OPTIMIZATION AND EVALUATION OF A REVERSE TRANSCRIPTASE LOOP AMPLIFICATION TEST FOR O'NYONG'NYONG VIRUS

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Optimization and evaluation of a reverse transcriptase loop amplification test for O'nyong'nyong virus

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

I dedicate this work to my beloved grandmother the late Martha Nafula Maero Ndombi. She was my source of inspiration since my childhood.

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TABLE OF CONTENTS

LIST OF TABLES

.LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

An	Anopheles mosquito
BIP	Backward Inner Primer
BLP	Backward Loop Primer
bp	Base pair
CDC	Centres for Disease Control
CGA	Arginine Codon
CHIKV	Chikungunya Virus
CNS	Central Nervous System
CO_2	Carbon dioxide gas
ddH_2O	Double Distilled Water
DMEM	Dulbeccos' Minimum Essential Medium
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate.
DTT	Dithiothreitol
E1 and E2	Envelope Proteins
EDTA	Ethylenediaminetetraacetic acid
EMEM	Eagles' Minimum Essential Medium
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FIP	Forward Inner Primer

FLP	Forward Loop Primer
GDD	General Duplication Divergence
HCl	Hydrochloric Acid
HIV	Human Immunodeficiency Virus
JEV	Japanese Encephalitis Virus
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KCl	Potassium Chloride
KEMRI	Kenya Medical Research Institute
L	Litre
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulphate
Min	Minute
MM	Maintenance Media
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
$(NH_4)_2SO_4$	Ammonium Sulphate
NPV	Negative Predictive Value
NUITM	Nagasaki University Institute of Tropical Medicine
NsP	Non-structural Proteins
ONNV	O'nyong'nyong Virus
P	Polyprotein
PA	Plaque Assay

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
pmol	Picomol
Poly (A)	Polyadenine tail
PPV	Positive Predictive Value
RNA	Ribonucleic Acid
RNase	Ribonuclease,
RT-LAMP	Reverse Transcriptase Loop Amplification Test
RT PCR	Reverse Transcriptase Polymerase Chain Reaction
RTase	Reverse Transcriptase
ssRNA	Single stranded ribonucleic acid
s	Second
SDS	Sodium Dodecyl Sulfate
SFV	Semliki Forest Virus
TBE / TAE	Tris Borate EDTA /Tris Acetate EDTA
T Cells	Thymus Derived Cells
UGA	Opal Termination Code
µl	Microlitre
USD	United States Dollar
UV	Ultra Violet
WN	West Nile

ABSTRACT

O'nyong'nyong fever, caused by infection with a mosquito-borne East African alphavirus, is an acute, non-fatal illness characterized by fever and polyarthralgia. Alpha viruses such as Chikungunya and O'nyong'nyong are endemic in East Africa and have caused extensive epidemics and therefore their rapid diagnosis is a priority. In this study, a novel, cheap and rapid method of gene amplification known as Reverse Transcriptase Loop Amplification Test was optimized for ONNV detection. Other methods such as; RT-PCR, Cell culture, plaque assay were also performed for purposes of comparison with RT-LAMP. The objective of this work was to optimize and evaluate an RT-LAMP assay for detection of O'nyong'nyong virus. One hundred samples were prepared. 40 of the samples were spiked with a high concentration of ONNV antigen (1:1000) while 40 these samples were spiked with low concentration of ONNV antigen (1:10000) and 20 few of the samples were not spiked with the ONNV antigen. All the samples were blinded and coded to avoid bias. These samples were analyzed using plaque assay, RT-PCR, and RT-LAMP. Plaque assay was used as the gold standard in this study. Out of the 100 samples tested by plaque assay 40 (11-30 pfu/ml) tested positive, 30 (0 pfu/ml) negative and 30 (1-10 pfu/ml) indeterminate. Using conventional RT-PCR 40 samples were positive, 30 negative and 30 indeterminate. By RT-LAMP 40 samples were positive, 23 negative and 37 indeterminate. RT-LAMP detected 7 samples as indeterminate that conventional PCR and plaque assay did not detect. The sensitivity and specificity of RT-PCR and RT-LAMP was calculated using plaque assay as the gold standard. The sensitivity of RT-

LAMP was 100% whereas that of RT-PCR was 85%. The specificity of RT-LAMP was 77% whereas that of RT-PCR was 100%. RT-LAMP is the best screening test for sensitivity whereas RT-PCR is the best screening test for specificity. Reliability testing was done by calculating the positive (PPV) and negative (NPV) predictive values. PPV of RT-LAMP was 85% whereas that of RT-PCR was 100%. NPV of RT-LAMP was 100% and that of RT-PCR was 77%. In terms of cost and more detailed analysis RT-LAMP emerged as the best test amongst the three assays carried out in this study. The research findings reported in this work will contribute towards the diagnosis of viral infections especially ONNV and other emerging arboviruses particularly in outbreaks. Further optimization and evaluation should be done to establish RT-LAMP as a tool for surveillance of not only O'nyong'nyong virus but also other emerging viruses.

CHAPTER ONE

1. INTRODUCTION

1.1 Background to the study

The term “O’nyong’nyong” was derived from a Northern Ugandan dialect (Haddow *et al.,* 1960) and is generally meaningless in Southern Uganda, where more common local terms for ONN fever include “kyikonyogo” and “kyikutiiya.” (roughly translated as “beaten on the joints with a stick”).

O’nyong’nyong (ONN) virus is a mosquito-borne alphavirus that caused a major East African epidemic during 1959–1962. The epidemic began in Northern Uganda and involved an estimated 2 million patients in Kenya, Tanzania and Uganda (Williams *et al*., 1965). This self-limiting and previously unknown disease was characterized by debilitating polyarthralgia, often accompanied by rash and lymphadenitis (Shore, 1961). During 1959–1962, 46 strains of ONN virus were isolated from acutely ill patients in 15 locations in Uganda, Kenya and Tanzania (Williams *et al*., 1965). Although no fatal cases of ONN virus were documented, the epidemic was associated with substantial morbidity as a result labor-intensive production and agricultural industries were significantly affected (Johnson, 1988).

Two of the region's major malaria vectors, *Anopheles gambiae* and *Anopheles funestus,* were implicated as the primary epidemic vectors of this virus (Corbet *et al.,* 1961; Williams *et al.,* 1965). In 1978, a strain of ONN virus was isolated from

Anopheles funestus collected in western Kenya (Johnson *et al*., 1981). To date, the enzootic vectors and natural reservoir hosts of ONN virus remain unknown.

In mid 1996, after an apparent absence for 35 years, the first recognized re-emergence of ONN fever occurred in South-Central Uganda (Rwaguma *et al.,* 1997). The earliest cases were noted in the rural Rakai District. Within a few weeks, the epidemic had extended to the adjacent districts of Masaka and Mbarara. At that time, vertebrate studies conducted failed to incriminate rodents or mongooses as amplifying hosts of the virus (Ogen-Odoi *et al*., 1999). Although a strain of ONN virus was isolated from *Mansonia uniformis* mosquitoes, *Anopheles funestus* was implicated as the primary epidemic vector (Lutwama *et al.,* 1999). Results of molecular virologic studies suggested that a single genotype of ONN virus circulated during this epidemic and indicated that this genotype shared a high degree of genetic and protein sequence homology with an ONN virus strain isolated in Northern Uganda in 1959 during the previous epidemic (Lanciotti *et al.,* 1998).

In addition to ONN virus, emergence and reemergence of arboviral fevers caused by mosquito-borne viruses, such as dengue virus, Chikungunya (CHIK) virus and ONN virus have been frequently reported in East Africa in the past few years. From the clinical perspective, these infections have similar clinical manifestations and are difficult to distinguish from one another.

There is sufficient evidence to suggest that many arboviruses are endemic in Kenya. A serosurvey conducted during September 1987 for evidence of human arboviral

infections in the Coast Province of Kenya revealed the following seroprevalences: O'nyong'nyong 0.7%, Rift Valley fever, 2.8%; Sindbis, 2.6%; dugbe, 2.1%; dengue-2, 1.0%; West Nile, 0.9%; Chikungunya, 0.7% and Nairobi sheep disease, 0.3% (Morrill *et al*., 1991). These data suggest that several viruses could be circulating amongst certain Kenyan populations below the outbreak thresholds.

Various reasons have been advanced to explain the emergence of ONN and other arboviruses. These include increasing migration to the area by people from disease-endemic regions, a high level of vector infestation, the lack of eradication measures and changing climatic patterns. Most importantly, it may well be that the encroachment of human populations into bush and forest areas leads to increased exposure to sylvatic mosquitoes that vector these viruses.

Before 1985, extensive arbovirus surveillance was done by Metselaar *et al.*,1974 in Kenya (Henderson *et al*., 1968; Metselaar *et al*., 1974; Rodhain *et al.*, 1975; Metselaar, 1977; Metselaar, 1978). Currently there is very scanty information on prevalence of arbovirus in Kenya. However, in the very recent past, several research groups have begun to respond to the need for serosurveillance. This has been partly due to the outbreaks that have occurred with significant associated mortality. Most assays conducted in recent outbreaks used serological kits provided by the Centers for Disease Control and Prevention (CDC) that are often not readily available or accessible (Onyango *et al*., 2004a; Onyango *et al.*, 2004b).

