrated culture fluid at a flow rate of 2 drops/second, (3) 5ml of binding buffer to equilibrate the column and (4) 5ml of elution buffer. The monoclonal antibody 2D12 was collected in fractions of 1ml/tube that contained 75µl/tube of provided neutralizing buffer. The protein concentration of each fraction was determined using a GeneQuant Pro spectrophotometer (Amersham, Buckinghamshire, UK) and using the following formula:

$$Protein\ concentration\ (mg/ml) = (1.45\ OD_{280} - 0.74\ OD_{260}) \times Dilution\ factor$$

A curve was then drawn and the peak fractions that had a protein concentration above 1.0mg/ml were mixed, aliquoted into the cryogenic tubes and stored at -80 ℃.

An in-house protocol was followed to conjugate the monoclonal antibody 2D12. The HRPO (Sigma, St. Louis, USA) was activated by dissolving 4 mg in 1 ml of double distilled water. The dissolved HRPO was mixed with 200µl of freshly prepared 0.1M Sodium Periodate ($NaIO_4$) (Wako, Osaka, Japan). A rotator (Stuart, Staffordshire, UK) was used to mix the HRPO at room temperature for 2 h hence turning the colour from brown to green. Dialysis using a 10000 MW Slide-A-Lyzer dialysis cassette G2 (Thermo scientific, Rockford, USA) was then performed on the mixture versus 100 volumes of 1mM sodium acetate buffer, pH 4.4, at 4 ℃ for one overnight. To another dialysis cassette, 8 mg of monoclonal antibody 2D12 anti-YFV IgG was added and dialysed versus 10mM sodium carbonate buffer, pH 9.5, at 4 ℃ for one overnight. 20µl of 0.2M sodium carbonate buffer (pH 9.5) was added to the dialysed HRPO and immediately mixed with the dialysed anti-YFV IgG. The mixture was rotated for 2 h at room temperature while wrapped with an aluminium foil. One hundred microlitres of freshly prepared sodium tetrahydroborate ($NaBH_4$) (Wako, Osaka, Japan) was then added to the mixture and further rotated for 2 h at room temperature. A Slide-A-Lyzer

dialysis cassette G2 was used to dialyse the mixture versus $PBS^{(-)}$ at 4 ºC for one overnight.

Ammonium sulphate precipitation was performed on the HRPO conjugated monoclonal antibody, 2D12 to remove the un-conjugated HRPO. A 0.5 volume of saturated ammonium sulphate was added to the dialysed conjugated IgG and kept at r.t. for 15 min. The mixture was then centrifuged at 4,355 × *g* at 4 ºC for 15 min. The supernatant was discarded while the precipitate was re-suspended in the same original volume of $PBS^{(-)}$. There was then a repetition of saturated ammonium sulphate addition and centrifugation steps above. The HRPO conjugated 2D12 in $PBS^{(-)}$ was then dialysed versus $PBS^{(-)}$ at 4 ºC for one overnight. An equal volume of Pierce superfreeze peroxidise conjugate stabilizer and 40 mg of BSA (Sigma, St. Louis, USA) were added before dispensing into small aliquots the HRPO conjugated monoclonal antibody 2D12 and stored at -80 ºC.

3.15 Focus Reduction Virus Neutralization Test

A fifty percent focus reduction neutralization test ($FRNT_{50}$) was used to determine the presence of YFV specific neutralizing antibodies in test sera already regarded as positive by the IgM capture ELISA and/or IgG indirect ELISA. Briefly, Vero cells at a concentration 200,000 cells/ml were seeded into 96-well plates (Nunc) at a volume of 100 µl/well and cultured in growth medium (EMEM, 10% FCS, 0.02 mM NEAA, L-glutamine, PS supplemented) at 37 ºC for 1 day. Sera was diluted using maintenance

medium (EMEM, 2% FCS, 0.02 mM NEAA, L-glutamine, PS supplemented) in 1:10 then heat inactivated at 56 ºC for 30 min in a water-bath. The heat inactivated sera were then serially diluted using maintenance medium from 1:20 through to 1:1280. The serum was mixed with an equal volume of standard virus solution (100 FFU/0.1ml) and the virus-serum mixture incubated in a 5% CO_2 incubator at 37 ºC for 1 h.

One hundred microliters of virus-serum mixture was inoculated to Vero cells in triplicate wells, and allowed to adsorb at 37 ºC for 2 h in a CO_2 incubator. One hundred and fifty microliters of overlay medium (EMEM, 1.5 % FCS, 1.25 % Methylcellulose 4000, 0.02 mM NEAA, L-glutamine, PS supplemented) were added to each well, and the plates incubated at 37 ºC for 2-3 days. The plates were washed thrice with $PBS^{(-)}$ to remove methylcellulose, fixed with 5% formaldehyde (Wako, Osaka, Japan) in $PBS^{(-)}$for 30 min at r.t., rinsed and permeabilized with 200µl of 1% NP-40 solution in $PBS^{(-)}$ per well for 30 min at r.t. Three times washes of the plate with $PBS^{(-)}$ were followed by blocking using original concentration of Blockace for 30 min at r.t.. Focus immunostaining was performed after blocking. One hundred microliters of YFV specific monoclonal antibody 2D12 (diluted 1:200) was added for 1 h at 37 ºC, followed by a three times wash in $PBS^{(-)}$. Subsequently, 1:500 diluted HRPO conjugated goat anti-mouse IgG (American Qualex) was added to the plates at 100µl/well followed by incubation at 37 ºC for 1 h. The staining was visualized by the addition at 100µl/well of a 0.5 mg/ml solution of substrate 3,3'-diaminobenzidine tetrahydrochloride (DAB)

(Wako, Osaka, Japan) in $PBS^{(-)}$ with 0.03% of H_2O_2 added at room temperature for 30 min, and the staining reaction was allowed to proceed. After washing the stained cells with distilled water and air drying the plates, the number of foci per well were counted using a stereomicroscope microscope. The reciprocal of the endpoint serum dilution that provided a 50%, greater, reduction in the mean number of foci relative to control wells that contained no serum was considered to be the $FRNT_{50}$ titer.

3.16 Design and optimization of a Reverse Transcription Loop-Mediated Isothermal Amplification Assay (RT-LAMP) for yellow fever virus

3.16.1 RT-LAMP primer design

The RT-LAMP primers were designed from the non-structural protein 1 (NS1) region of the YFV strain: 17D (GenBank ID: P03314; Rice *et al*., 1985). Alignment of the NS1 nucleotide sequence with the available GenBank sequences of 20 YFV strains was performed using CLCBio MAINWORKBENCH Software V 6.01 (CLCBio, Aarhus, Denmark) to identify the conserved regions. Six primers were designed and comprised of the following: forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP), backward inner primer (BIP), forward loop primer (FLP), and backward loop primer (BLP) following a described criteria (Notomi *et al*., 2000). The primers were designed using Eiken's LAMP software Primer Explorer V4 (Eiken Chemical Co. Ltd., Tokyo, Japan).

3.16.2 RNA extraction

Genomic viral RNA was extracted from 140 µl of the ICF of Vero cells using a QIAmp Viral RNA Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. The extracted RNA was eluted in a total volume of 60 µl and stored at -80 °C for RT-PCR, RT-LAMP and real-time RT-PCR.

3.16.3 RT-PCR

The RT-PCR was performed using a Titan One Tube RT-PCR kit (Roche Diagnostics, Germany). The RT-PCR amplification was carried out in a 25 μl reaction mix containing 5 μl template RNA. The YFV specific primers used were: sense YFVP1 (TAC CCT GGA GCA AGA CAA GT) (nucleotide number: 1042-1061) (Eldadah *et al*., 1991) and antisense YFVC10 (AGC AAG AAA CAT GTC CAC CA) (nucleotide number: 1823-1804) targeting a 782 bp fragment of the envelope region (Tanaka, 1993). The RT-PCR reaction profile included a reverse transcription step at 37 °C for 1 h, with the following cycling times and temperatures: one cycle at 94 °C for 3 min; 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and, one cycle at 72 °C for 10 min as a final extension step. Ten microliters of the RT-PCR amplicons were subjected to electrophoresis in a 2% agarose gel (SeaKem GTG, Lonza, USA) in Tris-Acetate-EDTA buffer and stained using 0.5 μg/ml ethidium bromide for visualization under UV light at a wavelength of 302 nm.

3.16.4 RT-LAMP

The RT-LAMP was carried out in a total reaction volume of 25 μl using a Loopamp RNA amplification kit (Eiken Chemical Co., Ltd. Tokyo, Japan) with 40 pmol of inner primers FIP and BIP, 20 pmol of loop primers LF and LB, and 5 pmol of outer primers F3 and B3. Five microliters of eluted viral RNA was used as the template per reaction. Real-time monitoring of the RT-LAMP was done using a LA-320 Loopamp Real-time Turbidimeter (Teramecs, Kyoto, Japan) by incubation at 62 °C for 60 min and

inactivation at the end of the reaction at 80 °C for 5 min. The generated data were collected using LA-320 software and then exported to Microsoft Office Excel 2010 (Microsoft, Washington, USA). The turbidity at OD 400 nm was plotted against time to positivity while the threshold was set at 0.1.

3.16.5 Real-time RT-PCR

The limit of detection of the developed RT-LAMP was compared with a real-time RT-PCR that targeted the 3′ untranslated region (Bae *et al.*, 2003) **(Table 3.3)**. Briefly, a one-step, real-time RT-PCR was performed using a SuperScript III Platinum One-Step kit (Life technologies, New York, USA) and an ABI 7500 real-time PCR machine (Life technologies, New York, USA). RNA was reverse transcribed at 50 °C for 30 min and initial denaturation was performed at 95 °C for 2 min followed by 40 cycles: 95 °C for 15 sec, 60 °C for 45 sec. Data generated by the real-time RT-PCR were collected using Sequence Detection Software (Life technologies, New York, USA).

Table 3.2: RT-PCR primers used for the amplification of YFV

Primer name	Sequence (5′-3′)
3' UTR sense	AACCCACACATGCAGGACAA
3' UTR antisense	GTTGCAGGTCAGCATCCACA
3' UTR TaqMan	**F-CC**ATTTAGTCATCCATCGTATCCGAACGC **T**

(Bae *et al.*, 2003)

3.16.6 Feasibility of RT-LAMP

Consenting human serum donors who had no YFV vaccination history as confirmed by screening using an indirect IgG ELISA were used. Eight of these samples were YFV 17D ICF spiked in tenfold serial dilutions ranging from 2,900 PFU/ml to 0.0029

PFU/ml. Two human serum samples were spiked with EMEM containing 2% FCS and used as a negative control. Two YFV strains obtained from the 1992-1995 outbreak in Kenya (Reiter *et al*., 1998) were also used for validation of the assay. Briefly, virus titration using a plaque assay was performed and the PFU/ml generated. The two strains were then equilibrated to 2,900 PFU/ml like the virus titer of YFV strain: 17D used in the study. The equilibrated Kenyan YFV strains were then serially diluted tenfold ranging from 2,900 PFU/ml to 0.0029 PFU/ml. RNA was extracted from the diluted ICF and then RT-PCR and RT-LAMP were performed simultaneously for the detection of viral RNA, as described above.

3.17 Development of non structural (NS1) recombinant antigen for yellow fever virus (YFV)

3.17.1 NS1 primer design

The RT-PCR primers were designed from the non-structural protein 1 (NS1) region of the YFV strain: 17D (GenBank ID: P03314; Rice *et al*., 1985) as was done for the YFV RT-LAMP primer design. Alignment of the NS1 nucleotide sequence with the available GenBank sequences of 20 YFV strains was performed using CLCBio MAINWORKBENCH Software V 6.01 (CLCBio, Aarhus, Denmark) to identify the conserved regions. The NS1 primers were also designed using same software.

3.17.2 RNA extraction and NS1 RT-PCR

YFV RNA extraction was performed as detailed in 3.15.2. The RT-PCR was performed using a Titan One Tube RT-PCR kit (Roche Diagnostics, Germany). The YFV NS1 RT-PCR amplification was carried out in a 25 µl reaction mix containing 5 µl template RNA. The YFV specific primers used were: sense YFVNS1F (5'-CAG TGG ATC CGG ATG GGA AGT CAT GAA GT-3') (nucleotide number: 3054-3072) and antisense YFVNS1R (5'-CTG AGT CGA CCT AGG GAT ACC AAC ACC CAT C-3') (nucleotide number: 3448-3431) targeting a 394 bp fragment of the NS1 region with BamH1 and Sal1 restriction enzyme sites underlined respectively. The two restriction enzymes also occur in the pET 28a(+), 5.4 kbp (Agilent technologies, Texas, USA) vector at the multiple cloning site as shown in appendix VIII. Notable, pET 28a carries an N-terminal His-tag and kanamycin coding sequence which confers kanamycin resistance. The RT-PCR reaction profile included a reverse transcription step at 37 °C for 1 h, with the following cycling times and temperatures: one cycle at 95 °C for 4 min; 35 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min; and, one cycle at 72 °C for 10 min as a final extension step. Ten microliters of the RT-PCR amplicons were subjected to electrophoresis in a 1% agarose gel (SeaKem GTG, Lonza, USA) in modified Tris-Acetate-EDTA with 0.1 mM Tris buffer and stained using 0.5 µg/ml ethidium bromide for visualization under UV light at a wavelength of 302 nm. The gel bands were excised using a sharp razor and purified using a QIAEXII gel extraction protocol (Qiagen, USA). The purified PCR products were then double digested using

BamH1 (TAKARA, Japan) and Sal1 (TAKARA, Japan). This was followed by PCR product purification using QIAquick PCR purification kit (Qiagen, USA) following manufacturer's instructions to stop the enzymatic reaction.

3.17.3 DNA ligation and transformation

DNA ligation was performed using DNA ligation kit ver. 2.1 (Takara Bio Inc., Shiga, Japan) that used T4 DNA ligase and proprietary optimized buffers. The NS1 purified fragment and vector DNA, pET 28a, were used in a ratio of insert:vector 9:1 respectively. The mixed DNA was further mixed with the ligation mighty mix in 1:1 ratio. The reaction mix was incubated at 16 °C for one overnight. Higher temperatures (<26 °C) inhibit the formation of circular DNA. The recombinant plasmid inserts were confirmed to be in frame by DNA sequencing. Transformation involved the use of 100 µl of thawed BL21-CodonPlus(DE3)-RIPL competent cells (Agilent Technologies, Texas, USA) and 1.7 µl of β-mercaptoethanol (Wako, Osaka, Japan). The contents were swirled intermittently and incubated on ice for 10 min followed by the addition of 10 µl of the ligation mix to the tube. The mix was incubated in ice for 30 min before performing heat-pulse in a 42 °C water bath for 45 sec. The tube was then incubated on ice for 2 min. Preheated SOC medium, 0.9 ml, was added to the tube and incubated at 37 °C for 1 h with shaking at 225 rpm in an INNOVA-40 shaking incubator (New Brunswick, Connecticut, USA). The turbid mixture was then centrifuged at 3900 x g for 10 min using an Eppendorf 5417R (Eppendorf, Hamburg, Germany) and the pellet suspended in 100 µl of SOC medium (Sigma, St. Louis, USA). Streaking on an LB-

Agar plate containing kanamycin antibiotic (50μg/ml) (Wako, Osaka, Japan) of 100 μl *E. coli* containing the YFV NS1 plasmid was then performed and incubated at 37 °C incubator for one overnight for colony formation.

Next morning, a single colony was pricked using a sterile toothpick and aseptically transferred to a 14 ml round bottomed tube containing 3 ml of LB-broth (Life Technologies, New York, USA) containing kanamycin antibiotic (50 μg/ml). This was repeated for several clones and after overnight shaking, plasmid DNA was prepared by extracting it from the *E.coli*. PCR was perfomed using the YFV NS1 designed primers to confirm presence of insert in the plasmid. Only clones that had a PCR positive result were used for starter culture using a 14 ml round bottomed tube containing LB-broth. The tube was shaken at 37 °C for 6 h as a pre-culture. In a baffled 1 liter conical flask containing 250 ml of LB-broth with kanamycin antibiotic (50 μg/ml) the turbid pre-culture was added and the mix shaken at 37 °C of 3 h in an INNOVA-40 shaking incubator. Once the turbidity had reached 0.6 at ODλ600, 1 ml of the culture was collected and stored as total extract. To induce protein expression 250 μl of 1M isopropyl-β-D-thiogalactopyranoside (IPTG) (Wako, Osaka, Japan) was added to the 250 ml of culture and shaken at 37 °C for 3 h. Similar to the 1 ml collected prior to protein expression, a similar amount was collected after IPTG induction. The 250 ml *E. coli* culture was then centrifuged at 10,000 x g for 10 min at 4 °C in an Avanti J26 large volume centrifuge (Beckman Coulter, California, USA). The supernantant was

discarded and the pellet stored at -80°C overnight.

The frozen pellet was resuspended in 20 ml of 1 x Bugbuster (Millipore, Massachusetts, USA) containing 4 µl Benzonase nuclease (Millipore, Massachusetts, USA) and 2 µl recombinant Lysozyme (Millipore, Massachusetts, USA) with vortexing to make a homogenous solution. The mixture was rotated at half speed for 30 min at room temperature. Centrifugation was then performed to pellet the inclusion bodies while the soluble extract was retrieved but with storing 1 ml for SDS-PAGE. The pellet was then resusupended in 20 ml of 1 x BugBuster solution (Millipore, Massachusetts, USA) and vortexed well to remove any soluble and contaminating proteins in a bid to achieve high purity protein preparation. Recombinant Lysozyme solution was added to the mixture to a final concentration of 1 KU/ml, vortexed and incubated at r.t. for 5 min. An equal volume of 1:10 BugBuster was then added, vortexed and incubate at r.t. for 1 min. The suspension was then centrifuged at 5,000 x g for 15 min at 4 °C to collect the inclusion bodies. The pelleted inclusion bodies were resuspended in 1:10 diluted BugBuster and the step repeated for a total of three washes. After the final wash, the remaining inclusion bodies suspension was then centrifuged at 16,000 x g for 15 min at 4 °C.