The absence of a regular supply of assay kits *adapted* to ONN virus makes routine survey, even though essential, much less feasible than is desired. To overcome this difficulty, assays have been optimized in the KEMRI laboratories for the survey and diagnosis of some of these viruses.

1.2 Statement of the problem

Although ONN virus is a public health concern in East Africa, assays that can rapidly detect it cheaply and specifically especially during outbreaks are lacking. Therefore, it is important to identify and quantify this infection for epidemiological studies. Some outbreaks get a diagnosis after weeks, when the damage is already done. Diagnosis must be made to enable treatment, disease control and prevention for example quarantine if necessary and provision of mosquito nets.

1.3 Justification

The RT-LAMP assay is easy to develop, simple, rapid, cost effective and highly sensitive. The specificity of RT-LAMP assay is validated by absence of any cross-reaction with other closely related members of Alphavirus group. The gold standard plaque assay method has one disadvantage in that it is time consuming and one requires more than seven days to make a definitive diagnosis. PCR needs expensive equipment, such as a thermocycler, and highly skilled personnel. RT LAMP, on the other hand, can often be read with the naked eye and conducted by medium cadre laboratory staff. More importantly, whereas a PCR diagnosis can cost 30-40 USD and is difficult to decentralize, RT LAMP is expected to cost 8 USD or less and can be done in the field so long as a water bath can be maintained between 60^{o}C and 65^{o}C.

1.4 Hypothesis

- RT-LAMP is not comparable in sensitivity and specificity to plaque assay and RT-PCR for the detection of O'nyong'nyong virus.

1.5 Objectives

1.5.1 General Objective

- To optimize and evaluate an RT LAMP Assay for O'nyong'nyong Virus.

1.5.2 Specific Objectives

- To design primers and optimize conditions for RT-LAMP assay.
- To compare RT-LAMP with plaque assay and RT-PCR for O'nyong'nyong virus in terms of cost, turn around time per sample, simplicity in resource poor settings.
- To assess the feasibility of RT-LAMP as a diagnostic tool for laboratory testing of O'nyong'nyong virus in Kenya.

CHAPTER TWO

2. LITERATURE REVIEW

2.1 Classification of O'nyong'nyong virus

The O'nyong'nyong virus was first isolated by the Uganda Virus Research Institute in Entebbe, Uganda in 1959. It is a togavirus (family *Togaviridae*, genus *Alphavirus*). O'nyong'nyong virus is in group IV ssRNA. The name comes from the Nilotic language of Uganda and Sudan which means "weakening of the joints." O'nyong'nyong (ONN) virus is a mosquito-borne alphavirus that belongs to the Semliki Forest serological complex (Calisher and Karabatsos, 1988). This virus is closely related both genetically and clinically to Chikungunya virus, that causes epidemic polyarthritis (Kay and Aaskov, 1989).

2.2 Virion Properties of O'nyong'nyong virus

2.2.1 Morphology of O'nyong'nyong virus

ONN virions are spherical and measure about 70 nm in diameter. They consist of an envelope and a nucleocapsid. During the replication cycle, virions have not been observed outside a cellular environment and thus, have a cell-associated cycle. The capsids appear round and enveloped (with a tight fitting membrane envelop). Surface projections are distinctive glycoprotein spikes covering the surface evenly.

2.2.2 ONNV genome, nucleic acid and proteins

The ONNV genome is a linear positive-sense, non-segmented, single-stranded RNA, 11,835 nucleotides in length, GenBank accession number NC-001512. The RNA has a 5'-terminal cap and a 3'-terminal poly (A) tract (Strauss *et al.*, 1994). The 5' two-thirds of the genome encode the nonstructural or replicase proteins, while the structural genes are encoded in the 3' one-third. Immediately after uncoating of the virion, four nonstructural proteins (nsP1 to nsP4) are translated from the genomic RNA. The Polyprotein P123 results when translation is stopped at an opal termination codon (UGA) between nsP3 and nsP4. The Polyprotein P1234 is produced when read-through of the stop codon occurs (Strauss *et al*., 1983). Read-through occurs with 5 to 20% efficiency as determined by using *in vitro* translation reactions (de Grout *et al*, 1990; Shirako and Strauss, 1994). The structural genes are translated from a sub-genomic mRNA (26S RNA) that is transcribed from an internal sub-genomic promoter present in a full-length negative sense RNA copy. The negative-sense copy also functions as the template for production of new genomes (49S RNA).

Although all functions of the individual nonstructural proteins have yet to be determined, the nsP1 protein is required for initiating synthesis of viral RNA of negative polarity and also plays a role in capping genomic and sub-genomic viral RNAs (Hahn *et al.*, 1989a; Wang *et al*., 1991; Ahola *et al.*, 1997,). The nsP2 protein possesses RNA helicase activity and also serves as the viral protease for the nonstructural polyprotein (Hardy *et al.*, 1989; Strauss *et al*., 1992; Gomez de Cedron

et al., 1999; Merits *et al.,* 2001; Vasiljeva *et al.,* 2001). The function of nsP3 is not completely understood, but it is essential to RNA replicase function and heavily phosphorylated (Hahn *et al*., 1989b; Li *et al.,* 1990; Lastarza *et al.,* 1994; Vihinen *et al*., 2001). The presence of a characteristic general duplication- divergence (GDD) motif has led to the assignment of nsP4 as the viral RNA polymerase and genetic studies are consistent with this assignment (Kamer and Argos., 1984; Hahn *et al*., 1989b).

Sequence information indicates the termination codon appears before nsP4 replacement with an arginine codon (CGA) in at least two alphaviruses, ONN virus and Semliki Forest virus (Takkinen, 1986; Strauss *et al.,* 1988). For the viruses mentioned above, it is presumed that only P1234 is generated during replication. However, passaging of ONNV prior to sequencing may determine the codon at this particular locus (Strauss *et al*., 1988). Consensus sequences obtained from later, lower passage, isolates indicate that ONNV possess both sense and stop codons at this position (Lanciotti *et al.,* 1998). Further, when ONNV isolates found to have an opal termination codon are passaged in vertebrate cells (Vero), they rapidly acquire in its place an arginine codon (Lanciotti *et al*., 1998). Thus, ONNV quasispecies populations include both stop and arginine codons in equilibrium at this locus and that both sequences are somehow required for the survival of these viruses in nature. Replication of ONNV in either vertebrate or arthropod hosts may determine the predominance of the codon at this particular locus in the quasispecies population. The

O’nyong’nyong virus has at least three major subtypes or strains whose genomic sequences are currently available on genome databases.

2.3 Epidemiology of O’nyong’nyong virus

The first virus isolates were obtained during the 1959-1962 outbreak from mosquitoes and human blood samples collected from Gulu in Northern Uganda in 1959 (William *et al*., 1965a).

Typical clinical features included low-grade fever, symmetrical polyarthralgia, lymphadenopathy (particularly of the posterior cervical region) and a generalized papular or maculopapular exanthem (Shore *et al.,* 1961). Although some patients experienced prolonged joint pain during the recovery phase, no fatal cases or permanent sequelae were observed. Two of the region's major malaria vectors, *Anopheles funestus* and *Anopheles gambiae,* were implicated as the principal epidemic vectors of ONN virus (Corbet *et al.,* 1961; Williams *et al.,* 1965b). The epidemic waned in Uganda in 1961, ONN fever cases were documented in an area extending from Mozambique to Senegal during 1960–1962 (Bres *et al.,* 1963). After the epidemic of 1959–1962, results of seroprevalence studies suggested that sporadic human infections with ONN virus continued to occur within the region, though no cases of ONN fever were documented after 1962 (Geser *et al.,* 1970; Tsai *et al.,* 1997).

The second epidemic in 1996-1997 affected 400 people and was confined to Uganda. Beginning in mid 1996, after an apparent absence of 35 years, the first recognized reemergence of ONN fever occurred in south-central Uganda (Rwaguma *et al.,* 1997). The epidemic was focused near lakes and swamps, where it was associated with high infection and attack rates and an apparent to in apparent infection ratio of 2:1 (Sanders *et al.,* 1999). *Anopheles funestus* was implicated as the primary epidemic vector (Lutwama *et al.,* 1999).

2.4 Transmission of O'nyong'nyong virus

O'nyong'nyong virus is transmitted by bites from an infected mosquito. It is the only virus whose primary vectors are anopheline mosquitoes (*Anopheles funestus* and *Anopheles gambiae*).

Maintenance of arboviruses in nature requires a biological transmission cycle that involves alternating virus replication in a susceptible vertebrate host and a blood feeding arthropod. Although the vertebrate infection is acute and often associated with disease, continual transmission of virus is dependent on the establishment of a persistently infected state in the arthropod host. The general features of mosquito infection with arboviruses have been described (Bres *et al.,* 1963). Susceptible mosquito vectors become infected with alphaviruses after ingestion of an infectious blood meal. The envelope glycoproteins present on the surface of the virion facilitate entry into the midgut epithelial cells, most likely through a specific receptor-ligand interaction (Bres *et al.,* 1963; Monath and Heinz, 1996; Jupp and McIntosh, 1988;

Tsai *et al.,* 1997). Replication of virus in midgut epithelial cells may be followed by escape into the hemolymph. As virus enters the hemolymph, other tissues become accessible. Replication of virus in secondary tissues serves to further amplify virus in the hemolymph. If the virus successfully infects and replicates in the salivary glands, virus can be transmitted through the saliva to a vertebrate host. Although alphaviruses are generally poorly vectored by anopheline mosquitoes, ONNV appears to represent an exception as *Anopheles gambiae* and *Anopheles funestus* mosquitoes have been implicated as primary vectors during periods of epidemic transmission (Mudge and Aaskov, 1983; Kay and Aaskov, 1989; Beaty *et al.,* 1995).

2.5 Signs and Symptoms of O'nyong'nyong virus

Whereas outbreaks of arthropod borne viruses cause dramatic symptoms and mass panic, the spectrum of clinical signs and symptoms associated with endemic arbovirus infection is large. Luckily, most arbovirus infections either cause no or mild symptoms. Common symptoms of infection with the ONN virus are polyarthritis, rash and fever. Other symptoms include eye pain, chest pain, lymphadenitis and lethargy. No fatalities due to infection are known. Fever, headache, joint pains and rash are the principal signs and symptoms (Shore *et al.,* 1961; Williams *et al.,* 1965b).

Fever and rashes are very common in many infections and therefore it is generally acknowledged that arbovirus infections could be misdiagnosed as malaria or typhoid quite often. In situations where an arbovirus infection is suspected, only very specialized laboratories such as those in KEMRI can make a diagnosis.

2.6 Pathogenesis of O'nyong'nyong virus

Following infection with ONNV, intense viraemia occurs within 48 hours. Viraemia begins to wane around day 3. The appearance of haemagglutination inhibition activity in neutralizing antibodies clears the viraemia. Involved skin shows erythrocyte extravasation from superficial capillaries and perivascular cuffing. The ONN virus adsorbs to human platelets causing aggregation, suggesting a mechanism for bleeding. The immune system plays an important role especially in clearing virus particles from an individual who is infected with O'nyong'nyong virus. The immune system induces the production of interferons, T cells and neutralizing antibodies that prevent further infection and spread.

2.7 Diagnosis, prevention and control of O'nyong'nyong virus

The diagnosis of most alphaviruses is dependent upon virus isolation and detection of genomic sequences by nucleic acid amplification techniques. Virus isolation through Vero cell lines from acute-phase serum samples is the method of choice although it has the disadvantage that more than 7 days is required to make a definitive diagnosis. Both virus isolation and Plaque assays are time-consuming and tedious and require more than one week for completion. Thus virus isolation has a lessened impact on patient management and the control measures exercised by medical and public health personnel. Therefore, there is a great demand for the rapid detection of ONNV infection in acute phase of illness in order to provide timely clinical treatment, etiologic investigation and disease control.

Molecular techniques based on genomic sequence detection by reverse transcription (RT) PCR, nested PCR, Nucleic Acid Sequence Based Amplification (NASBA) and real-time PCR are therefore assumed to be significant for the rapid diagnosis and identification of ONNV in acute-phase serum samples (Kuno, 1998; Leon *et al*., 1998; Chan and Fox, 1999). Each of these amplification methods has its own innovation to re-initiate new rounds of RNA/DNA synthesis. For example, PCR uses the heat denaturation step of double-stranded DNA products to promote the next round of synthesis. NASBA eliminates the heat denaturation step using a set of transcription and reverse transcription reactions to amplify target sequence. These methods can amplify target nucleic acids to a similar magnitude, all with a detection limit of less than 10 copies within hours, but still have shortcomings.

Nucleic acid sequence based assays might eventually replace virus isolation and conventional RT-PCR as the new gold standard for the rapid diagnosis of virus infection in acute-phase serum samples. However, all these nucleic acid amplification methods have several intrinsic disadvantages, in that they require either a high precision instrument for amplification or an elaborate, complicated method for detection of amplified products. They also present a high risk of contamination. The instruments required to perform the real-time assays are expensive; this restricts their use to laboratories with good financial resources or research settings.

The present study describes the optimization and tests of a simple, rapid and cost-effective one-step, real-time and qualitative reverse transcription-loop-mediated isothermal amplification (RT-LAMP) assay for the rapid detection of ONNV. The RT-LAMP assay is a novel approach to nucleic acid amplification and is based on the principal of a strand displacement reaction. The stem loop structure amplifies the target with high degrees of specificity and selectivity with rapidity under isothermal conditions, thereby obviating the need for the use of a thermal cycler (Notomi *et al.*, 2000; Nagamine *et al.*, 2002).

The amplification efficiency of RT-LAMP is extremely high due to continuous amplification under isothermal conditions, which results in the production of a large amount of the by-product magnesium pyrophosphate, which leads to turbidity (Mori *et al*., 2001). Therefore quantitative detection of gene amplification is possible by real time monitoring of the turbidity in an inexpensive photometer. In addition, the higher amplification efficiency of the RT-LAMP method enables simple visual observation of amplification with the naked eye under UV lamp in presence of an intercalating dye, such as SYBR Green 1 or ethidium bromide. Avoiding the use of detection methods that use ethidium bromide, a carcinogen and use of expensive documentation systems.

The RT-LAMP assay has emerged as a powerful gene amplification technique for rapid identification of microbial infections and has been applied to the identification

of West Nile virus (WN) and Severe Acute Respiratory Syndrome – Associated Corona virus (Hong *et al.*, 2004; Parida *et al.*, 2004a). In the present study, the development and applicability of the RT-LAMP assay for rapid detection of ONNV is described. Data on specificity and sensitivity of the method are reported and the feasibility of the use of the technology for the clinical diagnosis of ONNV infection is discussed.

2.8 Control measures for O'nyong'nyong virus.

There are no alphavirus vaccines currently available. Vector control with repellents, protective clothing, breeding site destruction, and spraying are the preventive measures of choice.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Study design

This study involved number of experiments. Preparation of the 100 blinded samples was the first procedure to be done followed by cell culture, plaque assay, primer design, RNA extraction, RT-PCR and finally RT-LAMP.

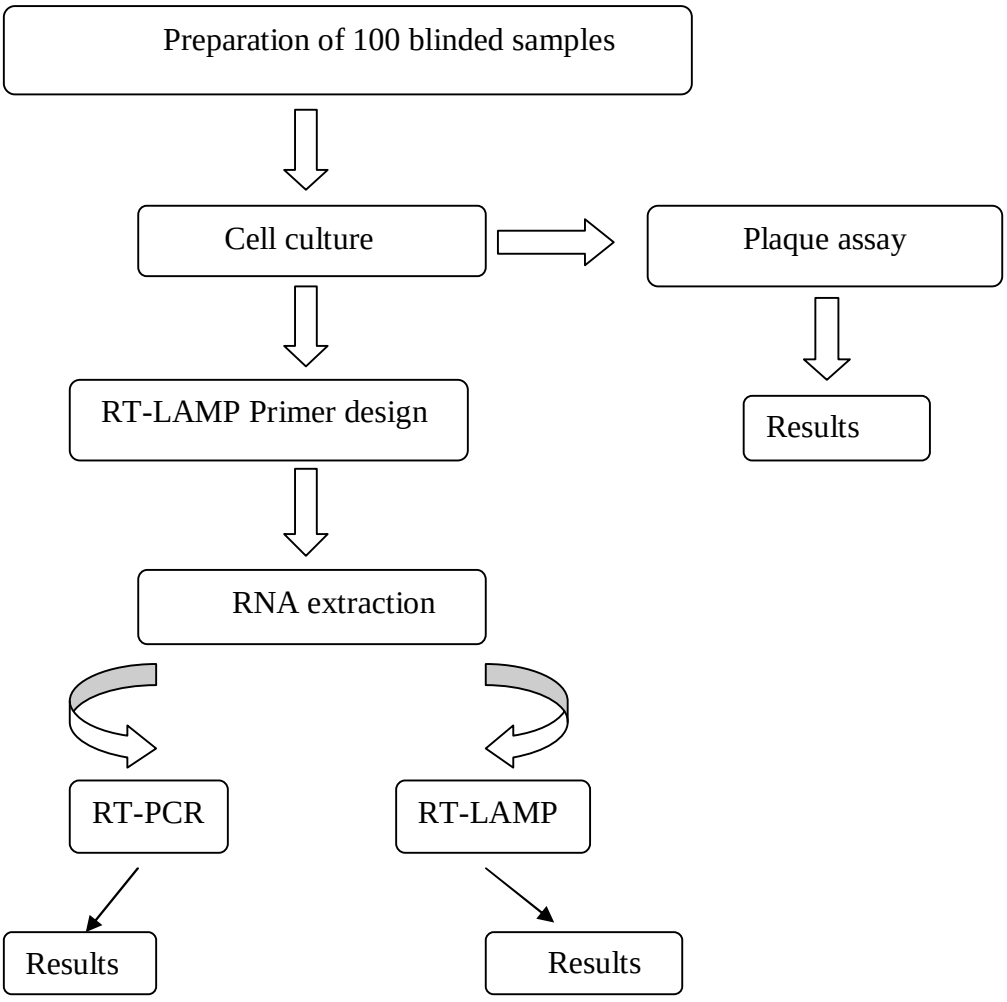

Figure 1: Study design

3.1.1 Sample size determination

Since there was no recorded current outbreak of ONNV, a total of 100 samples were prepared in the laboratory by spiking with ONNV antigen. The ONNV antigen originated from a positive sample isolated from 1996 ONNV second epidemic. Forty of the prepared 100 samples were spiked with a high concentration of the ONNV antigen (1:1000), 40 with lower concentration of ONNV antigen (1:10000) and 20 were not spiked with any ONNV antigen. This study also involved optimization of RT-LAMP and therefore did not involve use of sample size determination formula. The sample size in this study was based from previous studies carried out by other researchers based on the same assay (RT-LAMP).