The inclusion bodies pellet was then resuspended in 10 ml of 8M Urea pH 7.8 with 1 µl of 1M dithiothreitol. The suspension was rotated at 35 x g for 1 hr at r.t. to solubilize

the inclusion bodies. The suspension was then centrifuged at 24,000 x g for 30 min at 4 °C using an eppendorff table top centrifuge 5810R. The protein was in the supernatant due to denaturation. The resultant supernatant was then be applied to a Ni-NTA Agarose (Life Technologies, New York, USA) column, a highly selective immobilized metal ion affinity chromatography (IMAC) that has a ligand that is specific for polyhistidine-tagged recombinant proteins. Purification entailed washing the recombinant antigen in the Ni-NTA Agarose column with 10 mM PBS-500 mM NaCl containing 20 mM imidazole (Wako, Osaka, Japan). The purified recombinant antigen was eluted using 10 mM PBS (pH 7.5)-500 mM NaCl containing 250 mM imidazole. The purified recombinant antigen was aliquoted and stored in final concentrations of 10 % glycerol at -80 °C. The purity of the protein was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

3.17.4 Western Blot of the YFV NS1 recombinant antigen

The total extracts from the pre and post IPTG steps were centrifuged at 13,000 x g for 3 min at r.t. The pellet was then dissolved using LDS NuPAGE sample buffer (Life technologies, California, USA) and sonicated to break the genomic DNA. The sonicated mixture was then heat treated at 95 °C for 5 min and immediately placed on ice until loaded to the precast SDS-PAGE gel. The recombinant antigen was separated by electrophoresis using two pieces of 12 % polyacrylamide gel. The first gel was Coomassie Brilliant Blue (CBB) stained and immersed in staining solution containing 40% methanol, 10% acetic acid and 0.25% (v/v) CBB-R250 (Bio-Rad, Marnes-la-

Coquette, France) in water. The gel in the staining solution was gently shaken for 2 h. A de-staining solution containing 10% methanol, 7 % acetic acid in water was used to de-stain the gel until the bands became clear. Several washes of the gel using sterile distilled water were performed to remove methanol and acetic acid. The bands were then exposed in a LAS500 machine (GE Healthcare, Uppsala, Sweden) to get colorimetric image. The second gel was used for Western Blotting by electrotransfer using 1 x Tris-CAPS, 15 % (v/v) methanol in water to a polyvinylidene difluoride (PVDF) membrane (Millipore) using a semidry electroblotter (Bio-Rad, Marnes-la-Coquette, France). Blockace was then used to block the membrane overnight at 4 °C. The PVDF membrane was then placed in a sealable plastic bag and anti-his-tag HRP-DirecT (MBL) 1:5000 in 1x Tris buffered saline with Tween (TBST) (25mM Tris, pH 7.4, 3.0mM KCl, 140mM NaCl and 0.05% Tween 20) added followed by incubation at 37 °C for 1 h. The reaction will be visualized using ECL reagent (GE healthcare, Uppsala, Sweden) following the manufacturer's instruction. The PVDF membrane was then exposed in an ImageQuant LAS 500 chemiluminescence machine (GE healthcare, Uppsala, Sweden) using the IQ LAS 500 control software ver. 1.0.

3.18 Data management

The labels placed on the samples collected had Out Patient/In Patient Numbers, but no patient names. Laboratory workers were not able to identify the patients, but the clinician seeing the patients was able to link the result. Patient details included history of yellow fever virus vaccination, and also exact domicile for purposes of mapping

probable clusters of high arbovirus prevalence. These data was linked to the results of the laboratory tests we conduct and was stored in an MS Access 2007 and Spinet database and analysed using SPSS for Windows. All data was stored in a secure computer with a backup located in the CIPDCR Server.

CHAPTER FOUR

4.0 RESULTS

4.1 Development and optimization of an in-house indirect IgG ELISA, antigen detection ELISA and IgM-capture ELISA system using anti-YFV monoclonal antibody (2D12).

4.1.1Production of purified virus

Sucrose density gradient ultracentrifugation was used to prepare the YFV 17D purified virus at KEMRI-PD facility, Nairobi. Four batches were prepared to accumulate the required purified virus for indirect IgG ELISA **Figure 4.1**. The yields were 0.65 mg/ml, 0.80 mg/ml, 1.50 mg/ml and 1.15 mg/ml.

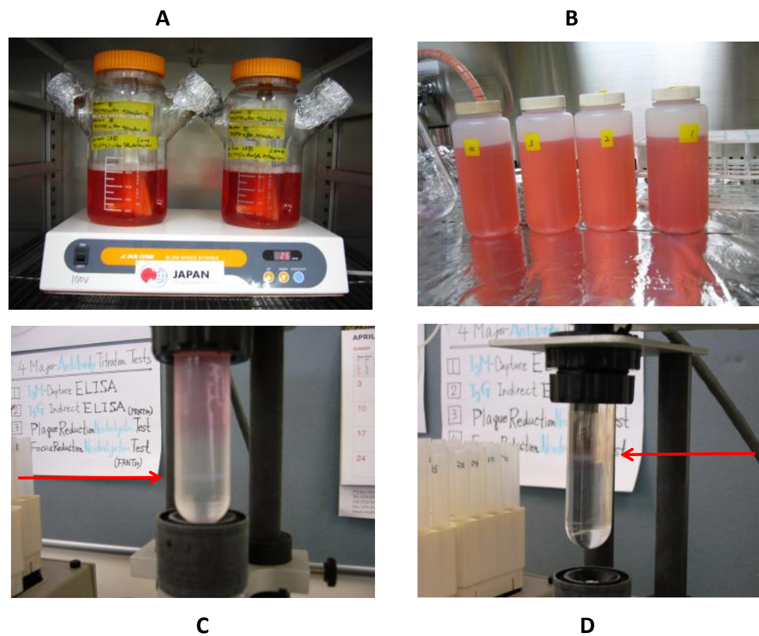


Figure 4.1: Production of purified virus.

(A) Two magnetic stirrer bottles culturing YFV 17D in a 37 °C incubator; (B) Four 500ml centrifuge tubes containing the YFV 17D infected culture fluid; (C) precipitate band after a sucrose gradient density sedimentation; (D) supernatant band after a sucrose gradient density sedimentation

4.1.2 Monoclonal antibody 2D12 propagation, purification and conjugation

The hybridoma was first cultured in complete RPMI 1640, transferred to Hybridoma SFM media and purification was performed via Protein G affinity chromatography using a commercial MAbTrap kit. Five batches were prepared yielding; 2.30 mg/ml, 1.96 mg/ml, 2.15 mg/ml, 2.08 mg/ml, 1.87 mg/ml and 2.24 mg/ml after spectrophotometry.

Figure 4.2 shows one batch purification using the commercial MAbTrap kit. Conjugation of HRP was attempted thrice using 4 mg/total 2D12 MAb per batch following an in-house protocol for use in YFV IgM capture ELISA yielding 1 ml in per batch in protein stabilizer.

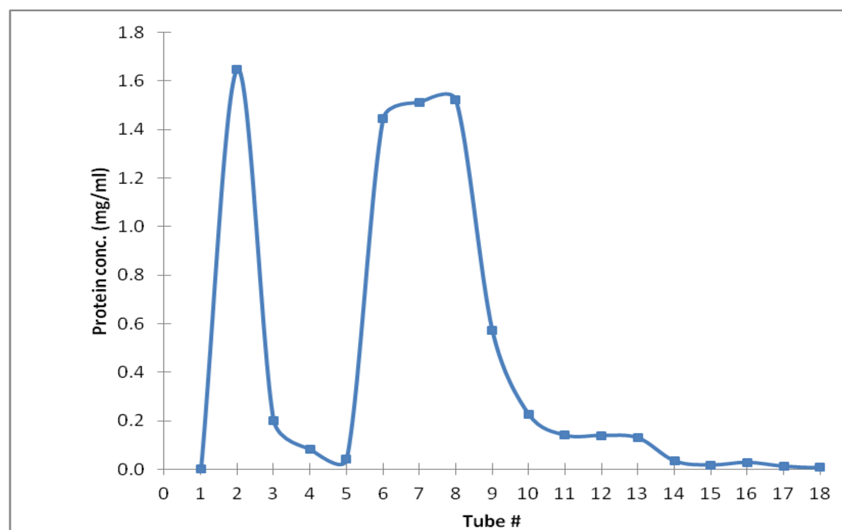


Figure 4.2: Monoclonal antibody 2D12 anti-YFV culture fluid fraction purification using MAbTrap kit

4.1.3 Sociodemographic charactersistics

A total of 469 human whole blood samples were collected from the following healthcare facilities in Kenya; Andersen Medical Centre (AMC), Endebess Sub District Hospital (END) and Kitale District Hospital (KDH) in Trans Nzoia County, Centre for Infectious and Parasitic Diseases Control Research, Kenya Medical Research Institute (KEMRI)

clinic (CIP) located in Busia County and Kapluk Dispensary (KAP) in Baringo County. The total samples included 57% female and 43% male in the present study. The highest study participants, 237 (51%), were from END, while the lowest study participants, 13 (3%), were from KAP. According to **Table 4.1** the largest age group was 16-30 years, 218 (47%), while the lowest age group was 1-15 years, 23 (5%). Most of the study participants (70%) had other forms of occupation other than farming (16%) and home staying (15%).

Table 4.1: Sociodemographic characteristics of study participants by facility

County	Trans Nzoia			Baringo	Busia		
Facility	AMC (n=94)	END (n=201)	KDH (n=125)	KAP (n=13)	CIP (n=36)	Total (n=469)	ANOVA
Sex							
Female	52(3)	109(2)	74(9)	5(1)	22(4)	262(19)	0.835**
Male	42(2)	93(3)	50(2)	8	14(2)	207(9)	
Age							
1-15	4	11	10	0	36(6)	61(6)	0.857**
16-30	34(1)	113	59(**7**)	6	0	212(8)	
31-45	43(3)	30(3)	33(2)	2	0	108(8)	
46-60	8(1)	34	11(1)	4	0	57(2)	
>61	5	13(2)	12(1)	1(1)	0	31(4)	
Occupation							
Others	81(4)	81(2)	122(9)	7	36(6)	327(21)	0.496**
Farmer	6(1)	61(2)	2(2)	6(1)	0	75(6)	
Home stay	7	59(1)	1	0	0	67(1)	
Seropositivity							
IgM (%)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	
IgG (%)	5(5.3)	5(2.5)	11(8.8)	1(7.7)	6(16.7)	28(6.0)	0.022*

AMC, Andersen Medical Center, CIP, KEMRI-Alupe clinic, END, Endebess District Hospital, KDH, Kitale District Hospital, KAP, Kapluk Dispensery. () indicate YFV IgG seropositive samples per site.* statistically significant, ** not statistically significant

4.1.4 IgM capture ELISA and Indirect IgG ELISA

Recent YFV exposure was checked using an in-house IgM capture ELISA following Bundo and Igarashi, 1985, protocol. A P/N (positive control (or sample) OD_{492}/negative control OD_{492}) ratio ≥2.0 was considered positive. In the present study none of the samples tested positive for YFV IgM. Twenty eight samples (6%) had YFV IgG titres 3,000-12,000 (cut-off point was set as ≥1:3,000) (**Table 4.1**) & (**Figure 4.3**). CIP showed the highest seropositivity among the five facilities, however, the GMT of YFV IgG titre of positives was not statistically significant. There was a significant difference in YFV IgG seropositivity between the study sites (p=0.022). In addition, there was no significant difference in YFV IgG between the different age groups (p=0.857), sex (p=0.835) nor occupation (p=0.496) (**Table 4.1**).

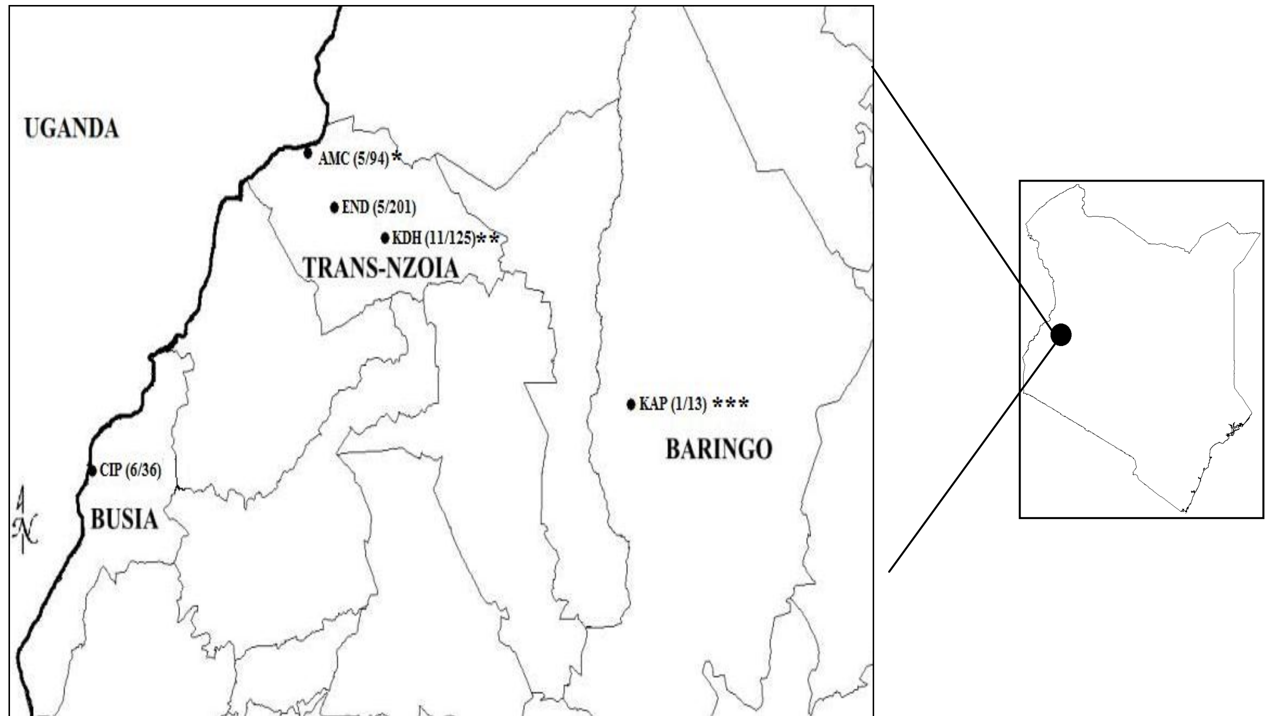
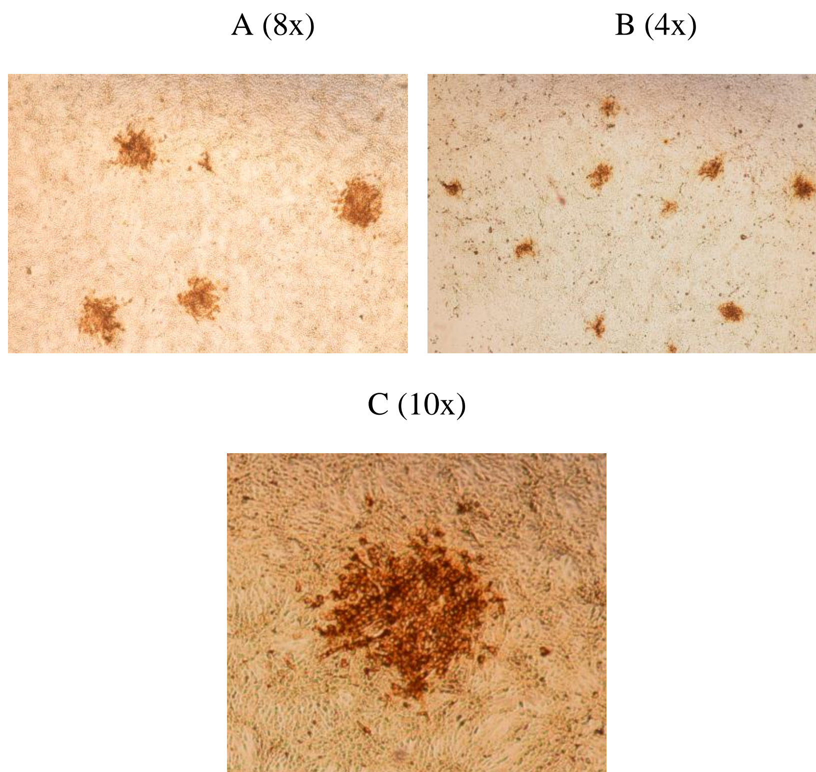


Figure 4.3: Map showing seropositivity in the hospital based sampling sites.

AMC, Andersen Medical Center, CIP, KEMRI-Alupe clinic, END, Endebess District Hospital, KDH, Kitale District Hospital, KAP, Kapluk Dispensery. () indicate YFV IgG seropositive samples per site. *, one YF confirmed case by YFV $FRNT_{50}$, **, two YF confirmed cases by YFV $FRNT_{50}$ and one WNV confirmed case by WNV $FRNT_{50}$, ***, one YF confirmed case by YFV $FRNT_{50}$. Source DIVA-GIS v 7.5.0.0.

4.1.5 Focus Reduction Neutralization Test (FRNT)

Twenty eight samples that had a YFV IgG indirect ELISA titre (≥1:3,000) were further analysed by $FRNT_{50}$, **Figure 4.4**, for confirmation of YFV infection using YFV, DENV1, DENV2, DENV3, DENV4 and WNV.



YFV infected Vero cells (Immunostaining)

Figure 4.4: Microscopic photos of YFV17D infected Vero cells after FRNT immunostaining using DAB

Four samples out of the 28 YFV IgG positive showed YFV specific neutralization titres above 1:10. Notable sample S0004 showed neutralization titre at 1:640 while the other

three samples, A0716, K0870 and K0960 showed neutralization titres below 1:100 as shown in **Table 4.2**. In addition, none of the four samples showed above 1:10 neutralization titre against DENV1, DENV2, DENV3, DENV4 and WNV. Interestingly, K1013 showed neutralization titre at 1:610 against WNV but showed below 1:10 neutralization titre against YFV, DENV1, DENV2, DENV3, and DENV4. The flavivirus indirect IgG ELISA using JEV, yielded an all positive result for the 28 YFV IgG positive samples as shown in **Figure 4.5**.