3.1.2 Preparation of samples

Preparation of Minimum Essential Medium (MEM) was supplemented 2% FBS, 2% L-glutamine and 1% antibiotic/antimycotic solution to make maintenance media. Samples were made by aliquoting 1 ml (1000ul) maintenance media in each of the 100 cryovials representing the 100 samples used in this study. Some of these 100 samples were spiked with high concentration of ONNV (1:1000) while other samples were spiked with low concentration of ONNV antigen (1:10000) and a few samples were not spiked with the ONNV antigen. These samples were then blinded and coded for confidentiality purposes and stored at - 80°C freezer until used.

3.1.3 Cell culture

This study involved the use of Vero Cells derived from African green monkey kidney cell line. Vero cells were grown in 6-well plates. The wells were seeded at a density of 2 x 10^5 cells per well in 4 ml of growth medium containing 10 % FBS, 2 % L-glutamine and 2 % antibiotic/antimycotic solution.

3.1.4 Plaque assay

Plaque assay was used as the gold standard assay in this study to quantify the amount of virus in each sample.

3.1.4.1 Inoculation of cells

Serial dilutions of ONNV were prepared in the laboratory. 1:100 dilution was made by adding 10 µl viral stock to 990 µl MM. Starting with a 1:100 dilution, 1:10 dilution was prepared by transferring 100 µl of diluted virus to 900 µl MM.

Cell culture plates were removed from the incubator and the wells inspected to ensure that the cells attached to form an even monolayer that was about 80–90% confluent. Growth medium was removed from the cell cultures and 0.1 ml of ONNV added to each positive control well. Sample dilutions (0.1 ml) was also added in each well taking care not to dislodge any cells. Plates were tipped to spread the virus evenly over the monolayer. The plates were covered and the cells incubated in a humidified CO_2 (5%) incubator for 60 min at 37°C to allow the virus to adsorb the cells.

3.1.4.2 Preparation of methylcellulose overlay medium.

Methylcellulose overlay medium (EMEM, 1.4% Methylcellulose, 2% FCS) was prepared as follows; 450 ml Milli-Q water (ddH_2O) (90% of final volume) was put into 1 litre bottle where 7 g of Methylcellulose was added into the bottle. Magnetic stirrer bar was applied in the bottle and the solution autoclaved. After cooling, the bottle was placed on to magnet stirrer until Methylcellulose dissolved. Approximately 3ml of methyl cellulose solution was added gently to each well. When the methylcellulose had set, the plates were incubated at 37°C in a humidified CO_2 (5%) incubator.

3.1.4.3 Fixing, staining of the plates and counting of plaques

Harvesting of the methylcellulose from the plate was done on the second day. Approximately 3ml of the overlay medium was removed with plastic pipette. Plates were fixed by adding 1 ml of 10% formaldehyde in PBS (1/10 diluted Formalin with PBS) over the cell and incubated for 1 hour at room temperature in the safety cabinet with UV light on to fix the plate. Formaldehyde was removed with plastic pipette and discarded into the specific waste bottle. The surface of the plates was wiped out with 70% ethanol. Plates were washed gently with tap water, followed by absorption of the plates on paper and staining solution added (0.5 ml of 1% Crystal Violet solution in water). Incubation for 5 to 10 minutes at room temperature was done. Crystal violet 1% was discarded and plates washed gently with tap water then air-dried at room temperature.

3.1.4.4 Formula for calculating plaque forming unit per ml

Pfu/ml (of original stock) = 1/dilution factor x number of plaques x 1/ (ml of inoculum/plate) (Bundo and Igarashi, 1985).

3.1.5 Reverse Transcriptase – Loop Amplification Test

3.1.5.1 Primer design

The oligonucleotide primers used for RT-LAMP amplification of ONNV were designed to target the structural (E1) gene. The nucleotide sequence of the E1 gene of the S-27 African ONNV prototype strain were retrieved from GenBank (accession no NC-001512). A set of six primers comprising two outer, two inner, and two loop primers that recognize eight distinct regions on the target sequence were designed by employing the Primer Explorer V4, Eiken Chemicals software program;

(https://primerexplorer.jp/lamp4.0.0/index.html).

The primers were selected based on criteria described previously by (Notomi *et al*., 2000). The two outer primers described as being forward outer primer (F3) and backward outer primer (B3). The inner primers described as forward inner primer (FIP) and backward inner primer (BIP). Furthermore, two loop primers, forward loop primer (FLP) and backward loop primer (BLP), were designed to accelerate the amplification reaction. FIP consisted of a complementary sequence of F1 and a sense sequence of F2. BIP consisted of a complementary sequence of B1 and a sense sequence of B2.

Table 1: RT-LAMP E1 gene primer sets designed for rapid detection of ONNV

Primer	Length of oligonucleotide	Sequence
FIP	41	5'GCCAAATGGTGCAGTATGCTGCTTACTCCCAGGCACCTTCG3'
BIP	42	5'ACCCGGTAAGAGCAGTGAACTGCCTGGTGAAAGCTGCATCTG3'
F3	18	5'CGGCAGGAGCAATACACG3'
B3	19	5'CAGGACATGTCTGTGATGG3'
FLP	20	5'ATGCCCCTTTTTCCTTGAGC3'
BLP	22	5'GCAACATACCAGTCTCCATTGA3'

The above primers were selected based on criteria described previously by (Notomi *et al*., 2000).

3.1.5.2 RT-LAMP

The RT-LAMP reaction was carried out in a 25-µl total reaction mixture volume with a Loop amp DNA amplification kit (Eiken Chemical Co. Ltd.) containing 40 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 20 pmol each of loop primers loop F and loop B, 1,400 µM each deoxynucleoside triphosphate, 0.6 M betaine (Sigma Chemical Co., St. Louis, Mo.), 40 mM Tris-HCl (pH 8.8), 20 mM KCl, 20 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Triton X-100, 0.125 U of avian ONN virus RTase (Invitrogen), 8 µl of DNA polymerase (large fragment; New England Biolabs), and the specified amounts of target RNA. The mixture was incubated at 61°C for 60 minutes in a heating block and then heated at 80°C for 5 min to terminate the reaction.

3.1.5.3 Analysis of RT-LAMP product

Following amplification by the RT-LAMP method, the tubes were inspected for white turbidity using naked eye after spin to deposit the precipitate in the bottom of the tube. The inspection was also performed through observations of colour change following the addition of 1µl of SYBR Green I dye to the tube. Positive amplification was noted by the change of the original orange colour to green dye under natural light as well as ultra violet light (302nm). Retention of the orange colour indicated no amplification.

3.1.6 Reverse Transcriptase –Polymerase Chain Reaction

3.1.6.1 RNA extraction

The genomic viral RNA was extracted from 140µl of all the 100 prepared samples using the QIAamp viral RNA mini kit (QIAGEN, Germany) in accordance with manufactures protocol. The RNA was eluted from QIAspin columns in a final volume of 60µl of elution buffer and stored at -80^{o}C until used.

3.1.6.2 RT –PCR

One-step RT-PCR was performed by employing the two primers; forward and reverse primer targeting the E1 gene of ONNV. The amplification was carried out in a 50-µl total reaction volume using a Promega Access Quick One-Step RT-PCR kit with 50 pmol of forward and reverse primers and 2 µl of RNA template. The thermal profile of RT-PCR was 35 cycles of 94°C for 3s, 94°C for 30 min, 55°C for 30 min, 68°C for 45min, 72°C for 7s and a final extension cycle at 4°C. After RT-PCR was performed, the products were electrophoresed on a 2.5% agarose gel in Tris-acetate EDTA buffer

followed by staining with ethidium bromide and visualization on a UV transilluminator at 302nm.

CHAPTER FOUR

4. RESULTS

4.1 Determination of RT-LAMP, RT-PCR and plaque assay.

4.1.1 RT-LAMP

The monitoring of RT-LAMP assay amplification was carried out by naked – eye inspection following addition of fluorescent dye to the amplified reaction master mix. In case of positive amplification, the colour of the reaction mixture changed to green whereas in case of no amplification, the original orange colour of the dye was retained, as observed with the negative controls with no RNA template as shown in the Figure 2 below.

Figure 2: Monitoring of ONNV RT-LAMP amplification

(A) Naked eye inspection under normal light. The original orange colour of the SYBR green I changed to yellow in the case of positive amplification, whereas in the negative control having no amplification, the original orange colour was retained. **(B)** Visual observation of green fluorescence of DNA binding SYBR green I under UV light.

4.1.2 Reverse Transcriptase –Polymerase Chain Reaction

The visualization of RT-PCR products was carried out using gel electrophoresis. After RT-PCR was performed, a 10µl portion was analyzed by agarose gel electrophoresis on a 2.5% agarose gel in Tris-acetate EDTA buffer followed by staining with ethidium bromide and visualization on a UV transilluminator at 302nm. In case of positive amplification there was formation of clear bands between 100bp to 200bp whereas in case of no amplification, there was no band formation as shown in the Figure 3.

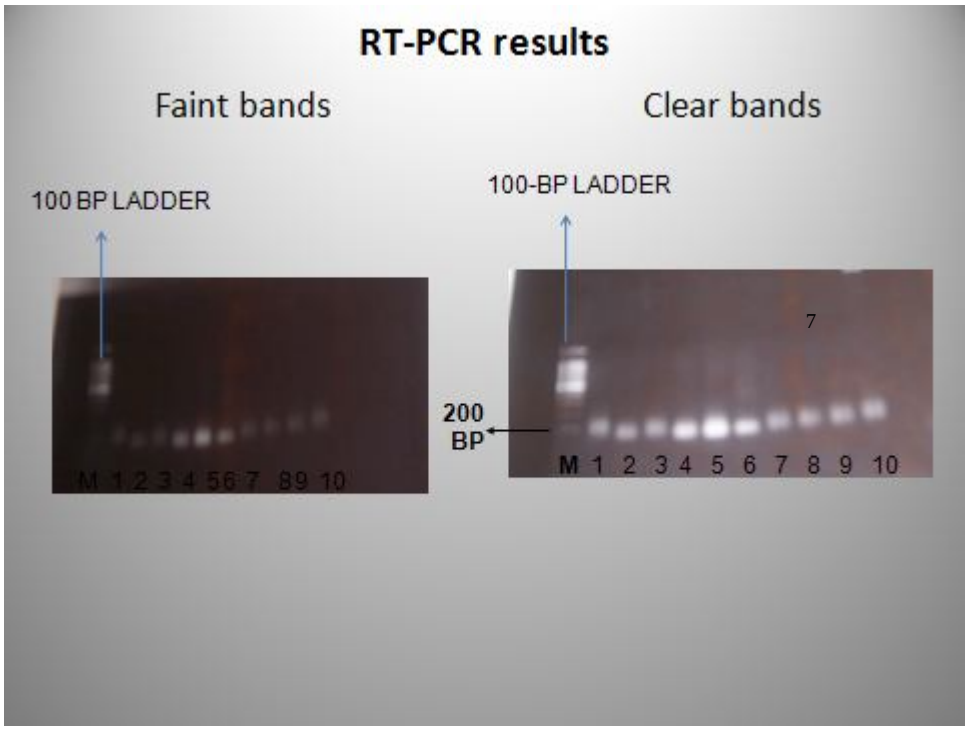

Figure 3: Agarose gel electrophoresis of RT-PCR product of the ONNV E1 gene.

The first lane (M) shows the 100-bp DNA ladder (Sigma Genosys). In **(A)** Faint bands indicate some of the samples which had been spiked with low concentration of ONNV antigen and no bands indicate

some of the different samples which had not been spiked with the ONNV antigen (1,2,3,4,5,6,7,8,9,and 10). In **(B)** very clear bands indicate some of the different samples that were spiked with high concentration of ONNV antigen (1,2,3,4,5,6,7,8,9 and 10)

4.1.3 Plaque assay

Plaque assay was used as the gold standard in this study. Plaque assay was performed to quantify the amount of ONNV titer in the samples that were spiked with ONNV antigen. Some of the samples that were spiked with ONNV antigen showed plaques whereas samples that had not been spiked with the ONNV antigen showed no plaques. An example is shown in Figure 4.

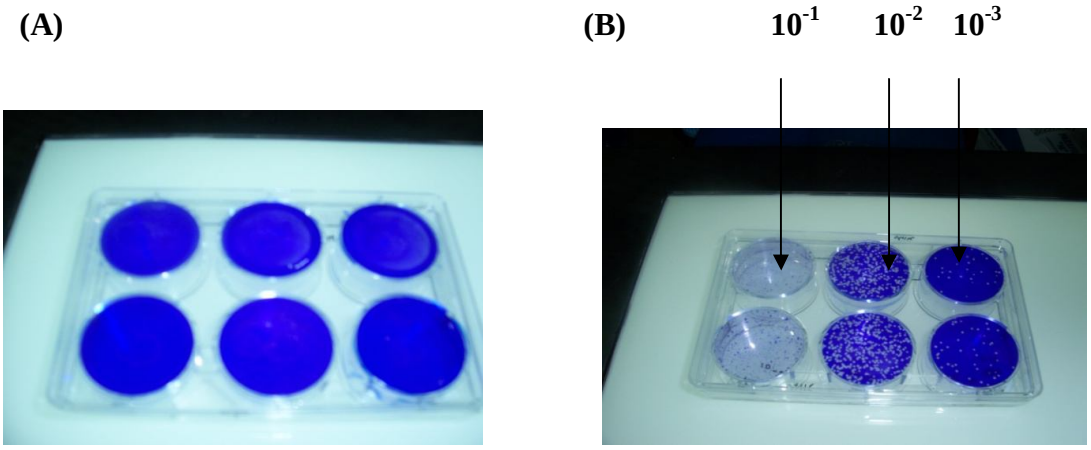

Figure 4: Plaque assay for a negative and positive sample.

(A) Shows the negative control 6-well plate of Vero cells which contained serial dilutions of negative sample that had not been spiked with the ONNV antigen. In this plate, no plaques were formed indicating that there was no ONNV present in the sample to cause plaques. **(B)** Shows a 6 well plate containing serial dilutions of a sample that had been spiked with ONNV antigen. In this plate there was formation of plaques indicating that there was ONNV in the sample.

Virus dilution 10^{-1} showed no plaques due to destruction of the whole monolayer by the virus. Virus dilution 10^{-2} showed confluent plaques therefore counting was impossible because the virus concentration was also high but not as high as 10^{-1} virus dilution. The plaques were too numerous to count thus increasing the chances of not getting accurate results. The dilution 10^{-3} virus dilution had the best plaques since the virus concentration in the sample was not high thus the plaques were easy to count. Table 1 illustrates the steps followed to arrive at the final plaque forming unit per ml of the ONNV positive samples.

Table 2: Different dilutions of virus inoculated and number of plaques in each well.

Virus dilution inoculated	**Number of plaques on each of two plates**	
10^{-1}	C*	C*
10^{-2}	C*	C*
10^{-3}	27	25

C* = Confluent plaques; counting not possible or too numerous to count

Innoculum volume = 0.1 ml

0.1ml of 10^{-1} dilution virus contains (27 + 25) = 52pfu

Total number of plaques = 52 pfu

Therefore 0.1ml of undiluted virus contains (52 pfu X 10) pfu = 52 X 10 pfu

Therefore 1 ml of undiluted virus contains; $\frac{(1X\ 52\ X10)\ pfu}{0.1}$ = 5200 pfu/ml

4.2 RT-LAMP, RT-PCR and plaque assay

4.2.1 RT-LAMP

Table 3: RT-LAMP assay

Reverse Transcriptase Loop Amplification Test results were obtained from a total of a hundred prepared samples. The results indicated that out of the 100 samples, 40 samples tested positive, 23 negative and indeterminate 37.

RT-LAMP results	**Positive**	**Negative**	**Indeterminate**	**Total**
Number of samples	40	23	37	100

4.2.2 RT-PCR

Table 4: RT-PCR assay

Reverse Transcriptase Polymerase Chain Reaction results were obtained from a total of a hundred prepared samples. The results indicated that out of the 100 samples, 40 samples tested positive, 30 negative and 30 indeterminate.

RT-PCR results	**Positive**	**Negative**	**Indeterminate**	**TOTAL**
Number of samples	40	30	30	100

4.2.3 Plaque assay

Table 5: Plaque assay

Plaque assay results were obtained from a total of a hundred prepared samples. The results indicated that out of the 100 samples, 30 samples had 0 pfu/ml, 30 samples ranged between 1 to 10 pfu/ml, 27 samples ranged between 11 to 20 pfu/ml and 13 samples ranged between 21 to 30 pfu/ml.

Plaque assay results	**0 pfu/ml**	**1 to 10 pfu/ml**	**11 to 20 pfu/ml**	**21 to 30 pfu/ml**
Number of samples	30	30	27	13

4.3 Comparative analysis of RT-LAMP, RT-PCR and plaque assay results

Analysis of the three assays; RT-LAMP, RT-PCR and plaque assay was based on the number of true negatives, true positives, positives by each test, and negatives by each test, Indeterminate, percentage positive and percentage negative.

Table 6: Comparative analysis of the three assays; RT-LAMP, RT-PCR and plaque assay

TEST	**N**	**True positive**	**True negative**	**Positive by test**	**Negative by test**	**Indeterminate**	% **positive**	% **negative**
RT-LAMP	100	40	23	40	23	37	40%	23%
Plaque assay (pfu/ml)	100	40	30 (0)	40 (11-30)	30 (0)	30 (1-10)	40%	30%
RT-PCR	100	40	30	40	30	30	40%	30%

4.4 Analysis of specificity and sensitivity of RT-LAMP and RT-PCR

Table 7: Specificity and sensitivity of RT-LAMP test using plaque assay as the gold standard.