Table 4.2: Neutralization test against six different flaviviruses using $FRNT_{50}$ of all the 28 YFV IgG ELISA positive samples

County	Sample	IgG Titres	GMT of IgG	YFV	DENV1	DENV2	DENV3	DENV4	WNV
Trans Nzoia	A0112	3,500	4,917	<10	<10	<10	<10	<10	<10
	A0130	6,000		<10	<10	<10	<10	<10	<10
	A0132	3,800		<10	<10	<10	<10	<10	<10
	A0716	9,000		**35**	<10	<10	<10	<10	<10
	A0724	4,000		<10	<10	<10	<10	<10	<10
	E0046	9,000	6,146	<10	<10	<10	<10	<10	<10
	E0094	7,000		<10	<10	<10	<10	<10	<10
	E0097	4,000		<10	<10	<10	<10	<10	<10
	E0103	6,000		<10	<10	<10	<10	<10	<10
	E0416	5,800		<10	<10	<10	<10	<10	<10

County	Sample	IgG Titres	GMT of IgG	YFV	DENV1	DENV2	DENV3	DENV4	WNV
	K0116	5,000		<10	<10	<10	<10	<10	<10
	K0135	8,000		<10	<10	<10	<10	<10	<10
	K0145	3,600		<10	<10	<10	<10	<10	<10
	K0169	4,000		<10	<10	<10	<10	<10	<10
	K0870	10,200		**80**	<10	<10	<10	<10	<10
	K0936	7,500	6,280	<10	<10	<10	<10	<10	<10
	K0960	9,000		**60**	<10	<10	<10	<10	<10
	K0983	4,000		<10	<10	<10	<10	<10	<10
	K0990	3,000		<10	<10	<10	<10	<10	<10
	K1011	10,500		<10	<10	<10	<10	<10	<10
	K1013	12,000		<10	<10	<10	<10	<10	**610**
Baringo	S0004	8,000	8,000	**640**	<10	<10	<10	<10	<10
	C0206	5,000		<10	<10	<10	<10	<10	<10
	C0235	10,000		<10	<10	<10	<10	<10	<10
Busia	C0239	5,500	5,343	<10	<10	<10	<10	<10	<10
	C0272	3,000		<10	<10	<10	<10	<10	<10
	C0275	6,000		<10	<10	<10	<10	<10	<10
	C0278	4,700		<10	<10	<10	<10	<10	<10

4.1.6 Flavivirus cross reactivity

To check the cross reactivity of YFV IgG against flavivirus the 28 YFV IgG positive samples were examined by flavivirus IgG indirect ELISA. The result, **Figure 4.5**, indicated IgG can cross-react between YFV and other flaviviruses in IgG indirect ELISAs.

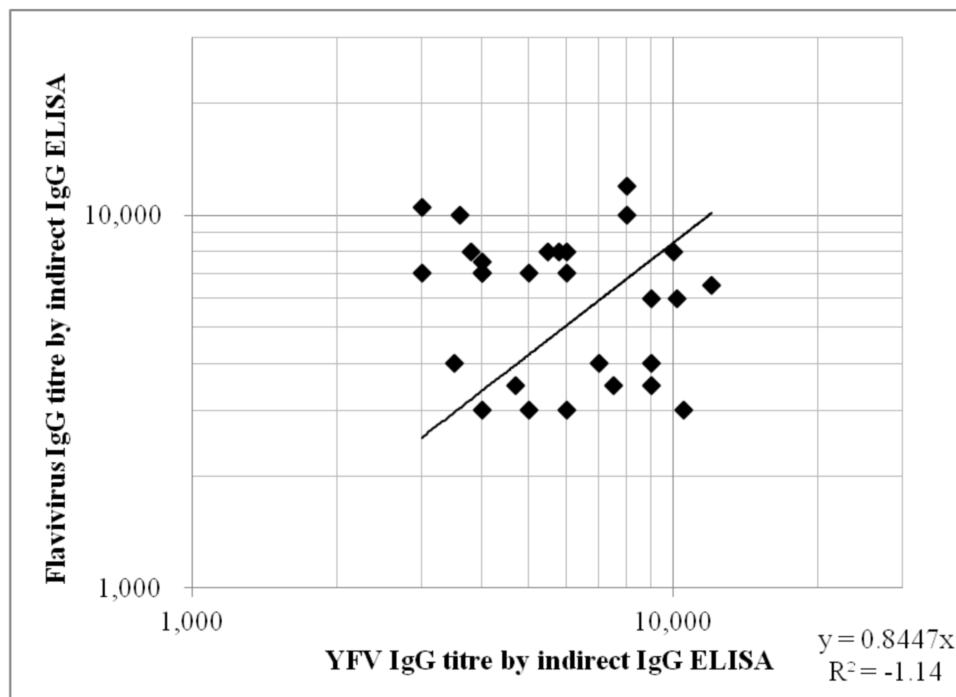


Figure 4.5: Flavivirus IgG indirect ELISA using JEV antigen versus YFV IgG indirect ELISA using YFV antigen for 28 Western Kenya samples. Cut-off value was set at 1:3,000 IgG titre.

4.2 The design, optimization and testing of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid diagnosis of YFV.

4.2.1 YFV RT-LAMP primer design

The designed primer sequences targeting the NS1 region of the YFV were successfully developed, as shown in **Table 4.3**.

Table 4.3: Details of the RT-LAMP primers used for the amplification of the YFV NS1 region

Primer name	Type	Length	Genome position[a]	Sequence (5′-3′)
F3	Forward outer	20	3090-3109	TCCACACCTTGGAGGCATTA
B3	Reverse outer	20	3331-3313	GTCCATCACAGTTGCCATCA
FIP	Forward inner	42	3200-3181 (F1c) + TTTT	GGCCTCCGATTGATCTCGGC
			3121-3138 (F2)	GTGTGAGTGGCCACTGAC
BIP	Reverse inner	44	3235-3256 (B2) + TTTT	GGTTCAGACGAACGGACCTTGG
			3298-3281 (B1c)	CCCTGGGCAAGCTTCTCT
LF	Forward loop	25	3164-3140	CTTCAACTGATGTTCCAATCGTATG
LB	Reverse loop	22	3257-3278	ATGCAGGTACCACTAGAAGTGA

[a] Genome position according to the YFV strain: 17D (GenBank accession number P03314)

4.2.2 YFV RT-LAMP limit of detection

RT-LAMP was performed using a real-time turbidimeter (**Figure 4.6**) and turbidity in the positive tubes was evident at the end of reaction as shown in **Figure 4.6**. Furthermore, the time to positivity was below 30 minutes with the inclusion of loop amplification primers. The RT-LAMP depicted an exponential rise in the turbidity recorded in under a 20-minute period regardless of the varying virus titre at the beginning of the reaction. In the RT-LAMP, the attainment of the turbidity peak did not vary while using the different RNA templates of a diluted ICF.

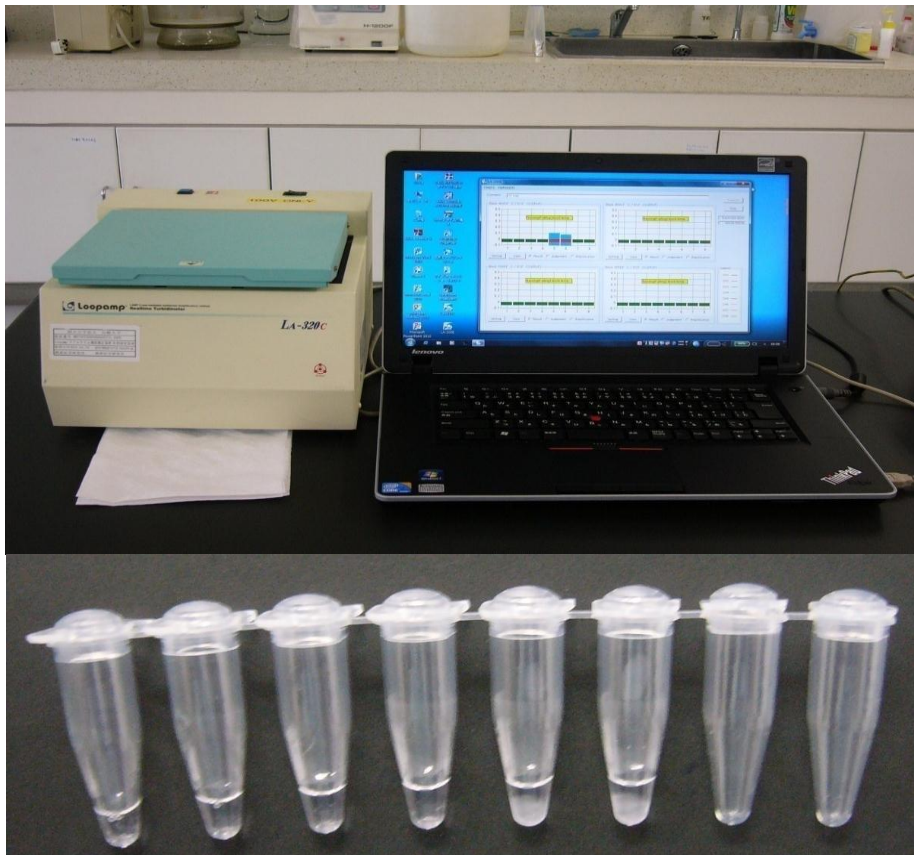


Figure 4.6: The RT-LAMP turbidimeter and EIKEN's reaction tubes showing turbidity.

However, there was a peak variation in the real-time RT-PCR using the same RNA templates, which made it difficult to interpret the results. The limit of detection of the RT-LAMP for the detection of YFV RNA was determined using tenfold serial dilutions and was assessed in parallel with real-time RT-PCR and RT-PCR. The detection limit of the RT-LAMP (**Figure 4.7**) and real-time RT-PCR (**Figure 4.8**) was 0.29 PFU/ml while that of the RT-PCR was 2.9 PFU/ml (**Figure 4.9**).

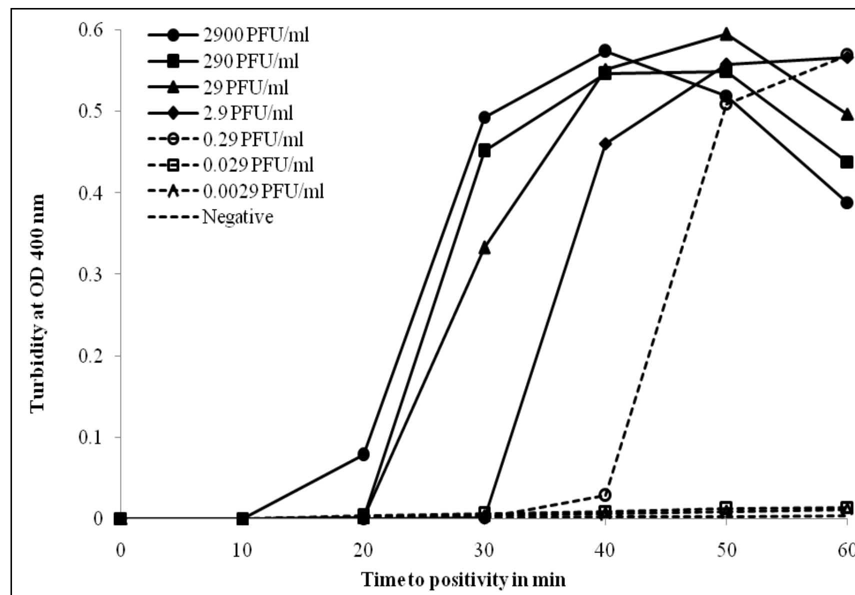


Figure 4.7: Analytical sensitivity of YFV RT-LAMP on YFV 17D ICF.

Tenfold diluted serially YFV17D from 2900 PFU/ml was used and detected using a real-time turbidimeter, LA 320; plaque forming unit (PFU); yellow fever virus: strain 17D (YFV 17D); infected culture fluid (ICF).

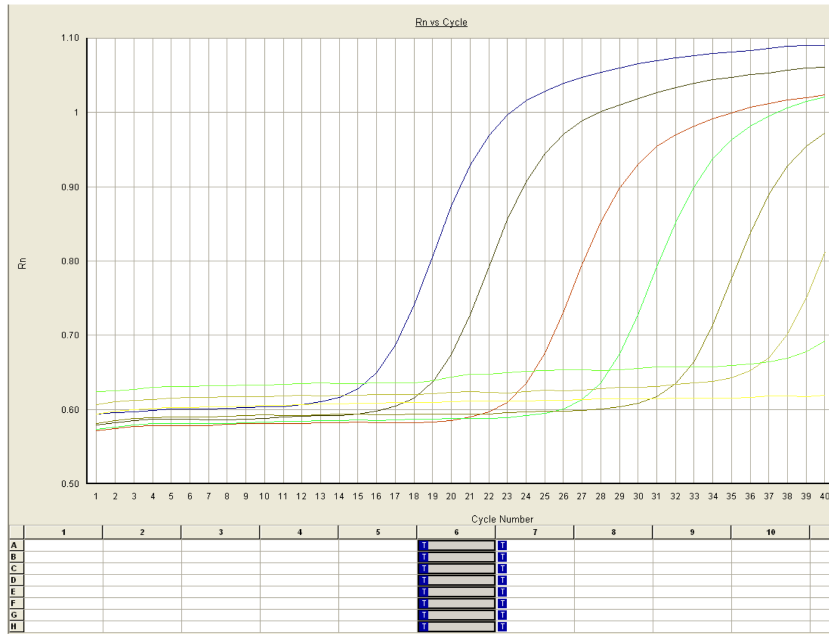


Figure 4.8: Detection limit of YFV using real-time RT-PCR. Tenfold diluted serially YFV17D from 2900 PFU/ml was used and detected using a real-time turbidimeter, LA 320; plaque forming unit (PFU); yellow fever virus: strain 17D (YFV 17D); infected culture fluid (ICF).

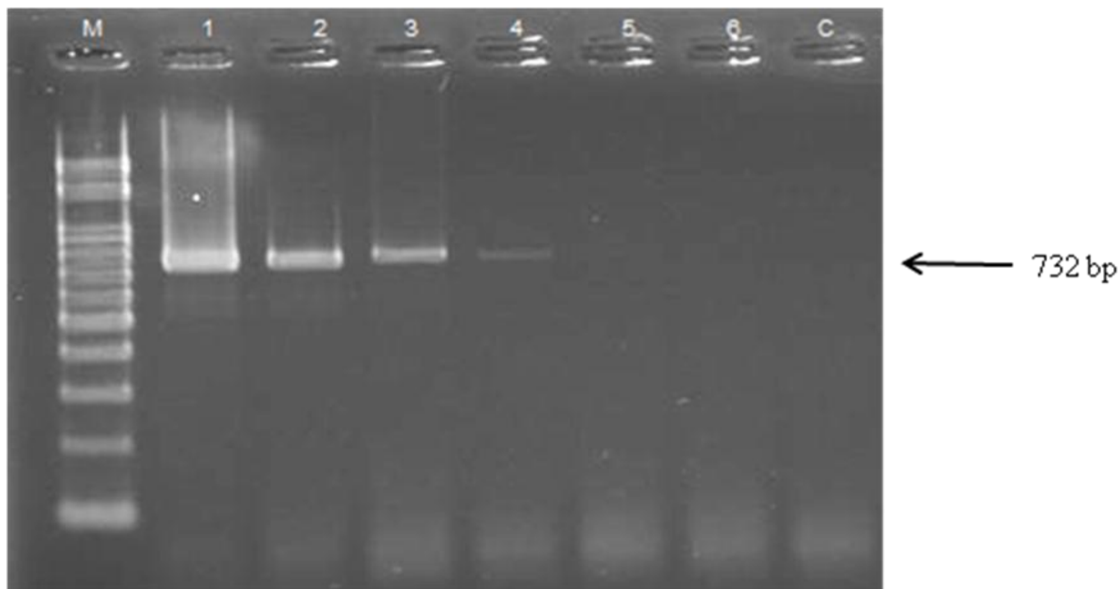


Figure 4.9: Detection limit of YFV using RT-PCR.

M (Molecular marker), 1 (2900 PFU/ml), 2 (290 PFU/ml), 3 (29 PFU/ml), 4 (2.9 PFU/ml), 5 (0.29 PFU/ml), 6 (0.029 PFU/ml), C (Negative control)

4.2.3 YFV RT-LAMP specificity

The specificity of the developed YFV RT-LAMP shown in **Figure 4.10** depicts that only YFV was amplified against JEV, DENV1-4, WNV, CHIKV and RVFV to assess the cross-reactivity of the test.

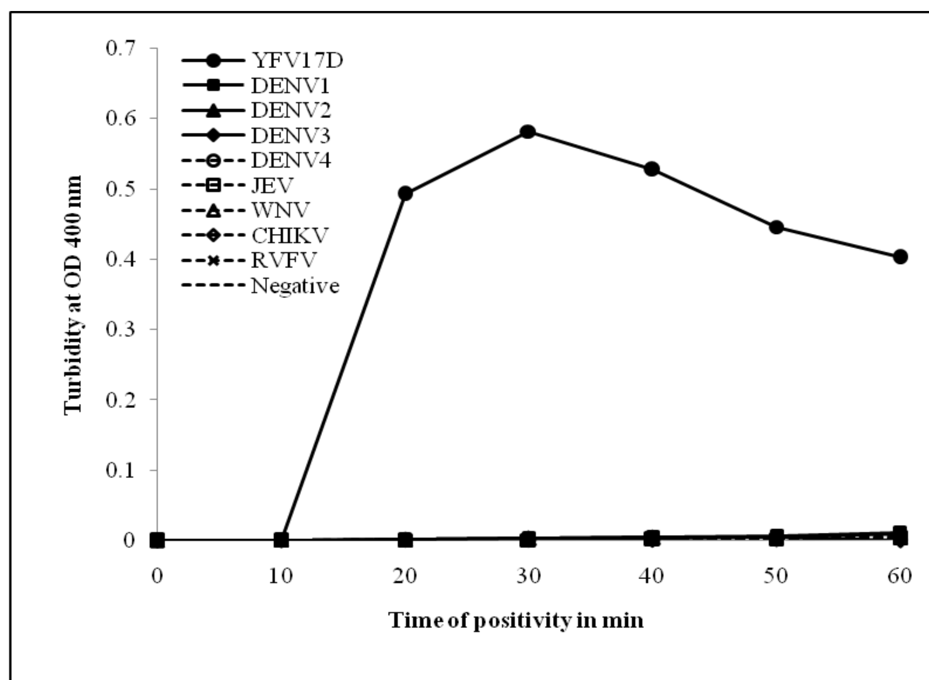


Figure 4.10: Detection specificity of the YFV RT-LAMP.