			PLAQUE ASSAY (GOLD STANDARD)		
			Positive	Negative	Total
RT-LAMP (TEST)		Positive	40	7	47
		Negative	0	23	23
		Total	40	30	70

SENSITIVITY = $\frac{40 \text{ X } 100\%}{40+ 0} = 100\%$

SPECIFICITY = $\frac{23 \text{ X } 100\%}{23+ 7} = 77\%$

Table 8: Specificity and sensitivity of RT-PCR test using plaque assay as the gold standard.

			PLAQUE ASSAY (GOLD STANDARD)		
			Positive	Negative	Total
RT-PCR (TEST)		Positive	40	0	40
		Negative	7	23	30
		Total	77	23	70

SENSITIVITY = $\frac{40 \text{ X } 100\%}{40 + 7} = 85\%$

SPECIFICITY = $\frac{23 \text{ X } 100\%}{23 + 0} = 100\%$

4.5 Calculation of the positive predictive value (PPV) and negative predictive values (NPV) for RT-LAMP and RT-PCR using plaque assay as the gold standard.

a) PPV% = $\frac{\text{True positives x 100}}{\text{True positives + False positives}}$

i) PPV for RT-LAMP = $\frac{40 \times 100}{40 + 7} = 85\%$

ii) PPV for RT-PCR = $\frac{40 \times 100}{40 + 0} = 100\%$

b) NPV% = $\frac{\text{True negatives x 100}}{\text{True negatives+ False negatives}}$

i) NPV for RT-LAMP = $\frac{23 \times 100}{23 + 0} = 100\%$

ii) NPV for RT-PCR = $\frac{23 \times 100}{23 + 7} = 77\%$

4.6 Distribution of test outcome by different screening tests

Using 70 samples the gold standard plaque assay detected accurately 40 samples as positive and 30 negative. However plaque assay was not able to distinguish clearly whether the remaining 30 samples (indeterminate) were either positive or negative. On the other hand, RT-LAMP assay was able to detect accurately a total of 63 samples of which 40 were positive and 23 were negative. However this test was not able to distinguish clearly whether the remaining 37 samples (indeterminate) were either positive or negative. In general the level of agreement in detecting the positive and negative with both tests was 66.5% and that of indeterminate was 33.5%.

Table 9: Comparison between the level of agreement of RT-LAMP and Plaque assay in terms of positives/negatives and indeterminate using Pearson chi-square test.

			Outcome		
			Positive/ Negative	In-determinate	Total
Test	RT-LAMP	Count	63	37	100
		% within Test	63.0%	37.0%	100.0%
	P/A	Count	70	30	100
		% within Test	70.0%	30.0%	100.0%
Total		Count	133	67	200
		% within Test	66.5%	33.5%	100.0%

There was no significant difference between the distributions of outcome of the tests using the gold standard which is plaque assay compared to RT-LAMP using Pearson chi-square test because the P value was calculated as 0.369. This explains that the difference of seven between the number of positives, negatives and indeterminate between plaque assay and RT-LAMP is not statistically significant.

Table 10: The distributions of outcome using RT-LAMP compared to the gold standard plaque assay using chi-square test. (P= 0.369).

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	1.100(b)	1	.294		
Continuity Correction(a)	.808	1	.369		
Likelihood Ratio	1.101	1	.294		
Fisher's Exact Test				.369	.184
Linear-by-Linear Association	1.094	1	.296		
N of Valid Cases	200				

Table 10 shows that the gold standard plaque assay detected accurately a total of 70 samples of which 40 were positive and 30 negative. However plaque assay was not able to distinguish clearly whether the remaining 30 samples (indeterminate) were either positive or negative. On the other hand, RT-PCR assay was able to detect accurately a total of 70 samples of which 40 were positive and 30 were negative. However this test was not able to distinguish clearly whether the remaining 30 samples (indeterminate) were either positive or negative.

Table 11: Comparison between level of agreement of RT-PCR and plaque assay in terms of positives/negatives and indeterminate using chi-square test.

			Outcome		
			Positive/ Negative	In-determinate	Total
Test	RT-PCR	Count	70	30	100
		% within Test	70.0%	30.0%	100.0%
	P/A	Count	70	30	100
		% within Test	70.0%	30.0%	100.0%
Total		Count	140	60	200
		% within Test	70.0%	30.0%	100.0%

A test of difference between distributions of test outcomes by different screening tests was performed using Pearson chi-square test. The level of agreement between the gold standard plaque assay and RT-PCR was 100% (perfect agreement {P= 1}).

Table 12: The level of agreement between the gold standard plaque assay and RT-PCR.

	Value	Df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.000(b)	1	1.000		
Continuity Correction(a)	.000	1	1.000		
Likelihood Ratio	.000	1	1.000		
Fisher's Exact Test				1.000	.561
Linear-by-Linear Association	.000	1	1.000		
N of Valid Cases	200				

4.7 Comparative analysis of RT-LAMP and other diagnostic techniques

This analysis was based on the cost of equipment, cost of reagents, cost to perform a single test, manpower, specificity, sensitivity, time spent to carry out the test and benefits of each test.

Amplification efficiency of LAMP was extremely high because there was no time loss for thermal change because of its isothermal reaction. Of particular importance was the substantial reduction in time required for confirmation of results by the RT-LAMP assay, 1hour compared to 3 to 4 hour in case of RT-PCR. More importantly, whereas a PCR diagnosis can cost 30-40 USD in KEMRI, and is difficult to decentralize, RT LAMP is expected to cost 8 USD and can be done in the field so long as a water bath can be maintained at between 60^{o}C for 60 minutes and

monitoring of gene amplification with naked eye through color changes. Plaque assay was time consuming since it took 4 days for the results to be read.

Table 13: Cost benefit analysis of the three assays performed in this study that is; plaque, RT-PCR and RT-LAMP.

ASSAY	EQUIPMENT COST	REAGENT COST/100 SAMPLES	TURN AROUND TIME/ SAMPLE	SIMPLICITY IN RESOURCE POOR SETTING (1-10)	PERSONNEL TRAINING
PLAQUE	10,000 USD	300 USD	4 DAYS	8	HIGH
RT-PCR	16, 000 USD	1000 USD	1 DAY	9	HIGH-MEDIUM
RT-LAMP	12, 000 USD	1000 USD	1 DAY	1	MEDIUM

RT-LAMP requires an inexpensive turbidimeter for monitoring. Cost of turbidimeter is 10,000USD which is very low compared to that of the ABI prism 7700 sequence detection system instrument (PE Applied Biosystems) required for the real-time TaqMan RT-PCR and NASBA assay, which is $100,000. RT-LAMP does not require sophisticated equipment i.e. thermocycler thus, has potential usefulness for clinical diagnosis and surveillance of ONNV in developing countries. The execution of RT-LAMP reaction and the measurement of its turbidity are extremely simple compared to those of the existing RT-PCR (TaqMan RT-PCR) that requires flourogenic primers and probes as well as expensive detection equipment. RT-LAMP requires only one type of DNA polymerase with strand displacement activity. RT-LAMP is a simple, economic molecular test and is easy to detect (detection sensitivity). High amplification efficiency of the RT-LAMP reaction yields a large amount of by-

product, pyrophosphate ion leading to a white precipitate of Magnesium pyrophosphate in the reaction mixture.

High cost of equipment required for performing RT-PCR assay restricts its use to laboratories with good financial resources. Not a method of choice in basic clinical settings in developing countries or in field situations because of the requirement for sophisticated instrumentation and expensive reagents. Requires high precision instruments for amplification or elaborate methods for detection of amplified products. PCR-based methods are often cumbersome to adapt for routine clinical use especially in peripheral healthcare settings and private clinics. Complicated method for detection of amplified products. Labour intensive and time consuming since it can take 3 to 4 hours to complete a set test. There is high risk of contamination.

Plaque assay is time consuming in that it requires more than one week for completion. It has less impact on patient management and control measures exercised by medical and public health personnel thus cannot provide timely clinical treatment and etiologic investigation and disease control in acute phase of illness. It requires viable virus in samples.

Table 14: Comparison in benefit analysis amongst the three assays.

RT-LAMP	**RT-PCR**	**Plaque assay**
Results obtained within 1 hour.	Results obtained within 3 to 4 hours.	Results obtained after 2 to 3 days for fast growing viruses like ONNV.
Results can be visualized by naked eye by examining the colour change of the fluorescent dye.	Results can only be visualized by gel electrophoresis not by naked eye visualization	Results visualized by naked eye through counting of the plaques formed.
Can be applied in field situations especially during outbreaks.	Strictly applied in well-equipped laboratories with financial resources	Applied in laboratories which deal with cell culture only.

CHAPTER FIVE

5. DISCUSSION, CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Introduction

This study demonstrates a quicker, cheaper and convenient assay for detection of O'nyong'nyong virus which is an alpha virus of public health importance. The RT-LAMP assay is a simple diagnosis tool in which the reaction is carried out in a single tube by mixing the buffer, primers, reverse transcriptase, and DNA polymerase and incubating the mixture at 61°C for 1 h in a regular laboratory water bath or heat block that provides a constant temperature of 61°C. The amplification can be performed in a shorter time than amplification by RT-PCR because there is no time loss due to thermal cycling. A one-step single-tube accelerated real-time quantitative RT-LAMP assay for the early and rapid diagnosis of ONNV was developed, optimized and compared to other existing virus detection assays. RT-LAMP assay was found to be the best suited method compared to the gold standard plaque assay and RT-PCR due to its simple operation, rapid reaction, and easy detection. The higher sensitivity of the RT-LAMP reaction was attributed to continuous amplification under isothermal conditions employing six primers that recognize eight distinct regions of the target gene (Monath and Heinz 1996; Mori *et al.*, 2001). Besides, the higher amplification

efficiency of RT-LAMP yielded a large amount of a by-product, pyrophosphate ion, leading to white precipitate of magnesium pyrophosphate in the reaction mixture. Since the increase in the turbidity of the reaction mixture according to the production of precipitate correlates with the amount of DNA synthesized, real-time monitoring of the RT-LAMP reaction can be achieved by real-time measurements of turbidity (Mori *et al.,* 2001; Nagamine *et al.,* 2002, Parida *et al*., 2004a, Parida *et al*., 2005,).

5.1.2 Design of primers and standardization of conditions for RT-LAMP assay

The RT-LAMP assay was standardized for rapid detection of ONN virus by targeting highly conserved regions of the E1 gene based on multiple sequence alignments of all circulating strains. The details of each primer with regard to their positions in the genomic sequences were shown in Table 14. The detection of gene amplification was accomplished by monitoring of turbidity at 61^{o}c for 1 hour. The results indicated that the minimum time required for the initiation of amplification was 10 minutes with viral RNA preparations. Compared to RT-PCR and plaque assay, RT-LAMP has the advantages of reaction simplicity and detection sensitivity. The higher sensitivity was attributed to continuous amplification under isothermal conditions by employing six primers that recognize eight distinct regions of the target.

5.1.3 Comparison of RT-LAMP with plaque assay and RT-PCR for O'nyong'nyong virus in terms of cost, turn around time per sample, simplicity and personnel training

This study emphasizes on the comparative analysis of RT-LAMP and other diagnostic techniques performed under this study that is; RT-PCR and Plaque assay. This analysis was based on the cost of equipment, cost of reagents, cost to perform a single test, manpower, specificity, sensitivity, time spent to carry out the test and the benefits of each test. Plaque assay was the cheapest assay in terms of equipment cost, reagent cost per 100 samples though its turn around time is longest of the three assays since it required 4 days for a definitive diagnosis to be made. RT-PCR and RT-LAMP were similar in terms of reagent cost per 100 samples and turnaround time per sample was the shortest in both assays (1 day). The difference between RT-LAMP and RT-PCR was in the equipment cost, simplicity in resource poor setting and personnel training. RT-PCR was the most expensive of the three assays since it employed the use of a thermocycler which was very expensive (10,000USD). RT-LAMP employed use of the loop amp turbidimeter which costs 6,000USD. RT-LAMP can be performed in resource poor setting and does not require highly trained staff. Plaque assay requires highly trained staff. In general RT-LAMP had the most advantages compared to plaque assay and RT-PCR.

5.1.4 Assessment of the feasibility of RT-LAMP as a diagnostic tool for laboratory testing of O'nyong'nyong virus in Kenya

The evaluation of the RT-LAMP assay for detection of viral RNA in virus infected samples was validated using 100 prepared and blinded samples and the results were compared with those of plaque assay and conventional RT-PCR. The data presented in this study suggested that the RT-LAMP assay was more sensitive than RT-PCR as it was able to detect 7 additional indeterminate cases that were negative by RT-PCR. The RT-LAMP assay demonstrated exceptionally higher sensitivity than conventional RT-PCR. The RT-LAMP assay was found to be 100% sensitive than RT-PCR, which was 85% sensitive. PPV of RT-LAMP was 85% whereas that of RT-PCR was 100%. NPV of RT-LAMP was 100% and that of RT-PCR was 77%. As demonstrated in this study, the RT-LAMP assay has demonstrated higher sensitivity by correctly picking up additional indeterminate with low concentration of virus that were missed by RT-PCR.

Plaque assay was used as the gold standard in this study. A plaque assay was used in this study to quantify the amount of the ONNV in each sample. Out of the 100 samples tested by plaque assay 40 (11-30 pfu/ml) samples tested as positive, 30 (0 pfu/ml) samples as negative and 30 (1-10 pfu/ml) indeterminate. Conventional RT-PCR 40 samples were positive, 30 negative and 30 indeterminate. By RT-LAMP all 40 samples were positive, 23 samples negative and 37 indeterminate. RT-LAMP detected 7 samples as indeterminate that conventional PCR and plaque assay did not detect.

The sensitivity and specificity of RT-PCR and RT-LAMP was calculated using plaque assay as the gold standard. The sensitivity of RT-LAMP was 100% whereas that of RT-PCR was 85%. The specificity of RT-LAMP was 77% whereas that of RT-PCR was 100%. RT-LAMP is the best screening test when it comes to sensitivity whereas plaque RT-PCR is the best screening tests when it comes to specificity. A test of difference between distributions of test outcomes by different screening tests was performed using Pearson chi-square test. The results indicated that plaque assay could be a better technique compared to RT-LAMP since it had lesser number of indeterminate by 7 and more numbers of positives and negatives by 7.

The results showed that both plaque assay and RT-PCR had similar outcome in terms of the number of positives, negatives and indeterminate. The level of agreement between the gold standards PA and RT-PCR was 100%. After comparing both RT-LAMP and RT-PCR with the gold standard plaque assay the level of significance at 95% confidence interval was $P>0.05$ meaning that the difference in 7 samples being negative and indeterminate was not statistically significant. This implies that reliability of the results using RT -LAMP is as good as using RT-PCR or Plaque assay.

One of the most powerful features portrayed by RT-LAMP assay is that it has great advantage in terms of monitoring of amplification that can be accomplished by SYBR Green I dye-mediated naked eye visualization. Of particular importance is the substantial reduction in the time required for the confirmation of results by the RT-LAMP assay, 1 hour compared to 3 to 4 hour in case of RT-PCR. Thus, the RT-

LAMP assay reported in this study allows rapid, real-time detection as well as quantification of ONNV in acute-phase serum samples without requiring sophisticated equipment and has potential usefulness for clinical diagnosis and surveillance of ONNV in developing countries.

5.2 Conclusions

The combined data reported here indicate that the RT-LAMP assay is extremely rapid, cost-effective, and highly sensitive and could be used along with virus isolation and/or serology as a comprehensive ONNV detection system in the diagnostic laboratory. The RT-LAMP assay developed in this study allows the rapid and accurate identification of ONNV. Due to its simple operation without the need for sophisticated equipment, it will be a valuable tool for the rapid detection of ONNV in well equipped laboratories, small-scale clinical laboratories, as well as field situations, like peripheral health care settings in developing countries. RT-LAMP is the method of choice in basic clinical settings in developing countries or in field situations because of the requirement for sophisticated instrumentation and expensive reagents. It is therefore critical to develop simple and economical molecular tests for the above scenarios.

5.3 Limitations

5.3.1 Availability of time and funds

This study involved a number of experiments mostly molecular diagnostic techniques which were very expensive in terms of equipment and reagent cost. Virus isolation techniques such as cell culture and plaque assay were time consuming since it took more than seven days to get results of these assays.

5.3.2 Quantification of RT-LAMP results.

Lack of L-200 loop-amp turbidimeter made it difficult to quantify the level of turbidity in each sample.

5.4 Recommendations

Further evaluation and validation of this study should be done with a more defined panel of serum to establish RT-LAMP assay as a tool for surveillance of not only O'nyong'nyong virus, but also other emerging viruses. The surveillance work will form part of the long term KEMRI-NUITM project, and it may be possible to build capacity at the district level to detect arboviruses without the need to ship samples to the central reference laboratory at KEMRI.

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APPENDICES

APPENDIX I

6.1 ONNV genome

ORIGIN

LOCUS NC_001512 3744 bp ss-RNA linear
DEFINITION O'nyong-nyong virus, complete genome.
ACCESSION NC_001512 REGION: 7670...11413
VERSION NC_001512.1 GI: 9627007
SOURCE O'nyong-nyong virus
ORGANISM O'nyong-nyong virus
Viruses; ssRNA positive-strand viruses, no DNA stage;
Togaviridae;
Alphavirus; SFV complex.
REFERENCE 1 (bases 1 to 3744)