Flaviviridae; yellow fever virus (YFV), dengue viruses (DENV 1-4), Japanese encephalitis virus (JEV), West Nile virus (WNV), *Togaviridae*; chikungunya virus (CHIKV), and *Bunyaviridae*; Rift Valley fever virus (RVFV) were used.

4.2.4 YFV RT-LAMP sensitivity

Through a limiting dilution assay, one human serum sample spiked with YFV (strain: 17D) ICF was compared with YFV (strain: 17D) ICF in a bid to assess serum interference with the RT-LAMP. A 100% concordance between the spiked human serum sample and YFV 17D ICF with regard to sensitivity was observed (**Figures 4.7 & 4.11**).

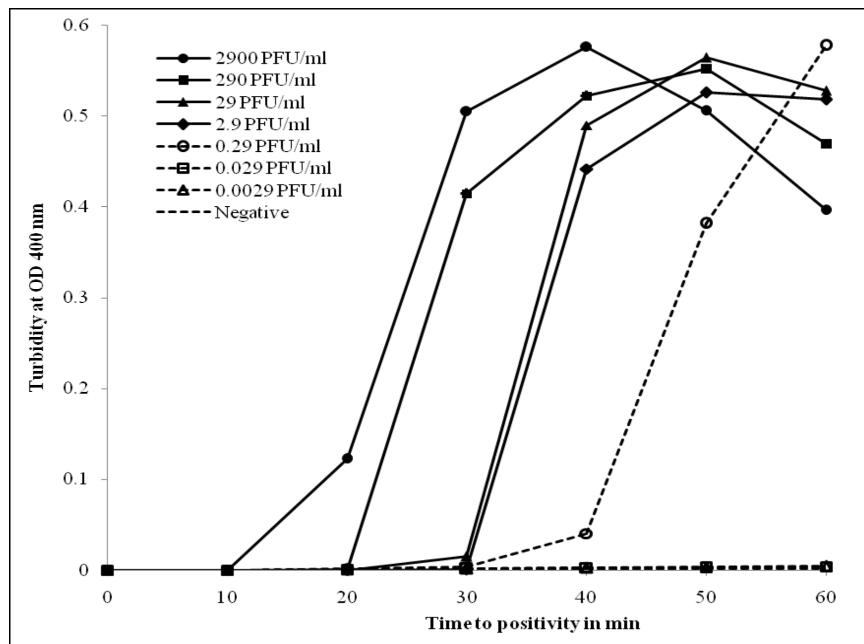


Figure 4.11: Analytical sensitivity of the YFV RT-LAMP on human serum spiked using YFV 17D.

Tenfold diluted serially YFV 17D from 2900 PFU/ml to 0.0029 PFU/ml was detected using a real-time turbidimeter, LA 320. Plaque forming unit (PFU); yellow fever virus strain: 17D (YFV 17D); infected culture fluid (ICF)

The time to positivity in both was below 30 minutes and the detection limit was 0.29 PFU/ml. The validation of the developed RT-LAMP was evaluated using two Kenyan strains from the YFV 1992-1993 Kenya outbreak (Sanders *et al*., 1998) isolated at the Viral Hemorrhagic Fever (VHF) laboratory at KEMRI, Kenya. The two Kenyan strains had earlier been laboratory confirmed using YFV RT-PCR at the VHF laboratory (Sang, R., *personal communication, 2011*). The performance of the RT-LAMP was compared to the established real-time RT-PCR and RT-PCR used for the diagnosis of YFV17D and the two Kenyan strains in a limiting dilution test. The YFV RT-LAMP further showed an equal detection limit sensitivity of 0.29 PFU/ml while using the two Kenyan strains and the vaccine strain (strain: 17D) (**Table 4.4**).

Table 4.4: Detection limit of three assays; RT-LAMP, Real-time RT-PCR and RT-PCR against yellow fever virus strains: 17D and two Kenya strains (1992-1993).

	17D	**Baringo 1**	**Baringo 2**
RT-LAMP	0.29 PFU/ml	0.29 PFU/ml	0.29 PFU/ml
Real-time RT-PCR	0.29 PFU/ml	0.29 PFU/ml	0.29 PFU/ml
RT-PCR	2.9 PFU/ml	2.9 PFU/ml	2.9 PFU/ml

4.3 Development of YFV NS1 recombinant antigen using an *E. coli* expression system.

4.3.1 YFV NS1 recombinant antigen RT-PCR

The YFV NS1 recombinant antigen was successfully amplified using RT-PCR and the PCR products gel purified for cloning as shown in **Figure 4.12**.

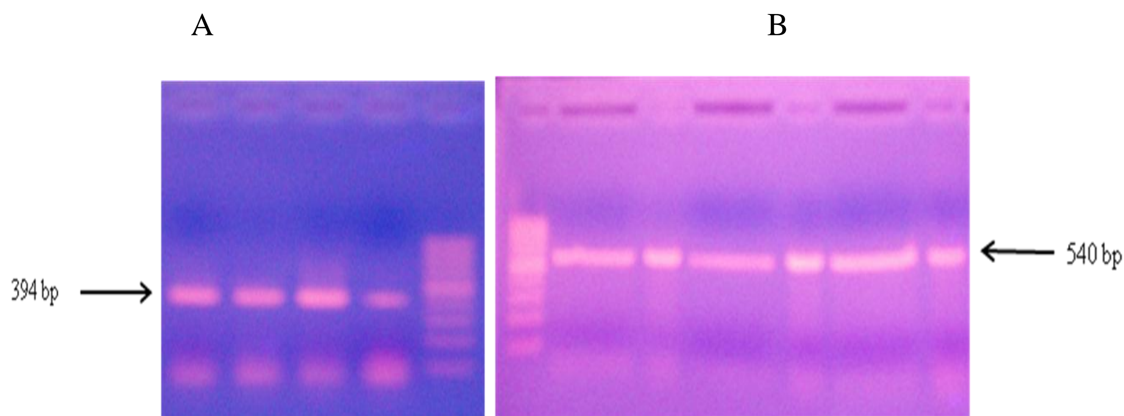


Figure 4.12: YFV NS1 RT-PCR agarose gel

(A) YFV RT-PCR agarose gel showing the 394 bp NS1 PCR product (B) YFV NS1 PCR products in a 1.8 % (w/v) agarose gel for gel purification.

4.3.2 YFV NS1 recombinant antigen cloning and protein expression

After gel purification the YFV NS1 gene was then inserted successfully into pET 28a, sequenced to ensure that it was in frame using an ABI 3500 genetic analyser (Life Technologies, New York, USA) as shown in appendix IV and cultured in LB agar containing kanamycin. No clones were observed in the negative control plate while several clones were observed in the kanamycin containing plates as shown in **Figure**

4.13. The picked clone was cultured in small scale and plasmid DNA extracted where only the PCR positive clone DNA was used for expression. A preculture was initiated and transferred into a largescale culture to increase the yield of the recombinant antigen using a shaking incubator.

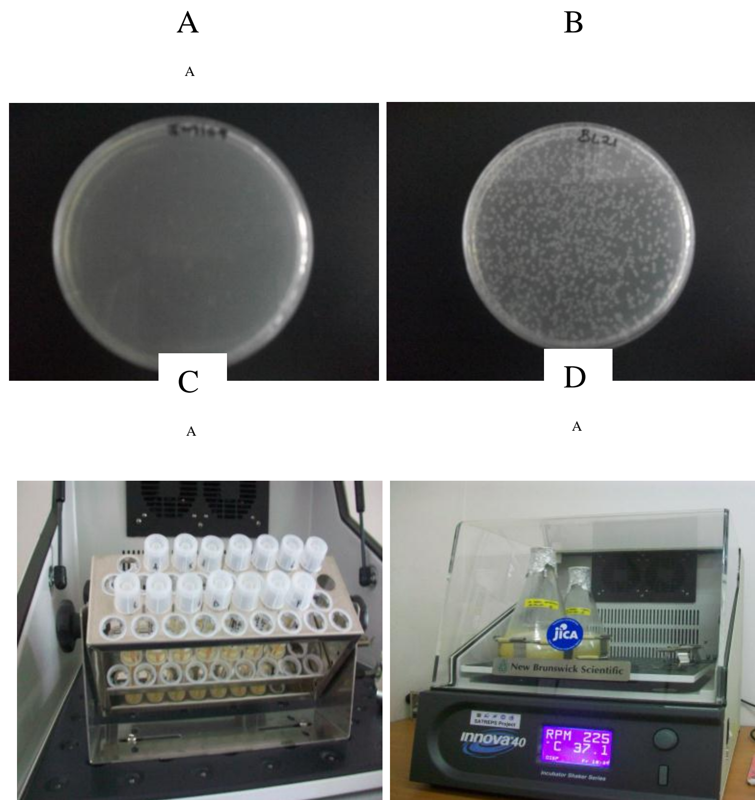


Figure 4.13: YFV NS1 cloning and recombinant antigen expression.

(A) Negative control LB-Agar plate without insert; (B) LB-Agar plate with YFV NS1 insert showing colonies; (C) Pre-cultures of YFV NS1 in LB-broth; (D) Large-scale culture of YFV NS1 for protein expression.

4.3.3 Western blot and CBB staining of the recombinant YFV NS1

The recombinant YFV NS1 protein was successfully expressed in the pET 28a system. The protein was expressed as an insoluble protein as shown in **Figure 4.14**. The YFV NS1 protein was expressed as a 15 KDa protein. Although CBB staining showed many bands in the soluble fraction the same was not observed in the Western blot.

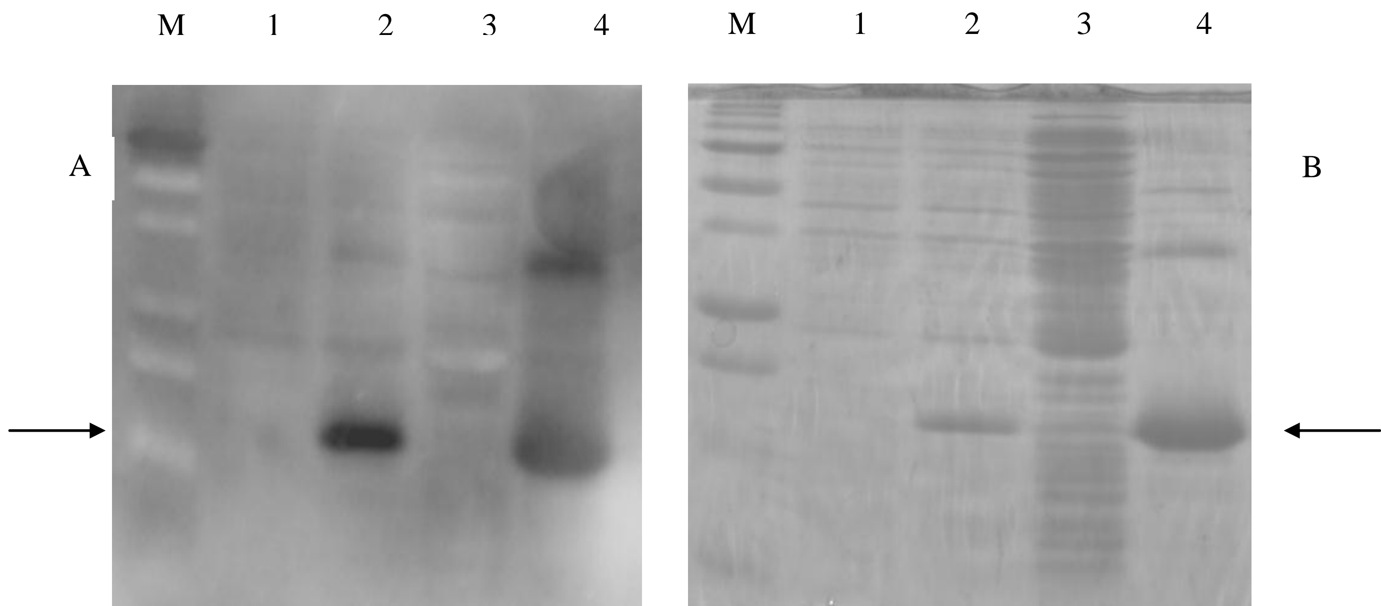


Figure 4.14: YFV NS1 recombinant antigen Western blotting and CBB staining.

A, Western blot gel and B, CBB stained SDS PAGE gel. M, protein marker; 1, pre-IPTG; 2, post-IPTG; 3, soluble fraction; 4, insoluble fraction; arrow indicates the 15 kDa recombinant protein.

CHAPTER FIVE

5.0 DISCUSSION

Yellow fever (YF), a disease caused by a mosquito-borne virus is an important viral heamorrhagic fever endemic in equatorial Africa and tropical South America (Gould *et al*., 1989). In this study the design and optimization of an RT-LAMP assay for the detection of YFV, development of in house indirect IgG, antigen capture and IgM capture ELISAs as well as the development of a recombinant YFV NS1 antigen were undertaken.

5.1 Seroprevalence of YFV in febrile patients visiting selected health facilities in Western Kenya

There was an overall YFV IgG prevalence rate of 6% in the present study, covering selected parts of Western Kenya, during the period 2010-2012. In an earlier study by Mease *et al*., 2011, an overall prevalence of 9.2% was recorded. Notable, in the two studies, Busia recorded prevalence rates of 3.2% in Mease *et al*., 2011 study and 16.7% in the present study. However, the sampled population in Mease *et al*., 2011 study was largely adults while in the present study both adults and children were included. The three sites in Trans Nzoia County offered a forested set up in END and AMC due to nearness to Mt. Elgon and the border to Uganda and KDH an urban set up. However, the seroprevalence rates and GMT of IgG titres did not significantly differ between the three facilities. KAP offered a relatively high prevalence rate of 7.7% even with the small sample set used in the present study.

Noteworthy, is its proximity to the last major YFV outbreak in Kenya 1992-3 (Okello *et al*., 1993).

Since there is cross reactivity among flaviviruses and more so when flaviviruses are endemic in the same region, the establishment of FRNT as a test will largely aid in confirmatory diagnosis of YFV. In the first index case, S0004, the sample was from a YFV vaccinated, 66 year old, female, from Kiptorenya village, Kapluk location of Baringo County. Her YFV indirect IgG ELISA titre was 1:8,000 hence $FRNT_{50}$ was performed using YFV, DENV 1-4 and WNV. Notable, a YFV $FRNT_{50}$ of 1:640 was obtained for this index case. This result was much higher than the average neutralizing antibody titre against YFV after vaccination by plaque reduction neutralization test (Neidrig *et al*., 1999). The increased neutralization result in the Kapluk index case could be a pointer to exposure during the 1992-93 outbreak or silent circulation of YF in the area. Since the IgM capture ELISA result was negative the exact exposure remains unexplained but the increased IgG titre level might not be from vaccination only. The YFV vaccination histories of the remaining three $FRNT_{50}$ positive samples (A0716, K0870 and K0960) are unknown hence not further discussed.

In the second index case, K1013, the sample was obtained from a non YFV vaccinated, 21 year old, female, from Sitatunga village, Kaplamai location of Trans Nzoia County. Her YFV indirect IgG ELISA was 1:12,000, however, YFV $FRNT_{50}$ was below 1:10 and the WNV $FRNT_{50}$ was 1:610. It might be linked to WNV

exposure in the past. This further underscores the need for differential diagnosis of arboviral infections in areas were more than one arbovirus is circulating. The remaining 23 samples could be due to other related flavivirus infections such as Zika virus especially in Busia. To check the cross reactivity of YFV IgG against flavivirus the 28 YFV IgG positive samples were examined by flavivirus IgG indirect ELISA. The result indicated IgG can cross-react between YFV and other flaviviruses in IgG indirect ELISAs. It suggests that various flaviviruses are circulating in this region.

5.2 A real-time reverse transcription loop-mediated isothermal amplification assay for the rapid detection of Yellow Fever virus

The resurgence of YF in the Eastern African region raises public health concerns, creating a need to improve surveillance, early warning systems and expanded immunization programs (Ellis & Barrett, 2008). The last YF outbreak in Kenya, 1992-1993, caused high mortality and morbidity since low immunity levels existed within the affected communities (Sanders *et al*., 1998; Reiter *et al*., 1998).

Although regular vaccination is the ideal policy for disease control of YF, often YF endemic countries are faced with frequent outbreaks. An alternative approach using early detection and containment followed by post-outbreak vaccination might reduce the disease burden. Thus, the time required for the diagnosis of YF is a critical factor for early detection and containment of the disease. The laboratory confirmation of suspected cases uses several YF diagnostic assays that include IgM-capture ELISA,

IFA, virus isolation, RT-PCR, and PRNT (De Brito *et. al*., 1992; Deubel *et. al*., 1997; Monath *et. al*., 1989; Tanaka, 1993; Drosten *et al*., 2002; Groot *et al*., 1962). Although IgM-capture ELISA is used as a standard serological test in reference laboratories, the detection of IgM relies mainly on convalescent phase samples. Furthermore, there is cross-reactivity of IgM amongst related flaviviruses that co-exist in the same region such as DENV and WNV, which makes YFV diagnosis difficult. Due to the genetic homology in the flavivirus group, YFV has shown cross reactivity with other members of the genus *flavivirus* - in particular, DENV (Houngton *et. al*., 2008) and West Nile virus (WNV) (Mansfield *et al*., 2011). Both virus isolation and PRNT are highly specific and are routinely used as confirmatory tests at the reference laboratories even if it takes one week to get results. Therefore, the advent of molecular-based assays, such as RT-PCR, has aided in the rapid detection of YFV (Tanaka, 1993; Drosten *et al*., 2002) and real-time RT-PCR (Bae *et al*., 2003). Hence, an RT-LAMP assay used to detect YFV has augmented the existing real-time RT-PCR and conventional RT-PCR in the timely detection of YFV by reducing the time of reaction from more than 3 hours to less than 1 hour including the results observation via the real-time monitoring system.

The loop-mediated isothermal amplification (LAMP) is an alternate method for nucleic acid amplification that was developed by Notomi *et al*., (2000). The technique has been optimized for several arboviruses including DENV serotypes 1-4 (Parida *et al*., 2005), Japanese encephalitis virus (JEV) (Parida *et al*., 2006; Toriniwa & Komiya 2006), chikungunya virus (CHIKV) (Parida *et al*., 2007), Rift Valley

fever virus (RVFV) (Le Roux *et al*., 2009), and WNV (Parida *et al*., 2004), although YFV reverse transcription-LAMP (RT-LAMP) had not been described in literature. The present work, designed, optimized and tested an RT-LAMP for YFV in a bid to complete the panel of RT-LAMP for closely related flaviviruses. This is important for ease of differential diagnosis in areas where co-circulation of YFV, DENV or WNV may occur.

The sensitivity of RT-LAMP indicated that serum did not interfere with the assay hence it could be applied in the detection of YFV in clinically suspected patients. However, there is further need to intensively validate the assay using YF clinical samples from various endemic countries. The specificity of RT-LAMP was confirmed by amplification only of YFV but not DENV 1-4, WNV, JEV, CHIKV or RVFV tested using the developed YFV primer set. This high specificity could solve the cross reactivity problem of serological diagnosis among these viral diseases. The stable reactivity of the RT-LAMP assay of YFV might be attributed to the less than 5% mismatch of the nucleotides in the conserved NS1 region among 5 genotypes. Furthermore, the archiving of the samples over a ten-year period did not affect the detection efficacy of the assay, which might be due to the slow evolution speed of YFV (Sall *et al*., 2010).

5.3 Development of YFV NS1 recombinant antigen

In the present study, the development of a YFV NS1 recombinant antigen was undertaken. Cloning and protein expression was achieved through a pET28a

expression vector in an *E. coli* system. Successful expression of YFV NS1 was achieved as shown by Western blotting using the His-tag site and CBB stained SDS PAGE gel of the 15 KDa recombinant protein. Worldwide, the use of NS1 in the detection of DENV is commonly practiced although this has not been the same for YFV. The YFV non-structural protein offers a diagnostic window for detection of YFV antigen even after the viremic phase. Secretions of NS1 in the acute phase to convalescent phase of YFV infection offers a similar platform for detection as the same occurs in DENV.

During human flavivirus infection such as DENV, NS1 is a well established diagnostic target for antigen detection system since it is the only non structural protein secreted into the bloodstream (Macdonald *et al*., 2005). Although the precise role of secreted NS1 is not clearly understood, this unique property has made it a widely used tool for point of care diagnostics for other flavivirus: DENV (Young *et al*., 2000), WNV (Macdonald *et al*., 2005), JEV (Konishi, 2011) though not reported yet for YFV. This formed the basis of the present study. The YFV containment strategy can be through immunization services and interventions *via* vaccination however this is not always readily available in areas where YFV outbreaks occur. The provision of diagnostic platforms might offer detection capabilities that lead to targeted interventions. The recombinant antigens may be used to develop an easy to use rapid strip that could be used as a point of care tool or openly in the field even where resources are limited as is mostly the case in Sub Saharan Africa in future in a bid to improve patient management.

It is recommended that a further study be undertaken on YFV NS1 in generation of monoclonal antibodies and the development of an antigen detection ELISA and immunochromatographic test. Since NS1 is antigen based as opposed to IgM capture ELISA and indirect IgG ELISA, its use will improve the diagnostic value. The developed YFV monoclonal antibodies are anti-NS1 specific which will accord accurate detection of YFV infections in areas where other flaviviruses such as DENV and WNV are co-circulating. The strength of NS1 based assay lies on specificity as conventional serological ELISA though quantitative prone to cross-reactivity challenges (Konishi, 2011).

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

- There was no YFV IgM positive detected in this present study.
- There was an overall YFV IgG prevalence rate of 6% in the present study, covering selected parts of Western Kenya, during the period 2010-2012.
- Since there is cross reactivity among flaviviruses and more so when flaviviruses are endemic in the same region, the establishment of FRNT as a test will largely aid in confirmatory diagnosis of YFV.
- This study showed that YFV though genetically distant from JEV complex, serological cross reactivity was strong.
- The YFV RT-LAMP reduced the time of reaction from 3 h to 1 h and increased sensitivity tenfold compared to YFV RT-PCR.
- The YFV RT-LAMP offers a simple, rapid and reliable diagnostic tool for yellow fever when there are outbreaks of acute hemorrhagic fever in Kenya and other African countries.
- Since the burden of YF still exists, there is need for the development of cost effective diagnostic platforms such as the development of a YFV NS1 recombinant antigen.

6.2 RECOMMENDATION

The following recommendations emanate from the study:

1. The developed YFV RT-LAMP needs to be tested in the field environment to ensure domestication of the assay in outbreak prone areas in Kenya.

2. Since the developed $FRNT_{50}$ and YFV IgM ELISA was performed using 17D the vaccine strain there is need to use a local isolate to ensure there is increased sensitivity of the assays.

3. The packaging of the developed IgM capture ELISA in a ready to use kit should be undertaken to ensure reliable and cost effective options are availed to the Ministry of Health after validation of the assays using a WHO sample panel.

4. The development of monoclonal antibodies from the recombinant NS1 antigen should be undertaken to enable development of immunochromatographic tests. The use of a point of care tool will enable easy screening of field samples and suspected outbreak samples in remote areas of Kenya.

5. Both serological and molecular developed assays need to be used as research tools to better map the burden of YF in the Kenya as they are now available in-country to local researchers. This will involve routine surveillance for epidemiological purpose and vaccination status for immunization programs.

6. There is need to establish detection systems for closely related flaviviruses such as Zika and Uganda S as they are endemic in YF zones as well. This might resolve the unexplained anti-flavivirus IgG indirect ELISA positive but YFV IgG indirect ELISA negative samples.

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APPENDICES

Appendix I: SSC approval for SSC# 1829

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/9152 **18th March, 2011**

Allan B. Ole Kwallah

Thro'
Director, CIPDCR
P.O. Box 3
BUSIA

REF: SSC No.1829 (Revised) – Novel approaches in the detection of yellow fever virus in febrile patients visiting selected health facilities in Western Kenya. PI: Allan B. Ole Kwallah (CIPDCR)

I am pleased to inform you that the above-mentioned proposal, in which you are the PI, was discussed by the KEMRI Scientific Steering Committee (SSC), during its **176th meeting** held on **1st March, 2011** and has since been approved for implementation by the SSC.

Kindly submit 4 copies of your revised protocol to SSC for onward transmission to ERC.

The SSC however, advises that work on this project can only start when ERC approval is received.

Sammy Njenga, PhD
SECRETARY, SSC

In Search of Better Health

Appendix II: ERC approval for SSC # 1829

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 June 24, 2011

TO: ALLAN OLE KWALLAH
PRINCIPAL INVESTIGATOR

THRO': DR. MATILU MWAU
DIRECTOR, CIPDCR,
BUSIA

21/July/2011
Forwarded

RE: SSC PROTOCOL NO. 1829 (*RATIFICATION FOR INITIAL SUBMISSION*): NOVEL APPROACHES IN THE DETECTION OF YELLOW FEVER VIRUS IN FEBRILE PATIENTS VISITING SELECTED HEALTH FACILITIES IN WESTERN KENYA.

This is to inform you that the **provisional approval** granted by the Chair KEMRI/ERC for initial review was ratified by the full Committee during the 190th meeting of the KEMRI/ERC meeting held on 14th June 2011.

You may proceed with your study.

Yours sincerely,

Caroline Kithinji,
FOR: SECRETARY,
KEMRI/ ETHICS REVIEW COMMITTEE

Appendix III: Continued ERC approval for SSC # 1829

KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 **August 3, 2012**

TO: **MR. ALLAN OLE KWALLAH (PRINCIPAL INVESTIGATOR)**

THROUGH : **DR. MATILU MWAU,**
THE DIRECTOR, CIPDCR,
BUSIA

Dear Sir,

RE: SSC PROTOCOL No. 1829 (*REQUEST FOR ANNUAL RENEWAL*): NOVEL APPROACHES IN THE DETECTION OF YELLOW FEVER VIRUS IN FEBRILE PATIENTS VISITING SELECTED HEALTH FACILITIES IN WESTERN KENYA

Thank you for the Continuing Review Report for the period **June 2011** to **June 2012.**

This is to inform you that at the 205th meeting of the KEMRI Ethics Review Committee held on 31st July 2012, the Committee reviewed the above referenced application and made note of the following:

1. RT-LAMP assay for yellow fever has been designed and optimized.
2. The first and third objectives .e. to develop recombinant antigens for Yellow Fever Virus and to establish an ELISA-based system for Yellow Fever virus using monoclonal antibody 2D12 respectively, are yet to be realized.
3. An abstract titled "A Reverse Transcription Real-time Loop Mediated Isothermal Amplification Assay for the Rapid Detection of Yellow Fever" by A Ole Kwallah *et al* has been prepared.
4. The planned activities for the next project period include:
   (a) To continue with the project aims.
   (b) To conduct a field-based evaluation of the assays developed.
   (c) To embark on thesis writing and submission to JKUAT.
   (d) To present study findings at conferences.

The Committee was of the opinion that the progress made in the reporting period is satisfactory. Consequently, the study was granted approval for continuation effective the **31st day of July 2012.**Please note that authorization to conduct this study will automatically expire on **July 30, 2013.**

If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **June 18, 2013.**

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received.

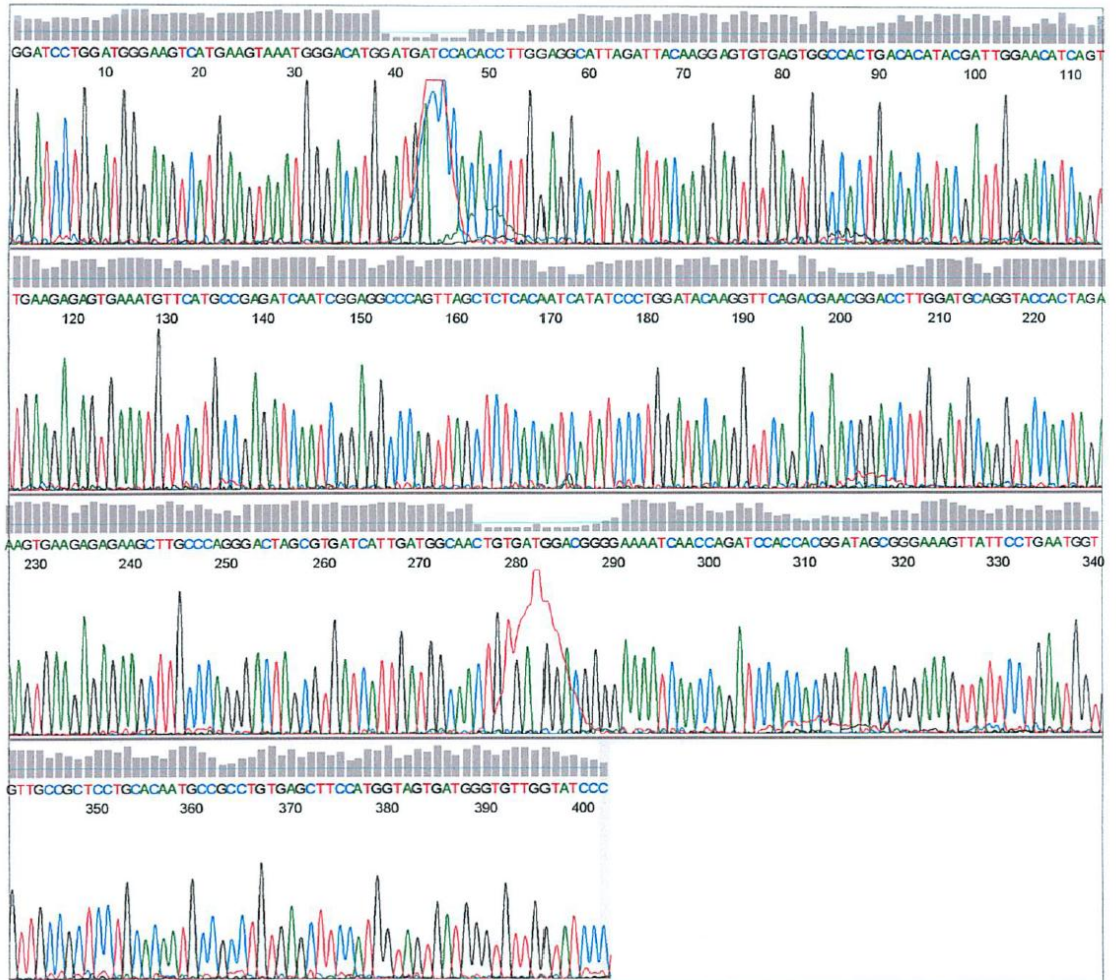
In Search of Better Health

Appendix IV: YFV NS1 recombinant antigen sequence

File: YFV NS1 Recombiant Antigen_allan.ab1

Sample Name: P1Fhalf
Mobility: KB_3500_POP7_BDTv3.mob
Spacing: 12.3971
Comment: n/a

Signal Strengths: A = 90, C = 110, G = 78, T = 133
Lane/Cap#: 8
Matrix: n/a
Direction: Native



Appendix V: Questionnaire

NOVEL APPROACHES IN THE DETECTION OF YELLOW FEVER VIRUS IN FEBRILE PATIENTS VISITING SELECTED HEALTH FACILITIES IN WESTERN KENYA

Please take time to read and understand the following information carefully. Filling up the questionnaire will take a maximum of 15 minutes. Feel free to ask as many questions as possible for clarification

Date of hospital visit

…………………..……….

Study Number

…..…………………..…

Village

…………………………...…
…

Sub-location

………………...……………….

Location

……..……………………….

Division

………..……………………………

District

……...…………………………
.

Province

…………...………………………

Gender:

Male ( ) Female( )

Age (years) ( ) Date of birth ( )

Have you ever been vaccinated for Yellow Fever?

Yes ( ) No ( )

If Yes, when?

Year ( ) Date ( ) Age ( )

Presenting Complaints:

Fever:

Yes ( ) No ( )

Duration of fever…………………………………..

Temperature in °C as measured by health worker.........................

Rash:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Eye infection:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Jaundice:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Headache:

Yes ( ) No ( )

Duration of symptoms…………………………………..

Symptoms suggestive of meningitis-meningoencephalitis

Yes ( ) No ( )

Duration of symptoms…………………………………..

Bleeding diathesis:

Yes ( ) No ( )

Duration of symptoms………………………………….

List any other symptoms

1. ……………………………….
2. ……………………………….
3. ……………………………….

Any other remarks:

……………………………………………………………………………………………
……………………………………………………………………………………………
……………………………………………………………………………………………
……………………………………………………………………………………………
…………

Name of person completing form: ………………………...

……………………………….

Signature ………………………….. Date ……………….. Time ……………………

Appendix VI: Informed consent forms for adults

Informed Consent for Adults aged 18 and above, in English.

My name is …..………and I work in this health facility. We think that Yellow Fever is an important cause of febrile illness in this area. In order to be sure about the exact magnitude of the problem, we are conducting a study to develop new assays for Yellow Fever. The information we gather is useful to the government and other policy makers who may consider preventative programs in this community or other communities in the future. We will summarize our findings from this study and disseminate it to various stakeholders including Ministry of Health, KEPI, KEMRI, and others. The KEMRI's ethical review committee, who are responsible for conducting such reviews at national level, has approved this study.

Research Procedures: You are here to have a little blood drawn from you because your clinician has recommended some tests**.** If you agree to be a participant in this study, we will ask you a few questions regarding where you reside and your vaccination status. Then we will take 5 mls (1 teaspoon) of blood during the procedure. We will use sterile and disposable instruments that are clean and safe. The extra blood taken from you will be transported to the KEMRI laboratories in Busia or/and Nairobi for analysis of Yellow Fever status. In order to ensure complete confidentiality of the test results, no names will be attached to the blood samples, but an identification number assigned to you will be used to label the sample. In addition we will take 15 minutes of your time to fill up a questionnaire that is related to this work.

Risk/benefits: During this procedure there will be no long-lasting effect. However, you may feel a brief moment of pain or fear. You will not be given any monetary benefits; neither will you incur any costs. The study will benefit your community since by helping us and the government to understand the problems your community is facing as a result of Yellow Fever, we will be able to recommend and design appropriate interventions to minimize the impact of this infectious disease.

Participant's Rights: Your participation in this study is voluntary and if you decline to participate, you will not be denied any services that are normally available to you.

Confidentiality: We will make every effort to protect your identity. You will not be identified in any report or publication of this study or its results.

Contact Information: If you have questions now or in the future regarding your rights or this study, you may ask any of the field officers involved in this study or contact

Allan Kwallah of KEMRI at 02027222541 ext 2256/2290.

Consent for the individual for blood sample:

May I now ask if you would like to participate in the study?

The above details about the study and the basis of participation have been explained to me and **I agree** to take part in the study. I understand that I am free to choose to be part of the study. I also understand that if I do not want to go on with the study, I can withdraw at any time. **I give my consent** for my blood to be tested for Yellow Fever.

Please sign here or put your right hand thumb mark if you agree:

Signature/ Thumb mark--- Date -------------------------

Ridhaa ya watu waliozidi umri wa miaka 18, kwa Kiswahili

Jina langu ni…...................................………..na ni mfanyakazi wa kituo hiki cha afya. Tunafanya utafiti juu ya ugonjwa uitwao Yellow Fever ambao tunafikiri ni mojawapo ya magonjwa yanayoambukiza jamii hii. Ili tujue umuhimu wa Yellow Fever hapa, tumeamua kufanya utafiti utakaolenga kuboresha upimaji wa virusi vya Yellow Fever. Matokeo ya utafiti huu yatasambazwa kwa serikali na wadau kama Wizara ya Afya, KEPI, KEMRI na wengineo, ambao watatengeneza miradi mbalimbali itakayolenga kupunguza maambukizi na kuboresha huduma za walioathirika katika jamii hii na nyingine hapo baadaye. Utafiti huu umepitishwa na kuruhusiwa na kamati inayohusika na utoaji wa vibali vya utafiti ya KEMRI, yaani ERC.

Utaratibu:

Daktari wako ameshakueleza kwamba anahitaji upimwe damu ili ajue kama una magonjwa fulani. Ikiwa utakubali kujihusisha na utafiti huu, kwanza tutakuuliza maswali machache juu ya umri wako, unapoishi, na kama umeshapewa chanjo ya yellow fever. Baadaye, tutatoa kiasi cha 5 mls (inawiana na kijiko kidogo cha chai kimoja) cha damu, zoezi ambalo litachukua muda mfupi tu. Tutatumia vifaa visafi na salama ambavyo vitafunguliwa mbele ya macho yako na vitatumika kwako tu na kutupwa mara tu baadaye. Baadaye, damu tutakayotoa kwa huu utafiti itapelekwa maabara Busia au/na Nairobi kwa upimaji wa Yellow Fever. Ili kuhakikisha usiri wa jina lako katika utafiti huu, jina na maelezo yako hayataandikwa kwenye sampuli ya damu, bali sampuli itatambulishwa na namba tu. Ni utaratibu wa utafiti huu kuwa watafiti hawatakujulisha majibu yako moja kwa moja ila kwa kupitia daktari wako wa matibabu hapa kliniki iwapo atayapokea matokeo ya sampuli yako toka kwetu haraka iwezekanavyo. Punde tu baadaye tutakuuliza maswali machache ambayo ni ya umuhimu katika kufanikisha utafiti huu ambayo yatanakiliwa mbele yako kwa muda wa dakika kumi na tano tu.

Faida/Mapungufu:

Utasikia maumivu kidogo ama woga wakati unachomwa sindano, lakini hutapata maumivu ya muda mrefu. Hutapata malipo yoyote ya kifedha, na pia hutatumia pesa zako mwenyewe katika utafiti huu. Utafiti huu utasaidia jamii yako kwa sababu tukifahamu matatizo ya jamii hii, tutaweza kushauri na kutengeneza miradi mbalimbali ya kupunguza athari za Yellow Fever.

Haki za mshirika:

Ushiriki wako katika utafiti huu ni wa hiari kabisa. Ukikataa kushiriki, hutanyimwa huduma zinazotolewa kwa kawaida.

Usiri/Utunzaji wa taarifa:

Katika utafiti huu, tutahakikisha kuwa maelezo yako, jina lako ni siri kabisa. Jina lako halitaandikwa au kuhusishwa kwa hii fomu, sampuli, na popote ndani ya ripoti nzima tutakayotoa baadaye.

Mawasiliano

Iwapo utakuwa na swali kuhusiana na haki zako ama utafiti huu, unaruhusiwa kuwasiliana na afisa yeyote wa utafiti au Allan Kwallah wa KEMRI, Nambari ya simu 0202722541 ext 2256/2290

Ridhaa ya kutolewa damu:

Napenda kukuuliza ridhaa yako ya ushiriki wako katika zoezi la utoaji damu

Nimeelewa maelezo ya hapo juu yanayohusu utafiti huu, na ninakubali kushiriki katika zoezi hili. Naelewa kuwa ushiriki wangu ni wa hiari, na pia kama ninabadili nia kuhusu ushiriki wangu muda wowote, naruhusiwa kujiondoa katika zoezi hili bila kushurutishwa kutoa sababu. Natoa ridhaa damu yangu itumike katika upimaji wa Yellow Fever. Sahihi/dole gumba………………………… Tarehe………………..

Appendix VII: Assent forms for minors

Informed Assent for minors

My name is…..………and I work in this health facility. We think that Yellow Fever is an important cause of febrile illness in this area. In order to be sure about the exact magnitude of the problem, we are conducting a study to develop assays for the detection of Yellow Fever. The information we gather is useful to the government and other policy makers who may consider preventative programs in this community or other communities in the future. We will summarize our findings from this study and disseminate it to various stakeholders including Ministry of Health, KEPI, KEMRI, and others. The KEMRI's ethical review committee, who are responsible for conducting such reviews at national level, has approved this study.

Research Procedures: Your child is here to have a little blood drawn because your child's clinician has recommended some tests**.** If you allow your child to participate in this study, we will ask you a few questions regarding where your child resides and his/her vaccination status. Then we will take 5 mls (1 teaspoon) of blood during the procedure. We will use sterile and disposable instruments that are clean and safe. The extra blood taken from your child will be transported to the KEMRI Busia or/and Nairobi laboratories for analysis of your child's Yellow Fever Status. The tests we conduct may also identify other infections related to Yellow Fever if these are present. In order to ensure complete confidentiality of the test results, no names will be attached to the blood samples, but a number assigned to your child will be used to label the sample. In addition we will take 15 minutes of your time to fill up a questionnaire that is related to this work.

Risk/benefits: During this procedure there will be no long-lasting effect. However, your child may feel a brief moment of pain or fear. Your child will not be given any monetary benefits; neither will he/she incur any costs. The study will benefit your community since by helping us and the government to understand the problems your community is facing as a result of Yellow Fever, we will be able to recommend and design appropriate interventions to minimize the impact of this infectious disease.

Your child's Rights: Your child's participation in this study is voluntary and if you disallow participation, you will not be denied any services that are normally available to you.

Confidentiality: We will make every effort to protect your child's identity. Your child will not be identified in any report or publication of this study or its results.

Contact Information: If you have questions now or in the future regarding your child's rights or this study, you may ask any of the field officers involved in this study or contact Allan Kwallah of KEMRI at 02027222541 ext 2256/2290.

Assent for the individual for blood sample:

May I now ask if you will allow your child to participate in the study?

The above details about the study and the basis of participation have been explained to me and **I allow my child** to take part in the study. I understand that I am free to callow my child to be part of the study. I also understand that if I do not want him/her to go on with the study, I can withdraw at any time. **I give my assent** for my child's blood to be tested for Yellow Fever.

Please sign here or put your right hand thumb mark if you agree:

Signature/ Thumb mark--   Date -------------------------

Ridhaa ya watoto walio chini ya miaka 18, kwa Kiswahili

Jina langu ni………..….. na ni mfanyakazi wa kituo hiki cha afya. Tunafanya utafiti juu ya ugonjwa uitwao Yellow Fever ambao tunafikiri ni mojawapo ya magonjwa yanayoambukiza jamii hii. Ili tujue umuhimu wa Yellow Fever hapa, tumeamua kufanya utafiti utakaolenga kuboresha upimaji wa virusi vya Yellow Fever. Matokeo ya utafiti huu yatasambazwa kwa serikali na wadau kama Wizara ya Afya, KEPI, KEMRI na wengineo, ambao watatengeneza miradi mbalimbali itakayolenga kupunguza maambukizi na kuboresha huduma za walioathirika katika jamii hii, na nyingine hapo baadaye. Utafiti huu umepitishwa na kuruhusiwa na kamati inayohusika na utoaji wa vibali vya utafiti ya KEMRI, yaani ERC.

Utaratibu:

Daktari wako ameshakueleza kwamba anahitaji mtoto wako apimwe damu ili ajue kama mtoto huyu ana magonjwa fulani. Ikiwa utakubali mtoto wako ahusike kwa utafiti huu, kwanza tutakuuliza maswali machache juu ya umri wake, anapoishi, na kama ameshapewa chanjo ya yellow fever. Baadaye, tutatoa kiasi cha 5 mls (inawina na kijiko kidogo cha chai kimoja) cha damu, zoezi ambalo litachukua muda mfupi tu. Tutatumia vifaa visafi na salama ambavyo vitafunguliwa mbele ya macho yako na vitatumika kwako tu na kutupwa mara tu baadaye. Damu tutakayotoa kwa huu utafiti itapelekwa maabara kwa upimaji wa Yellow Fever. Ili kuhakikisha usiri wa jina la mtoto wako katika utafiti huu, jina na maelezo yake hayataandikwa kwenye sampuli ya damu, bali sampuli itatambulishwa na namba tu. Punde tu baadaye tutakuuliza maswali machache ambayo ni ya umuhimu katika kufanikisha utafiti huu ambayo yatanakiliwa mbele yako kwa muda wa dakika kumi na tano tu.

Faida/Mapungufu:

Mtoto wako atasikia maumivu kidogo ama woga wakati anachomwa sindano, lakini hatapata maumivu ya muda mrefu. Hatapata malipo yoyote ya kifedha, na pia hatatumia pesa zako katika utafiti huu. Utafiti huu utasaidia jamii yako kwa sababu tukifahamu matatizo ya jamii hii, tutaweza kushauri na kutengeneza miradi mbalimbali ya kupunguza athari za Yellow Fever.

Haki za mtoto wako:

Ushiriki wa mtoto wako katika utafiti huu ni wa hiari kabisa. Ukikataa ashiriki, mtoto wako hatanyimwa huduma zinazotolewa kwa kawaida.

Usiri/Utunzaji wa taarifa:

Katika utafiti huu, tutahakikisha kuwa maelezo ya mtoto wako na jina lake ni siri kabisa. Jina lake halitaandikwa au kuhusishwa kwa hii fomu, sampuli, na popote ndani ya ripoti nzima tutakayotoa baadaye.

Mawasiliano

Iwapo utakuwa na swali kuhusiana na haki za mtoto wako ama utafiti huu, unaruhusiwa kuwasiliana na afisa yeyote wa utahini, au Allan Kwallah wa KEMRI, Nambari ya simu 0202722541 ext 2256/2290

Ridhaa ya kutolewa damu:

Napenda kukuuliza ridhaa yako ya ushiriki wa mtoto wako katika zoezi la utoaji damu

Nimeelewa maelezo ya hapo juu yanayohusu utafiti huu, na ninakubali mtoto wangu ashiriki katika zoezi hili. Naelewa kuwa ushiriki wa mtoto wangu ni wa hiari, na pia kama nabadili nia kuhusu kushiriki kwake muda wowote ule naruhusiwa kumuondoa katika zoezi hili bila kushurutishwa kutoa sababu. Natoa ridhaa damu ya mtoto wangu itumike katika upimaji wa Yellow Fever.

Sahihi/dole gumba…………………………

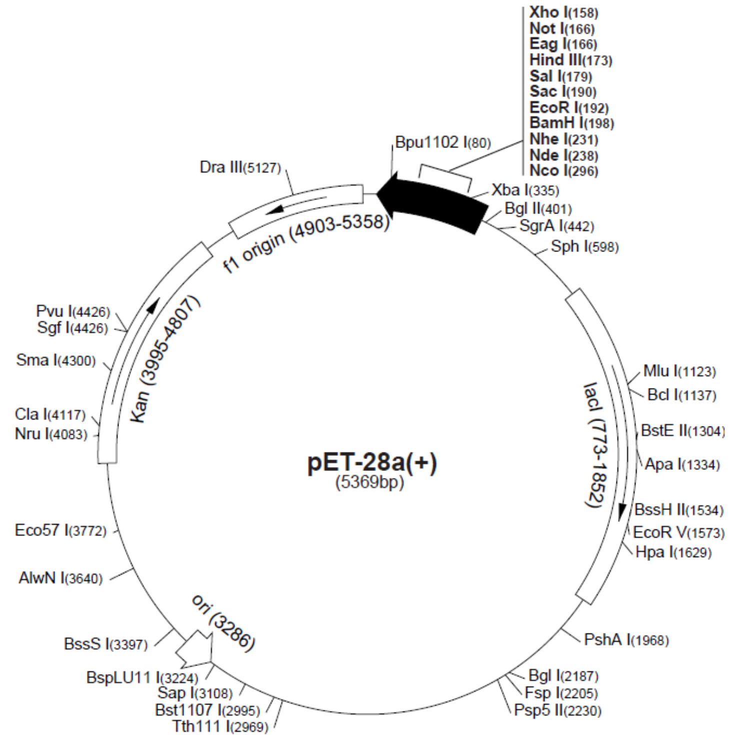
Tarehe………………

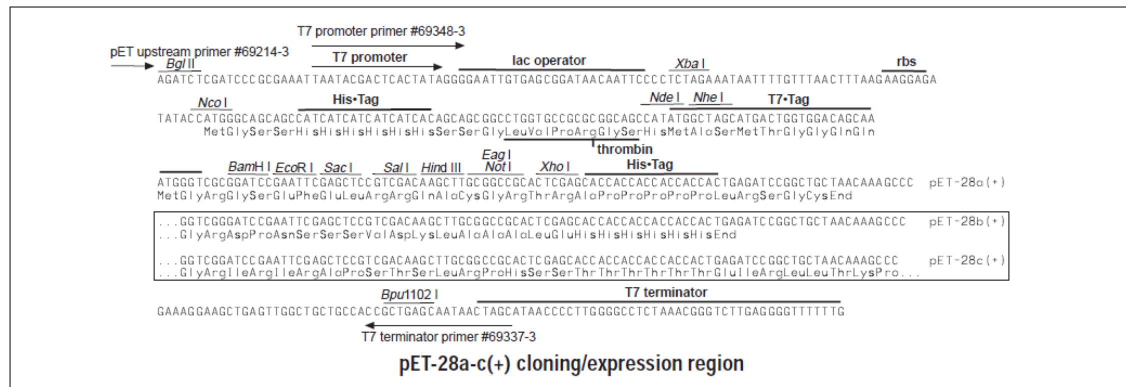
Appendix VIII: pET 28a showing BamHI and SalI sites

pET-28a(+) sequence landmarks

T7 promoter	370-386
T7 transcription start	369
His•Tag coding sequence	270-287
T7•Tag coding sequence	207-239
Multiple cloning sites	
(*Bam*H I - *Xho* I)	158-203
His•Tag coding sequence	140-157
T7 terminator	26-72
lacI coding sequence	773-1852
pBR322 origin	3286
Kan coding sequence	3995-4807
f1 origin	4903-5358

The maps for pET-28b(+) and pET-28c(+) are the same as pET-28a(+) (shown) with the following exceptions: pET-28b(+) is a 5368bp plasmid; subtract 1bp from each site beyond *Bam*H I at 198. pET-28c(+) is a 5367bp plasmid; subtract 2bp from each site beyond *Bam*H I at 198.





pET-28a-c(+) cloning/expression region

Appendix IX: Reprints of published papers

Journal of Virological Methods 193 (2013) 23–27

Contents lists available at SciVerse ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Short communication

A real-time reverse transcription loop-mediated isothermal amplification assay for the rapid detection of yellow fever virus

Allan ole Kwallah[a,d], Shingo Inoue[b,*], Anne W.T. Muigai[a], Toru Kubo[b], Rosemary Sang[c], Kouichi Morita[b], Matilu Mwau[d]

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A B S T R A C T

Yellow fever, a mosquito-borne disease, is an important viral hemorrhagic fever in Africa and South America where it is endemic. Detection of yellow fever virus (YFV) in Africa remains a challenge due to a lack of highly specific tests. The aim of this study was to develop and optimize a rapid detection reverse transcription loop-mediated isothermal amplification (RT-LAMP) for YFV. The RT-LAMP was done isothermally at 62 °C using a real-time turbidimeter that allowed detection within 1 h. Specificity of the RT-LAMP was determined using RNA from flaviviruses and other related viruses where only YFV RNA was detected: West Nile virus, dengue viruses, Japanese encephalitis virus, Rift Valley fever virus, and chikungunya virus. In addition, equal sensitivity was also observed when the RT-LAMP and the real-time RT-PCR were compared using YFV-spiked human serum samples with a detection limit of 0.29 PFU/ml. Two Kenyan YFV wild strains showed an equal detection limit as the vaccine strain 17D in this study. The RT-LAMP reduced the time of reaction from 3 h to 1 h and increased sensitivity tenfold compared to RT-PCR. Therefore, this test offers a simple, rapid and reliable diagnostic tool for yellow fever when there are outbreaks of acute hemorrhagic fever in Kenya and other African countries.

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Yellow fever (YF), a mosquito-borne disease, is an important viral hemorrhagic fever in Africa and South America where it is endemic (Gould et al., 1989). Yellow fever virus (YFV) is the prototype of the family *Flaviviridae* and the genus *Flavivirus* together with other arboviruses including dengue viruses (DENVs) (Monath and Nystrom, 1984). YFV is a non-segmented, single-stranded, positive-sense RNA of approximately 11 kb nucleotides in length encoding three structural (capsid, pre-membrane/membrane, and envelope) proteins and seven non structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Rice et al., 1985). There are three well described YFV transmission cycles: a sylvatic transmission cycle between non-human primates, *Aedes africanus* and canopy-dwelling mosquitoes such as *Hemagogus janthinomys* (Weaver and Reisen, 2010); an urban cycle between humans and *Aedes aegypti* in Africa and South America; and, a savannah cycle between humans and various *Aedes* mosquitoes reported only in Africa (Barrett and Monath, 2003). These three transmission cycles lead to the same clinical disease (Vainio and Cutts, 1998). After an incubation period of three to six days, there is a sudden onset of fever, chills, headache, viremia and neutropenia. Within a week symptoms such as nausea, hematemesis, melena, jaundice and distressing pain in the back, loins and limbs are observed with case fatality rates of more than 50% (Monath, 2008; WHO, 2010).

Africa continues to be faced with the risk of an YF outbreak as shown by events of the past three decades. In the Eastern African region outbreaks have been reported in Kenya (1992–93) (Sanders et al., 1998), Sudan (2003, 2005) (Onyango et al., 2004; Gould et al., 2008) and Uganda (2010–11) (WHO, 2011; McMullan et al., 2012). The resurgence of YF in the Eastern African region raises public health concerns, creating a need to improve surveillance, early warning systems and expanded immunization programs (Ellis and Barrett, 2008). The last YF outbreak in Kenya, 1992–1993, caused high mortality and morbidity since low immunity levels existed within the affected communities (Sanders et al., 1998; Reiter et al., 1998).

Due to the genetic homology in the flavivirus group, YFV has shown cross reactivity with other members of the genus *flavivirus* – in particular, DENV (Houghton et al., 2008) and West Nile virus (WNV) (Mansfield et al., 2011). Although several YFV serological diagnostic tests such as the IgM capture ELISA, the immunofluorescence antibody test (IFA), and the hemagglutination inhibition test (De Brito et al., 1992; Deubel et al., 1997; Monath et al., 1989;

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Monath and Nystrom, 1984) have been developed, these show cross reactivity among flavivirus antibodies. Virus isolation and plaque reduction neutralization tests (PRNT) have been used as confirmatory tests in reference laboratories, although the technical requirements are time-consuming (Groot and Ribeiro, 1962). Recently, molecular approaches, such as RT-PCR (Tanaka, 1993; Drosten et al., 2002) and real-time RT-PCR (Bae et al., 2003), have been used in the detection of YFV.

The loop-mediated isothermal amplification (LAMP) is an alternate method for nucleic acid amplification that was developed by Notomi et al. (2000). The technique has been optimized for several arboviruses including DENV serotypes 1–4 (Parida et al., 2005), Japanese encephalitis virus (JEV) (Parida et al., 2006; Toriniwa and Komiya, 2006), chikungunya virus (CHIKV) (Parida et al., 2007), Rift Valley fever virus (RVFV) (Le Roux et al., 2009), and WNV (Parida et al., 2004), although YFV reverse transcription-LAMP (RT-LAMP) has not been described in literature. The present study reports the design and testing of an RT-LAMP for the rapid detection of YFV.

The RT-LAMP primers were designed from the non-structural protein 1 (NS1) region of the YFV strain: 17D (GenBank ID: P03314; Rice et al., 1985). Alignment of the NS1 nucleotide sequence with the available GenBank sequences of 20 YFV strains was performed using CLCBio MAINWORKBENCH Software V 6.01 (http://www.clcbio.com/; CLCBio, Aarhus, Denmark) to identify the conserved regions. Six primers were designed following a described criteria (Notomi et al., 2000): the forward outer primer (F3), the reverse outer primer (B3), the forward inner primer (FIP), the reverse inner primer (BIP), the forward loop primer (FLP), and the reverse loop primer (BLP). The primers were designed using Eiken's LAMP software Primer Explorer V4 (http://primerexplorer.jp/e/; Eiken Chemical, Tokyo, Japan).

The YFV live attenuated vaccine strain 17D was propagated in Vero cells (African green monkey kidney cell line) maintained at 37 °C in Eagle's minimum essential medium (EMEM) supplemented with 2% fetal calf serum (FCS) and 0.2 mM nonessential amino acids (NEAA) and harvested after 7 days. In addition, YFV (strain: Baringo 1), YFV (strain: Baringo 2), DENV1 (strain: Hawaii), DENV2 (strain: 00St-22A), DENV3 (strain: SLMC50), DENV4 (strain: SLMC318), JEV (strain: ML-17), CHIKV (strain: S27), RVFV (strain: Smithburn) and WNV (strain: Eg101) viruses were propagated by regular sub-culturing in either *Aedes albopiticus* clone C6/36 cells (Igarashi, 1978) or Vero cells. A confluent monolayer of Vero or C6/36 cells was inoculated with the respective virus and then incubated at 37 or 28 °C, respectively. Cells were observed daily for cytopathic effect (CPE); at 80% or more CPE, the infected culture fluid (ICF) was harvested by centrifugation at 600 × *g* for 10 min at room temperature.

The propagated viruses were titrated using a modified plaque assay to establish the virus titer (De Madrid and Porterfield, 1969). Briefly, Vero cells were seeded in a 6-well plate and cultured until a monolayer of cells was observed. Tenfold serial dilutions of respective virus (1×10^1 to 1×10^7) were prepared in EMEM containing 2% FCS and added to each corresponding well in duplicate followed by an overlay of EMEM containing 2% FCS and 1.25% (w/v) methylcellulose. The plates were placed in a 5% CO_2 incubator at 37 °C for three to six days. The staining of cells was done using 0.5% (w/v) Crystal violet and the plaque forming units per ml (PFU/ml) were derived.

Genomic viral RNA was extracted from 140 μl of the ICF of Vero cells using a QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The extracted RNA was eluted in a total volume of 60 μl and stored at −80 °C for the comparative RT-PCR, RT-LAMP and real-time RT-PCR. The RT-PCR was performed using a Titan One Tube RT-PCR kit (Roche Diagnostics, Basel, Switzerland). The RT-PCR amplification was carried out in a 25 μl reaction mix containing 5 μl template RNA. The YFV specific primers used were: sense YFVP1 (TAC CCT GGA GCA AGA CAA GT) (nucleotide number: 1042-1061) (Eldadah et al., 1991) and antisense YFVC10 (AGC AAG AAA CAT GTC CAC CA) (nucleotide number: 1823-1804) targeting a 782 bp fragment of the envelope region (Tanaka, 1993). The RT-PCR profile included a reverse transcription step at 37 °C for 1hr, with the following cycling times and temperatures: one cycle at 94 °C for 3 min; 35 cycles at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min; and, one cycle at 72 °C for 10 min as a final extension step. Ten microliters of the RT-PCR amplicons were subjected to electrophoresis in a 2% agarose gel (SeaKem GTG, Basel, Switzerland) in Tris-Acetate-EDTA buffer and stained using 0.5 μg/ml ethidium bromide for visualization under UV light at a wavelength of 302 nm.

The RT-LAMP was carried out in a total reaction volume of 25 μl using a Loopamp RNA amplification kit (Eiken Chemical, Tokyo, Japan) with 40 pmol of inner primers FIP and BIP, 20 pmol of loop primers LF and LB, and 5 pmol of outer primers F3 and B3. Five microliters of eluted viral RNA was used as the template per reaction. Real-time monitoring of the RT-LAMP was done using a LA-320 Loopamp Real-time Turbidimeter (Teramecs, Kyoto, Japan) by incubation at 62 °C for 60 min and inactivation at the end of the reaction at 80 °C for 5 min. The generated data were collected using LA-320 software and then exported to Microsoft Office Excel 2010 (Microsoft, Washington, USA). The turbidity at OD 400 nm was plotted against time to positivity while the threshold was set at 0.1.

The limit of detection of the developed RT-LAMP was compared with a real-time RT-PCR that targeted the 3′ untranslated region (Bae et al., 2003). Briefly, a one-step, real-time RT-PCR was performed using a SuperScript III Platinum One-Step kit (Invitrogen, California, USA) and an ABI 7500 real-time PCR machine (Applied Biosystems, Foster City, USA). RNA was reverse transcribed at 50 °C for 30 min and initial denaturation was performed at 95 °C for 2 min followed by 40 cycles: 95 °C for 15 s, 60 °C for 45 s. Data generated by the real-time RT-PCR were collected using Sequence Detection Software (Applied Biosystems, Foster City, USA).

Ethical approval for human sample use was sought and granted by the Kenya Medical Research Institute's Ethical Review Board (SSC 1829). The consenting human serum donors had no YFV vaccination history as confirmed by screening using an indirect IgG ELISA. Eight of these samples were YFV 17D ICF spiked in tenfold serial dilutions ranging from 2900 PFU/ml to 0.0029 PFU/ml. Two human serum samples were spiked with EMEM containing 2% FCS and used as a negative control. Two YFV strains obtained from the 1992–1993 outbreak in Kenya (Sanders et al., 1998) were used for validation of the assay. Briefly, virus titration using a plaque assay was performed and the PFU/ml generated. The two strains were then equilibrated to 2900 PFU/ml like the virus titer of a YFV strain: 17D used in the study. The equilibrated Kenyan YFV strains were then diluted serially tenfold ranging from 2900 PFU/ml to 0.0029 PFU/ml. RNA was extracted from the diluted ICF and RT-PCR and RT-LAMP were performed simultaneously for the detection of viral RNA, as described above.

The designed primer sequences targeting the NS1 region of the YFV were successfully developed, as shown in Table 1. Furthermore, the time to positivity was below 30 min with the inclusion of loop amplification primers. The RT-LAMP depicted an exponential rise in the turbidity recorded in under a 20-min period regardless of the varying virus titres at the beginning of the reaction. In the RT-LAMP, the attainment of the turbidity peak did not vary while using the different RNA templates of a diluted ICF. However, there was a peak variation in the real-time RT-PCR using the same RNA templates, which made it difficult to interpret the results. The limit of detection of the RT-LAMP for the detection of YFV RNA was determined using tenfold serial dilutions and was assessed in parallel with real-time RT-PCR and RT-PCR. The detection limit of the RT-LAMP and

Table 1
Details of the RT-LAMP primers used for the amplification of the YFV NS1 region.

Primer name	Type	Length	Genome position[a]	Sequence (5′–3′)
F3	Forward outer	20	3090-3109	TCCACACCTTGGAGGCATTA
B3	Reverse outer	20	3331-3313	GTCCATCACAGTTGCCATCA
FIP	Forward inner	42	3200-3181 (F1c)+TTTT	GGCCTCCGATTGATCTCGGC
			3121-3138 (F2)	GTGTGAGTGGCCACTGAC
BIP	Reverse inner	44	3235-3256 (B2)+TTTT	GGTTCAGACGAACGGACCTTGG
			3298-3281 (B1c)	CCCTGGGCAAGCTTCTCT
LF	Forward loop	25	3164-3140	CTTCAACTGATGTTCCAATCGTATG
LB	Reverse loop	22	3257-3278	ATGCAGGTACCACTAGAAGTGA

[a] Genome position according to the YFV strain: 17D (GenBank accession number P03314).

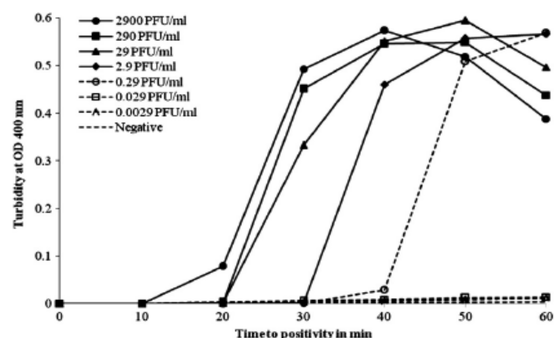

Fig. 1. Analytical sensitivity of YFV RT-LAMP on YFV 17D ICF. Tenfold diluted serially YFV17D from 2900 PFU/ml was used and detected using a real-time turbidimeter, LA 320; plaque forming unit (PFU); yellow fever virus: strain 17D (YFV 17D); infected culture fluid (ICF).

real-time RT-PCR was 0.29 PFU/ml while that of the RT-PCR was 2.9 PFU/ml (Figs. 1 and 2).

The specificity of the developed YFV RT-LAMP shown in Fig. 3 demonstrates that only YFV was amplified against JEV, DENV1-4, WNV, CHIKV and RVFV to assess the cross-reactivity of the test. Through a limiting dilution assay, one human serum sample spiked with YFV (strain: 17D) ICF was compared with YFV (strain: 17D) ICF in a bid to assess serum interference with the RT-LAMP. A 100% concordance between the spiked human serum sample and YFV 17D ICF with regard to sensitivity was observed (Figs. 1 and 4). The time to positivity in both was below 30 min and the detection limit was 0.29 PFU/ml. The validation of the developed RT-LAMP was evaluated using two Kenyan strains from the YFV 1992–1993

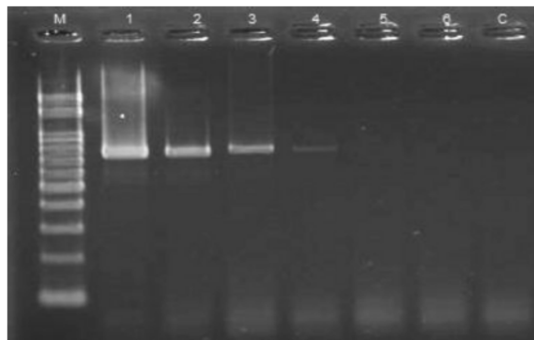

Fig. 2. Detection limit of YFV using RT-PCR. M (Molecular marker), 1 (2900 PFU/ml), 2 (290 PFU/ml), 3 (29 PFU/ml), 4 (2.9 PFU/ml), 5 (0.29 PFU/ml), 6 (0.029 PFU/ml), C (Negative control).

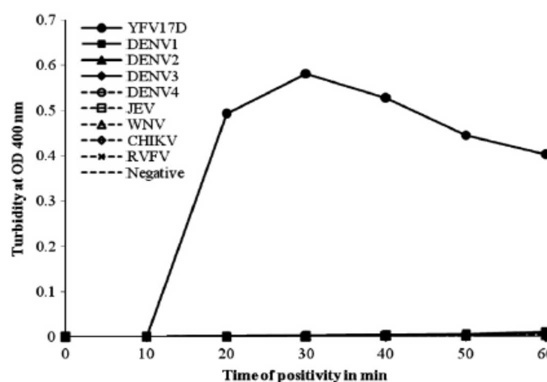

Fig. 3. Detection specificity of the YFV RT-LAMP. *Flaviviridae*; yellow fever virus (YFV), dengue viruses (DENV 1-4), Japanese encephalitis virus (JEV), West Nile virus (WNV), *Togaviridae*; chikungunya virus (CHIKV), and *Bunyaviridae*; Rift Valley fever virus (RVFV) were used.

Kenya outbreak (Sanders et al., 1998) isolated at the Viral Hemorrhagic Fever (VHF) laboratory at KEMRI, Kenya. The two Kenyan strains had earlier been laboratory confirmed using YFV RT-PCR at the VHF laboratory (Sang, personal communication). The performance of the RT-LAMP was compared to the established real-time

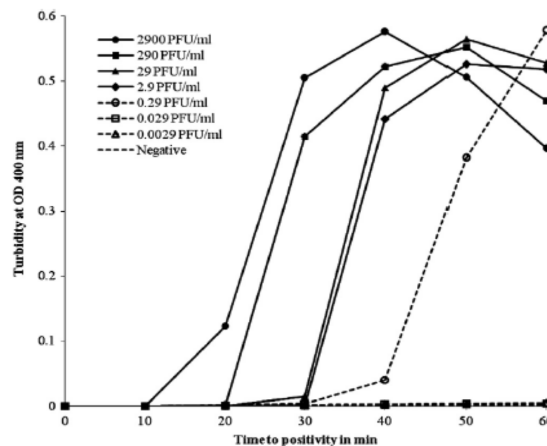

Fig. 4. Analytical sensitivity of the YFV RT-LAMP on human serum spiked using YFV 17D. Tenfold diluted serially YFV 17D from 2900 PFU/ml to 0.0029 PFU/ml was detected using a real-time turbidimeter, LA 320. Plaque forming unit (PFU); yellow fever virus strain: 17D (YFV 17D); infected culture fluid (ICF).

Table 2
Detection limit of three assays against yellow fever virus strains including 17D and two Kenya strains (1992–1993): RT-LAMP, Real-time RT-PCR, and RT-PCR.

	17D	Baringo 1	Baringo 2
RT-LAMP	0.29 PFU/ml	0.29 PFU/ml	0.29 PFU/ml
Real-time RT-PCR	0.29 PFU/ml	0.29 PFU/ml	0.29 PFU/ml
RT-PCR	2.9 PFU/ml	2.9 PFU/ml	2.9 PFU/ml

RT-PCR and RT-PCR used for the diagnosis of YFV17D and the two Kenyan strains in a limiting dilution test. The YFV RT-LAMP further showed an equal detection limit sensitivity of 0.29 PFU/ml while using the two Kenyan strains and the vaccine strain (strain: 17D) (Table 2).

Although regular vaccination is the ideal policy for disease control of YF, often YF endemic countries are faced with frequent outbreaks. An alternative approach using early detection and containment followed by post-outbreak vaccination might reduce the disease burden. Thus, the time required for the diagnosis of YF is a critical factor for early detection and containment of the disease. The laboratory confirmation of suspected cases uses several YF diagnostic assays that include IgM-capture ELISA, IFA, virus isolation, RT-PCR, and PRNT (De Brito et al., 1992; Deubel et al., 1997; Monath et al., 1989; Tanaka, 1993; Drosten et al., 2002; Groot and Ribeiro, 1962). Although IgM-capture ELISA is used as a standard serological test in reference laboratories, the detection of IgM relies mainly on convalescent phase samples. Furthermore, there is cross-reactivity of IgM amongst related flaviviruses that co-exist in the same region such as DENV and WNV, which makes YFV diagnosis difficult. Both virus isolation and PRNT are highly specific and are routinely used as confirmatory tests at the reference laboratories even if it takes one week to get results. Therefore, the advent of molecular-based assays, such as RT-PCR, has aided in the rapid detection of YFV (Tanaka, 1993; Drosten et al., 2002) and real-time RT-PCR (Bae et al., 2003). Hence, an RT-LAMP assay used to detect YFV would augment the existing real-time RT-PCR and conventional RT-PCR in the timely detection of YFV by reducing the time of reaction from more than 3 h to less than 1 h including the results observation via the real-time monitoring system.

The sensitivity of RT-LAMP indicated that serum did not interfere with the assay hence it can be applied in the detection of YFV in clinically suspected patients. However, there is further need to intensively validate the assay using YF clinical samples from various endemic countries. The specificity of RT-LAMP was confirmed by amplification only of YFV but not DENV 1–4, WNV, JEV, CHIKV or RVFV tested using the developed YFV primer set. This high specificity could solve the cross reactivity problem of serological diagnosis among these viral diseases. The stable reactivity of the RT-LAMP assay of YFV might be attributed to the less than 5% mismatch of the nucleotides in the conserved NS1 region among 5 genotypes. Furthermore, the archiving of the samples over a ten-year period did not affect the detection efficacy of the assay, which might be due to the slow evolution speed of YFV (Sall et al., 2010).

In summary, YFV RT-LAMP could be used as an extremely rapid and highly sensitive and specific technique along with virus isolation and IgM-capture ELISA as a comprehensive detection system in diagnostic laboratories in Africa and South America where YF and other viral hemorrhagic fevers are endemic.

Conflict of interest

We have no conflicting interest regarding this work.

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Short Communication

Seroprevalence of Yellow Fever Virus in Selected Health Facilities in Western Kenya from 2010 to 2012

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SUMMARY: Yellow fever (YF), which is caused by a mosquito-borne virus, is an important viral hemorrhagic fever endemic in equatorial Africa and South America. Yellow fever virus (YFV) is the prototype of the family *Flaviviridae* and genus *Flavivirus*. The aim of this study was to determine the seroprevalence of YFV in selected health facilities in Western Kenya during the period 2010–2012. A total of 469 serum samples from febrile patients were tested for YFV antibodies using in-house IgM-capture ELISA, in-house indirect IgG ELISA, and 50% focus reduction neutralization test ($FRNT_{50}$). The present study did not identify any IgM ELISA-positive cases, indicating absence of recent YFV infection in the area. Twenty-eight samples (6%) tested positive for YFV IgG, because of either YFV vaccination or past exposure to various flaviviruses including YFV. Five cases were confirmed by $FRNT_{50}$; of these, 4 were either vaccination or natural infection during the YF outbreak in 1992–1993 or another period and 1 case was confirmed as a West Nile virus infection. Domestication and routine performance of arboviral differential diagnosis will help to address the phenomenon of pyrexia of unknown origin, contribute to arboviral research in developing countries, and enhance regular surveillance.

Yellow fever (YF), a disease caused by mosquito-borne virus, is an important viral hemorrhagic fever endemic in equatorial Africa and South America (1,2). Yellow fever virus (YFV) is the prototype of the family *Flaviviridae* and genus *Flavivirus* (3). The genus *Flavivirus* contains approximately 70 viruses including YFV, dengue viruses (DENVs), Japanese encephalitis virus (JEV), and West Nile virus (WNV) that are of major human public health concern (4). An estimated annual incidence of 200,000 cases of YF causing 30,000 deaths is reported each affected individuals without treatment (4,5). In the eastern African region, YF outbreaks have been reported in Kenya (1992–1993) (6,7), South Sudan (2003, 2005) (8,9), Uganda (2010–2011) (10), Sudan (2012) (11), and Ethiopia (2013) (12).

YF cases are difficult to diagnose from the clinical symptoms in the early stages of the illness because of "non-specific influenza-like symptoms" that are similar to those of other febrile illnesses. Differential diagnoses may include malaria, viral hepatitis, dengue, leptospirosis, or other hemorrhagic fevers (13). WHO recommends case definition for suspected YF as "any case presenting with acute onset of fever, with jaundice appearing within 14 days after the onset of the first symptoms" (14). The aim of this study was to determine the seroprevalence of YFV in selected health facilities in Western Kenya during the period 2010–2012.

A total of 469 human whole blood samples were collected from the following healthcare facilities in Kenya: Andersen Medical Centre (AMC), Endebess Sub District Hospital (END), and Kitale District Hospital (KDH) in Trans Nzoia County; Centre for Infectious and Parasitic Diseases Control Research, Kenya Medical Research Institute (KEMRI) clinic (CIP) located in Busia County, and Kapluk Dispensary (KAP) in Baringo County. Ethical approval for human sample use was sought and granted by KEMRI Ethical Review Board (SSC No. 1829). In addition, positive control serum samples were kindly provided by Dr. R. Sang (KEMRI, Nairobi, Kenya) because Kenya has not had a recent YF outbreak other than the one from 1992–1993 (6,7). The negative controls used in the present study were generated from consenting healthy volunteers who had not received YFV vaccine or had no pre-exposure to YFV field infection. YFV (strain: 17D), DENV serotype 1 (DENV1) (strain: Hawaii), DENV serotype 2 (DENV2) (strain: 00St-22A), DENV serotype 3 (DENV3) (strain: SLMC50), DENV serotype 4 (DENV4) (strain: SLMC318), and WNV (strain: Eg101) were grown on either C6/36 (mosquito cell line) (15) or Vero cells (African green monkey kidney cell line).

Recent YFV exposure was checked using in-house IgM-capture ELISA following the protocol of Bundo and Igarashi (16). A P/N (positive control [or sample] OD_{492}/negative control OD_{492}) ratio of ≥ 2.0 was considered positive. In-house indirect IgG ELISA, which used sucrose-gradient purified YFV antigen, was performed for detection of YFV IgG (17). Each sample was tested in duplicate and measured for mean OD_{492}; The IgG titer was calculated using a positive standard curve.

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With a cut-off value of positive IgG of 1:1,000 + 3 standard deviations, a sample titer of ≥1:3,000 was considered IgG-positive for YFV (17,18).

A 50% focus reduction neutralization test ($FRNT_{50}$) was used to determine the presence of YFV-specific neutralizing antibodies in test serum samples that were regarded as positive by IgM-capture ELISA and/or indirect IgG ELISA. The reciprocal of the endpoint serum dilution that provided a ≥50% reduction in the mean number of foci relative to the control wells that contained no serum was considered to be the $FRNT_{50}$ titer (19). In-house flavivirus indirect IgG ELISA using a sucrose-gradient purified JEV antigen (strain ML17) was performed for the detection of flavivirus cross reactive IgG among the YFV indirect IgG ELISA positive samples (17). The mean OD_{492} for each serum sample was measured and IgG titers were calculated using a positive standard curve. With a cut-off value of positive IgG set at 1:1,000 + 3 standard deviations, a sample titer of ≥1:3,000 was considered IgG positive for flavivirus (17,18). Data were analyzed using SPSS 20 (SPSS for Windows) (SPSS, Chicago, IL, US). IgG titers were expressed as the geometric mean. Analysis of variance (ANOVA) was used to compare means among the facilities. Correlation of the IgG titer with sex, age, and occupation was expressed as *P* values of Pearson's correlation coefficients. The level of significance was set at $P < 0.05$.

In the present study, none of the samples tested positive for YFV IgM. Twenty-eight samples (6%) had YFV IgG titers of 3,000–12,000 (Table 1) (Fig. 1). Among the 5 facilities, CIP showed the highest seropositivity; however, the GMT of positive YFV IgG titers was not statistically significant. There was a significant difference in YFV IgG seropositivity among the study sites ($P = 0.022$). In addition, there was no significant difference in YFV IgG titer in terms of different age groups ($P = 0.857$), sex ($P = 0.835$), and occupation ($P = 0.496$) (Tables 1 and 2). Twenty-eight samples that had YFV indirect IgG ELISA titers of ≥1:3,000 were further analyzed by $FRNT_{50}$ for confirmation of YFV infection using YFV, DENV1, DENV2, DENV3, DENV4, and WNV. Four out of the 28 YFV IgG-positive samples showed YFV-specific neutralization titers of ≥1:10. Notably, sample S0004 showed a neutralization titer of 1:640, whereas the other 3 samples, A0716, K0870, and K0960, showed neutralization titers of <1:100 (Table 2). In addition, none of the 4 samples showed a neutralization titer of ≥1:10 against DENV1, DENV2, DENV3, DENV4, and WNV. Moreover, K1013 showed a neutralization titer of 1:610 against WNV but ≤1:10 against YFV, DENV1, DENV2, DENV3, and DENV4. All 28 YFV IgG-positive samples yielded positive results for flavivirus indirect IgG ELISA using JEV (Fig. 2).

In the present study, there was a 6% overall YFV IgG prevalence rate during the period 2010–2012 in selected parts of Western Kenya. In an earlier study by Mease et al. in 2011, an overall prevalence of 9.2% was recorded (20). Notably, Busia showed prevalence rates of 3.2%, in Mease's study and 16.7% in the present study. However, the sampled population in Mease's study included mostly adults; in contrast, in the present study, both adults and children were included. Of the 3 sites in Trans Nzoia County is close to the Uganda border; of the 3 sites, END and AMC have a forest set-up upon account of nearness to Mt. Elgon while KDH has an urban set-up. However, the seroprevalence rates and GMT of IgG titers did not significantly differ among the 3 facilities. In the present study, KAP showed a relatively high prevalence rate of 7.7% even with the small sample set used; notably, KAP is located in close proximity to the region of the last major YFV outbreak in Kenya in 1992–1993 (21).

Because there is cross-reactivity among flaviviruses, more so when flaviviruses are endemic in the same region, establishment of FRNT as a test will largely aid

Table 1. Sociodemographic characteristics of study participants by facility

County	Trans Nzoia			Baringo	Busia	Total	ANOVA
Facility	AMC ($n = 94$)	END ($n = 201$)	KDH ($n = 125$)	KAP ($n = 13$)	CIP ($n = 36$)	($n = 469$)	
Sex							
Female	52 (3)	109 (2)	74 (9)	5 (1)	22 (4)	262 (19)	0.835[2]
Male	42 (2)	93 (3)	50 (2)	8	14 (2)	207 (9)	
Age							
1–15	4	11	10	0	36 (6)	61 (6)	0.857[2]
16–30	34 (1)	113	59 (7)	6	0	212 (8)	
31–45	43 (3)	30 (3)	33 (2)	2	0	108 (8)	
46–60	8 (1)	34	11 (1)	4	0	57 (2)	
>61	5	13 (2)	12 (1)	1 (1)	0	31 (4)	
Occupation							
Others	81 (4)	81 (2)	122 (9)	7	36 (6)	327 (21)	0.496[2]
Farmer	6 (1)	61 (2)	2 (2)	6 (1)	0	75 (6)	
Home stay	7	59 (1)	1	0	0	67 (1)	
Seropositivity							
IgM (%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	
IgG (%)	5 (5.3)	5 (2.5)	11 (8.8)	1 (7.7)	6 (16.7)	28 (6.0)	0.022[1]

[1]: statistically significant.
[2]: not statistically significant.
( ) indicate YFV IgG seropositive samples per site. AMC, Andersen Medical Center; CIP, KEMRI-Alupe clinic; END, Endebess District Hospital; KDH, Kitale District Hospital; KAP, Kapluk Dispensery.

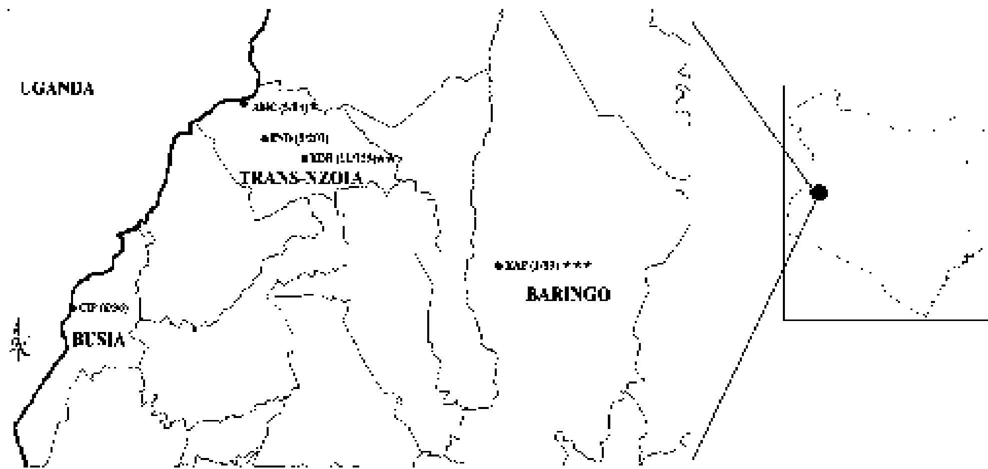

Fig. 1. Locations showing hospital based sampling sites in the study. *, 1 YF confirmed case by YFV $FRNT_{50}$; **, 2 YF confirmed cases by YFV $FRNT_{50}$ and one WNV confirmed case by WNV $FRNT_{50}$; ***, 1 YF confirmed case by YFV $FRNT_{50}$. AMC, Andersen Medical Center; CIP, KEMRI-Alupe clinic; END, Endebess District Hospital; KDH, Kitale District Hospital; KAP, Kapluk Dispensery. ( ) indicate YFV IgG seropositive samples per site. Source DIVA-GIS v 7.5.0.0.

Table 2. Neutralization test against six different flaviviruses using $FRNT_{50}$ of all the 28 YFV IgG ELISA positive samples

County	Sample	IgG titer	GMT of IgG	YFV	DENV1	DENV2	DENV3	DENV4	WNV
Trans Nzoia	A0112	3,500	4,917	<10	<10	<10	<10	<10	<10
	A0130	6,000		<10	<10	<10	<10	<10	<10
	A0132	3,800		<10	<10	<10	<10	<10	<10
	A0716	9,000		35	<10	<10	<10	<10	<10
	A0724	4,000		<10	<10	<10	<10	<10	<10
	E0046	9,000	6,146	<10	<10	<10	<10	<10	<10
	E0094	7,000		<10	<10	<10	<10	<10	<10
	E0097	4,000		<10	<10	<10	<10	<10	<10
	E0103	6,000		<10	<10	<10	<10	<10	<10
	E0416	5,800		<10	<10	<10	<10	<10	<10
	K0116	5,000	6,280	<10	<10	<10	<10	<10	<10
	K0135	8,000		<10	<10	<10	<10	<10	<10
	K0145	3,600		<10	<10	<10	<10	<10	<10
	K0169	4,000		<10	<10	<10	<10	<10	<10
	K0870	10,200		80	<10	<10	<10	<10	<10
	K0936	7,500		<10	<10	<10	<10	<10	<10
	K0960	9,000		60	<10	<10	<10	<10	<10
	K0983	4,000		<10	<10	<10	<10	<10	<10
	K0990	3,000		<10	<10	<10	<10	<10	<10
	K1011	10,500		<10	<10	<10	<10	<10	<10
	K1013	12,000		<10	<10	<10	<10	<10	610
Baringo	S0004	8,000	8,000	640	<10	<10	<10	<10	<10
Busia	C0206	5,000	5,343	<10	<10	<10	<10	<10	<10
	C0235	10,000		<10	<10	<10	<10	<10	<10
	C0239	5,500		<10	<10	<10	<10	<10	<10
	C0272	3,000		<10	<10	<10	<10	<10	<10
	C0275	6,000		<10	<10	<10	<10	<10	<10
	C0278	4,700		<10	<10	<10	<10	<10	<10

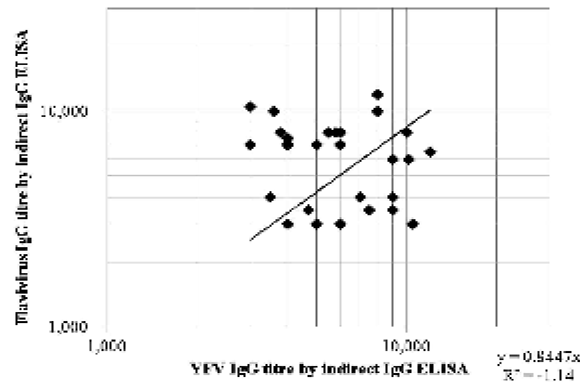

Fig. 2. Anti-flavivirus IgG ELISA using JEV antigen versus anti-YFV IgG ELISA using YFV antigen for 28 Western Kenya samples. Cut-off value was set at 1:3,000 IgG titer.

in confirming the diagnosis of YFV. In the first index case, S0004, the sample was from a 66-year-old YFV-vaccinated female from Kiptorenya village, Kapluk, Baringo County. She had a YFV indirect IgG ELISA titer of 1:8,000; hence, $FRNT_{50}$ was performed using YFV, DENV 1–4, and WNV. Notably, the YFV $FRNT_{50}$ titer was 1:640 for this index case. This titer was much higher than the average neutralizing antibody titer against YFV after vaccination by plaque reduction neutralization test (22). This increased neutralization in the Kapluk index case could indicate exposure during the 1992–1993 outbreak or silent circulation of YF in the area. The exact route of exposure remains unexplained because the IgM-capture ELISA result was negative; however, the increased IgG titer may not have resulted from vaccination only. The remaining 3 $FRNT_{50}$-positive samples, A0716, K0870, and K0960, are not further discussed because their YFV vaccination histories are unknown.

In the second index case, K1013, the sample was obtained from a 21-year-old non-YFV-vaccinated female from Sitatunga village, Kaplamai, Trans Nzoia County. Her YFV indirect IgG ELISA titer was 1:12,000; however, the YFV $FRNT_{50}$ titer was below 1:10 and WNV $FRNT_{50}$ titer was 1:610. This may be explained by past WNV exposure. These findings further underscore the need for differential diagnosis of arboviral infections in areas where more than 1 arbovirus is circulating. The remaining 23 samples could be due to other related flavivirus infections such as Zika virus, particularly from Busia. Furthermore, this study demonstrated that IgG can cross-react with YFV and other flaviviruses in indirect ELISA (Fig. 2), suggesting that various flaviviruses may be circulating in this region.

This study showed that even if genetically distant from JEV complex, YFV serological cross-reactivity was strong. There is need for further refinement of ELISA assays, and the cut-off value of 1:3,000 could probably be redetermined.

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Conflict of interest None to declare.

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Appendix X: Publications and presentations

Publications

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau. Seroprevalence of Yellow Fever virus in patients visiting selected health facilities in Western Kenya. *Japanese Journal of Infectious Diseases* (*in press*).

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Rosemary Sang, Toru Kubo, Kouichi Morita and Matilu Mwau (2013). A real-time reverse transcription loop-mediated isothermal amplification assay for the rapid detection of Yellow Fever Virus. *Journal of Virological Methods 193; 23-27.*

Presentations

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau (2014). Yellow Fever serosurveillance in Western Kenya. *JICA-JST SATREPS project symposium*. KEMRI training center, Nairobi, Kenya. 3 Jul 2014. (Oral presentation).

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau (2014). Novel approaches in the detection of Yellow Fever virus in Western Kenya. *Welcome Trust Emerging Scientific Researchers Conference*. Entebbe, Uganda. 19-22 May 2014. (Key-note speaker II)

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau (2012). Confirmatory diagnostic tests for YFV and RVFV - a progress report. *JICA-JST/Nagasaki University SATREPS project kick-off meeting*. 8 Jun 2012 (Oral presentation).

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau (2012). 1st Annual Hands-on workshop in Point-of-care diagnostic. *Institute of Primate Research, National Museums of Kenya*. Nairobi, Kenya. 25-29 Jun 2012. (Workshop participant).

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau (2011). A reverse transcription real-time loop mediated isothermal amplification assay for Yellow Fever Virus. Using Research Results for Dialogue, Action and Change - First National Health Research-to-Policy Dialogue, Windsor Golf and Country Club, Nairobi, Kenya. 21-22 Jun 2011

Allan ole Kwallah, Shingo Inoue, Anne Muigai, Kouichi Morita and Matilu Mwau (2011). One Health: Understanding human and veterinary diseases from molecular cell biology to successful interventions - International Workshop, Sokoine University, Morogoro, Tanzania. 1-7 Aug 2011. (Workshop participant).