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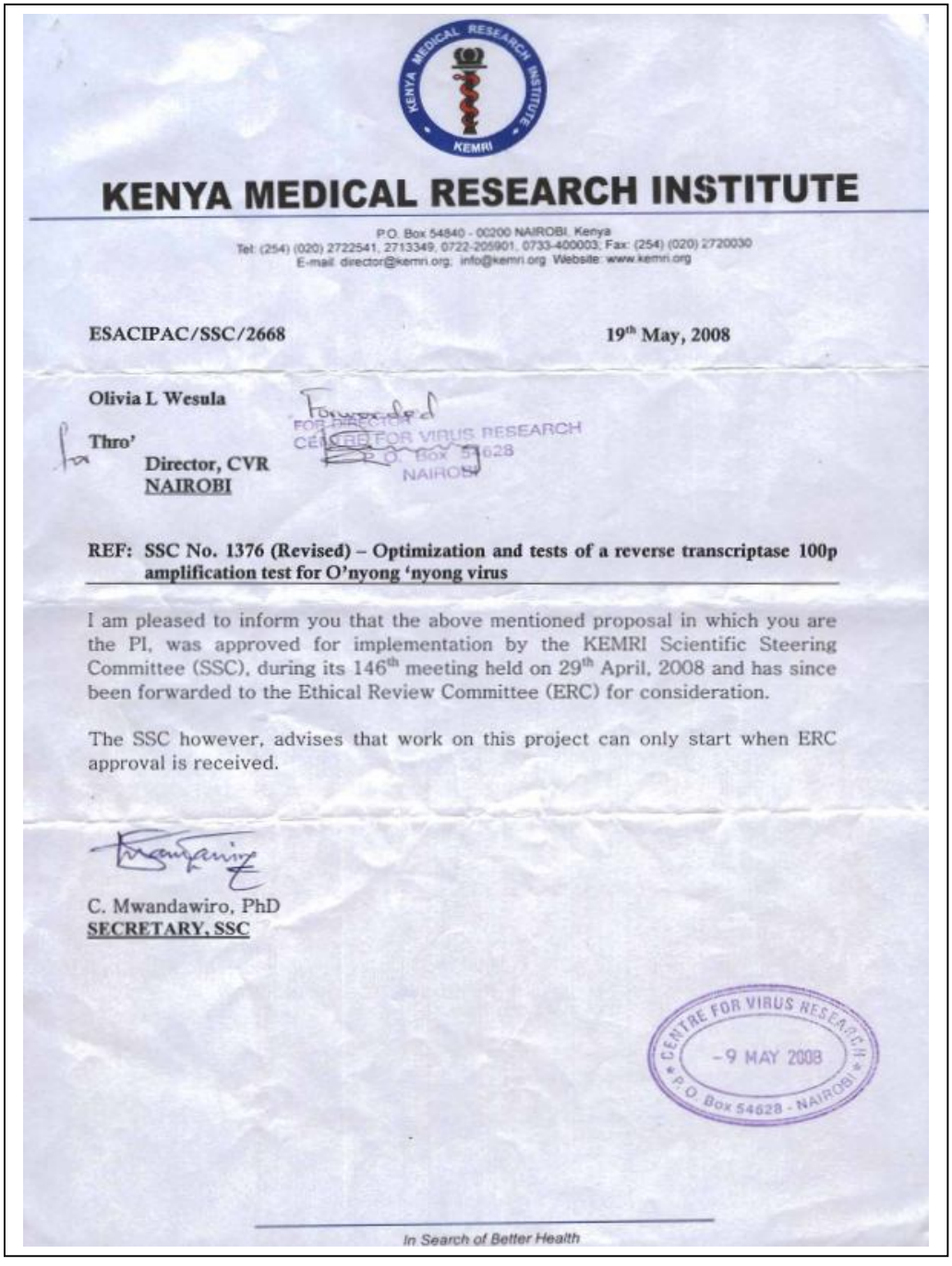
APPENDIX II

6.2 Approval from Scientific Steering Committee (SCC)



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ESACIPAC/SSC/2668 — 19th May, 2008

Olivia L Wesula

Thro'
Director, CVR
NAIROBI

Forwarded
FOR DIRECTOR
CENTRE FOR VIRUS RESEARCH
P.O. Box 54628
NAIROBI

REF: SSC No. 1376 (Revised) – Optimization and tests of a reverse transcriptase 100p amplification test for O'nyong 'nyong virus

I am pleased to inform you that the above mentioned proposal in which you are the PI, was approved for implementation by the KEMRI Scientific Steering Committee (SSC), during its 146th meeting held on 29th April, 2008 and has since been forwarded to the Ethical Review Committee (ERC) for consideration.

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

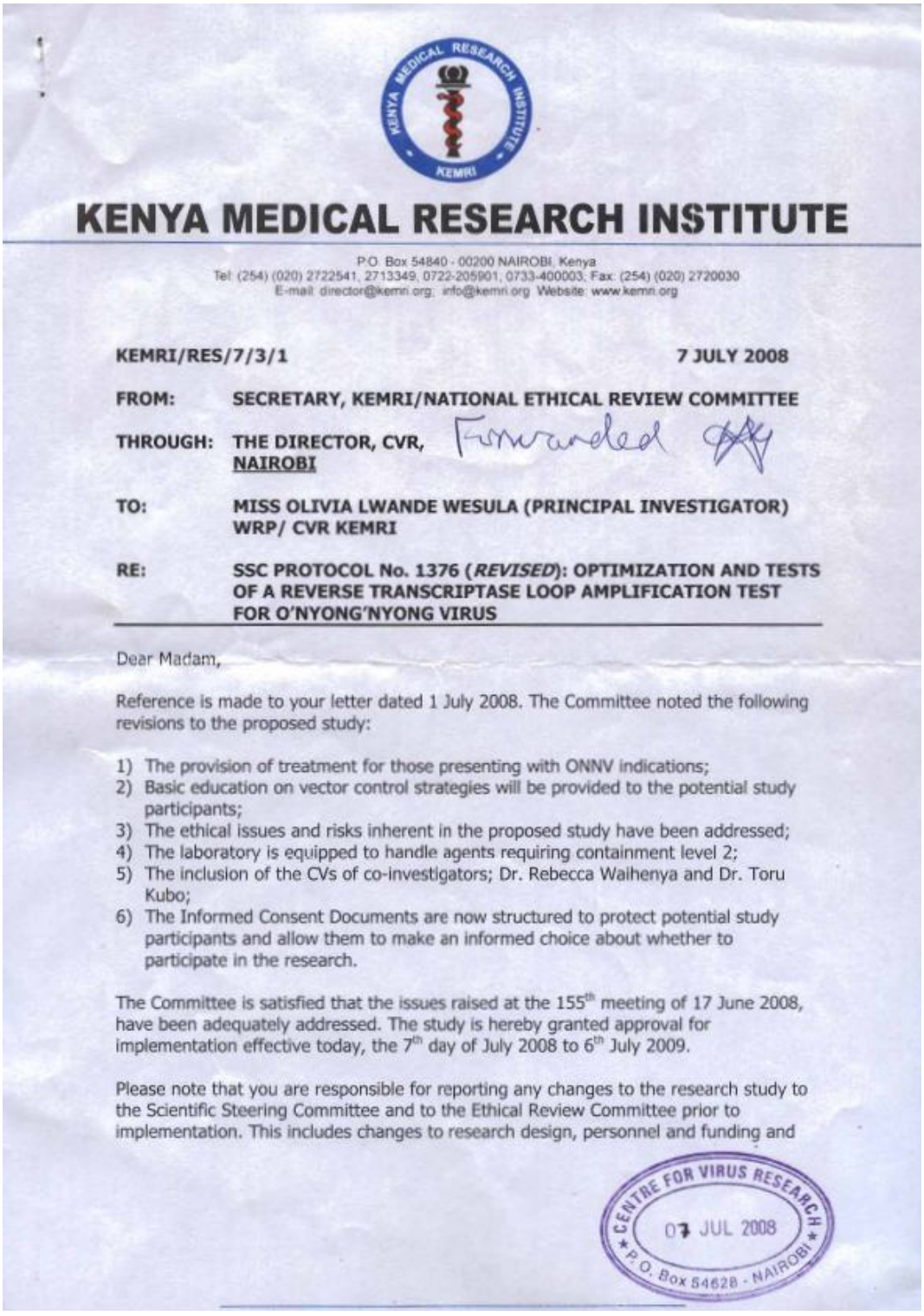
C. Mwandawiro, PhD
SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
-9 MAY 2008
P.O. Box 54628 - NAIROBI

In Search of Better Health

APPENDIX III

6.3 Approval from Ethical Review Committee (ERC)



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KEMRI/RES/7/3/1 **7 JULY 2008**

FROM: **SECRETARY, KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE**

THROUGH: **THE DIRECTOR, CVR, NAIROBI** Forwarded

TO: **MISS OLIVIA LWANDE WESULA (PRINCIPAL INVESTIGATOR) WRP/ CVR KEMRI**

RE: **SSC PROTOCOL No. 1376 (*REVISED*): OPTIMIZATION AND TESTS OF A REVERSE TRANSCRIPTASE LOOP AMPLIFICATION TEST FOR O'NYONG'NYONG VIRUS**

Dear Madam,

Reference is made to your letter dated 1 July 2008. The Committee noted the following revisions to the proposed study:

1) The provision of treatment for those presenting with ONNV indications;
2) Basic education on vector control strategies will be provided to the potential study participants;
3) The ethical issues and risks inherent in the proposed study have been addressed;
4) The laboratory is equipped to handle agents requiring containment level 2;
5) The inclusion of the CVs of co-investigators; Dr. Rebecca Waihenya and Dr. Toru Kubo;
6) The Informed Consent Documents are now structured to protect potential study participants and allow them to make an informed choice about whether to participate in the research.

The Committee is satisfied that the issues raised at the 155th meeting of 17 June 2008, have been adequately addressed. The study is hereby granted approval for implementation effective today, the 7th day of July 2008 to 6th July 2009.

Please note that you are responsible for reporting any changes to the research study to the Scientific Steering Committee and to the Ethical Review Committee prior to implementation. This includes changes to research design, personnel and funding and

CENTRE FOR VIRUS RESEARCH 07 JUL 2008 P.O. Box 54628 - NAIROBI

procedures that could introduce new or more than minimum risk to research participants. You may embark on the study.

Sincerely,

C. WASUNNA,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE