An evaluation of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) for diagnosis of rabies

By

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DECLARATION

I Golden Seulu Sandala, student number 2019104075 declare that the work presented in this dissertation, is my own work, carried out with the help of people acknowledged and that this work has not been submitted before for the award of a degree at any University.

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CERTIFICATE OF APPROVAL

This dissertation by Golden Seulu Sandala has been approved as fulfilling the requirements for the award of the degree Master of Science in One Health Laboratory Diagnostic Sciences by the University of Zambia

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ABSTRACT

Rabies is a neurological disease caused by a lyssavirus belonging to the family rhabdoviridae. The causative agent has a global distribution (except Antarctica) resulting in an estimated 59,000 human deaths worldwide. The true disease burden and public health impact due to rabies remain underestimated due to lack of sensitive laboratory diagnostic methods. This is particularly common in underdeveloped countries. This study was aimed at evaluating Reverse Transcriptase Loop-Mediated Isothermal Amplification (RT-LAMP) as a viable alternative method to RT-PCR and DFAT for diagnosis of rabies in Zambia. RT-LAMP primers were designed based on the most conserved region of N gene sequences covering various rabies virus isolates representative sequences from around Africa. Amplification products of the RT-LAMP assay was monitored by visual colour change of fluorescent dye and by agarose gel electrophoresis. To evaluate the RT-LAMP assay's applicability to rabies, archived samples stored at -80◦C at the University of Zambia repository were used. A total of 84 samples were tested using both RT-LAMP and RT-PCR, with 25 samples coming from Zimbabwe, 40 samples coming from Zambia, and 19 samples coming from Malawi. The results of the RT-LAMP were compared with RT-PCR assay. The RT-LAMP assay was successfully optimized with a turnaround time of about 2 hours and diagnostic sensitivity and specificity of 96.1% and 100%, respectively. The limit of detection (LOD) of the RT-LAMP was $2.19x10^{-5}$ ηg/µl of rabies sample RNA. In conclusion, RT-LAMP is a promising alternative to RT-PCR and DFAT for the diagnosis of rabies, offering several advantages in terms of cost-effectiveness, simplicity, and sensitivity. While further studies are needed to validate its performance in different settings, RT-LAMP has the potential to become a valuable tool in the global fight against rabies.

Keywords: rabies, sensitivity, specificity, optimization, reverse-transcriptase Loop-Mediated Isothermal Amplification

DEDICATION

This dissertation is dedicated to my dear parents, my siblings, and my dear wife, for their unfailing love, support, inspiration and encouragement right from my early school days and for making it all possible. For all that I am and will ever be, I owe it to you.

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ABBREVIATIONS

CSF	cerebrospinal fluid
CVRI	Central Veterinary Research Institute
DALY	disability-adjusted life–year
DFAT	Direct Fluorescent Antibody Test
DNA	Deoxyribonucleic acid
DRIT	direct rapid immunohistochemical test
ELISA	Enzyme-Linked Immunosorbent Assay
FAT	Fluorescent antibody test
FAVN	fluorescent antibody virus neutralization
GARC	Global Alliance for Rabies Control
ICTV	International Committee on Taxonomy of Viruses
NPV	Negative Predictive Value
PCR	Polymerase Chain Reaction
PEP	Post-Exposure Prophylaxis
PPV	Positive Predictive Value
RABV	Rabies virus
RFFIT	Rapid fluorescent focus inhibition test
RIG	Rabies immunoglobulin
RNA	Ribonucleic acid
RT-LAMP	Reverse Transcription Loop-mediated Isothermal Amplification
RT-PCR	Reverser Transcription Polymerase Chain Reaction
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
TAT	Turnaround time
UNZA	University of Zambia
WHO	World Health Organization
WOAH	World Organization for Animal Health

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Rabies is caused by a virus belonging to the genus Lyssavirus of *Rhabdoviridae* family (Tordo et al., 1986). All lyssaviruses are characterized by a bullet-shaped appearance when examined under the electron microscope. Rabies virus is a single-stranded, negative-sense, unsegmented, and enveloped RNA virus. The virus genome encodes five (5) genes namely, Nucleoprotein (N) which encapsulates the viral RNA, Glycoprotein (G) which aids in attachment to host cells, Matrix protein (M) responsible for virus budding and morphology, Phosphoprotein (P) and RNA polymerase (L) which form the viral replication complex (Tordo et al., 1986). Rabies has a worldwide distribution except in Antarctica. In Western Europe, Canada, the United States and Japan, dog-mediated rabies has been eradicated. Further, no human fatalities from dog-transmitted rabies have been reported in 28 of the 35 Latin American nations as of 2013 (Vigilato et al., 2013). The N (nucleoprotein) gene is commonly used for RT-LAMP primer design for rabies virus detection because it is a highly conserved gene among different rabies virus strains. This ensures that the designed primers can detect a broad range of rabies virus strains with high sensitivity and specificity. The expected band size of the RT-LAMP product for rabies virus detection varies depending on the primer design and the specific assay used. However, a typical size range for RT-LAMP products is between 150-300 bp (Itou, et al., 2014).

For over 4300 years, rabies has been known to be a public health problem (Takayama, 2005). In recent times, there has been an increase in development and establishment of several nucleic acid-based amplification methods for detection and clinical diagnosis of infection with rabies virus, among them include Nucleic-acid sequence based amplification (NASBA) and Polymerase Chain Reaction (PCR) (Chen et al., 2011, Biswal et al., 2012, Coertse et al., 2019, Camara, 2020). The few existing estimates on the burden of rabies documented in humans as part of research efforts can be completed by laboratory diagnosis of rabies in endemic settings, providing reliable, laboratory-confirmed data (Hampson et al., 2015). Findings from these studies are essential for raising policy makers' awareness of the rabies burden and for determining how to allocate resources most effectively for its control.

Rabies diagnosis in Zambia is mainly by FAT and PCR (Muleya et al 2012, Muleya et al., 2019). The assessment of the true burden of rabies in Zambia, has been severely hampered by a number of factors such as inadequate surveillance, lack of affordable, quick, and simple diagnostic tools and lack of public knowledge. Holistically, all these have led to the underreporting of cases, which has reduced the possibility of implementing efficient control strategies and interventions. Thus this study explored the development and assessment of a faster, cheaper and reliable RT-LAMP diagnostic assay in an effort to improve diagnosis and surveillance of rabies in Zambia.

Early diagnosis of the disease is critical for the timely administration of post-exposure prophylaxis to prevent the onset of the disease. Reverse transcription polymerase chain reaction (RT-PCR) and direct fluorescent antibody test (DFAT) are two commonly used methods for the diagnosis of rabies. However, there are certain challenges associated with the use of these methods for the diagnosis of rabies. One of the major challenges associated with the diagnosis of rabies using RT-PCR and DFAT is the possibility of false-negative results due to the viral load which may be below the detection limit of the test (Chen et al., 2011). Additionally, both RT-PCR and DFAT require technical expertise for accurate interpretation of results. Improper sample handling or processing can also lead to inaccurate results (Cohen et al., 2007). The sensitivity and specificity of both RT-PCR and DFAT can vary depending on the quality of the sample and the stage of the disease (Duong et al., 2016). Further, the cost of performing RT-PCR and DFAT can be high, especially in resource-limited settings, which can limit their availability and accessibility. Both RT-PCR and DFAT require specialized equipment and trained personnel, which can result in delays in obtaining results, especially in remote areas (Itou et al., 2014).

1.2 Statement of the problem

Despite rabies being a notifiable zoonotic disease in Zambia, there are various challenges in the diagnosis of rabies. This is principally due to lack of dependable and consistent supply of required diagnostic materials, maintenance and repair of machinery, and effective transportation of samples from the field to testing facilities. Additionally, laboratory diagnosis of rabies is conducted in two laboratories namely University of Zambia School of veterinary medicine and the Central Veterinary Research Institute, both in Lusaka province. In both labs, rabies diagnosis is done using DFAT and RT-PCR. These methods are often expensive and time consuming. In view of the aforementioned factors, the need for reliable and rapid

diagnostic techniques in both humans and animals is increasingly becoming important amidst the escalated cross-border trading, globalization, and upsurge at the human-animal interface. Therefore, improving the sensitivity, specificity, and availability of these tests can help improve the diagnosis and management of rabies in Zambia.

1.3 Study Objectives

1.3.1 General Objective

To evaluate RT-LAMP as a viable technique for diagnosis of rabies

1.3.2 Specific Objectives

1 To determine the optimal conditions for RT-LAMP diagnosis of rabies

2 To determine the accuracy indices (sensitivity and specificity) of RT-LAMP using RT-PCR as reference method

1.4 Research questions

1 Can Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) be an alternative method to RT-PCR for diagnosis of rabies?

2 What are the optimal conditions for RT-LAMP assay for rabies?

3 What is the sensitivity of the RT-LAMP for rabies?

4 What is the specificity of the RT-LAMP for rabies diagnosis?

1.5 Significance of the study

To a large extent, the underreporting of rabies cases in most underdeveloped countries due to limitations in laboratory facilities and surveillance systems. In Zambia, an attempt to establish RT-LAMP assay for rabies was made in 2012. However, up to now, the assay has not been evaluated and assessed against significant numbers of clinical samples to be considered an appropriate alternative to the DFAT or RT-PCR for rabies diagnosis despite it being cheaper than both DFAT and RT-PCR. The proposed RT-LAMP technique is a simple, and cost-effective method which will not require any specialized equipment for visualization of results, making it a better option, especially in resource-limited rural areas of Zambia. This in turn will aid in the establishment of an effective and efficient decentralized rabies surveillance system, which will enable the government to ascertain the true burden of rabies in Zambia and prevent human rabies deaths, ultimately helping to achieve the "Zero Human Deaths by 2030" global (WHO, 2018).

CHAPTER TWO

2. LITERATURE REVIEW

2.1 General Overview of Rabies

The increasing interactions between humans and animals at the human-animal interface has necessitated the adoption of One Health approach in addressing animal and public health challenges such as rabies. Rabies is a viral zoonotic disease with worldwide distribution with the exception of Antarctica and a few countries (Tordo et al., 1986). It is a continuous animal and public health hazard that causes about 59,000 human deaths annually worldwide, particularly in developing countries (Hampson et al., 2015) with 95% of these deaths occurring in Africa and Asia (Rupprecht et al., 2008). The disease is nearly 100% fatal, though death can be prevented by a complete course of post-exposure prophylaxis (PEP) prior to the onset of symptoms (Hampson et al., 2015). In order to overcome host species, geographic, and ecological obstacles, the rabies virus (RABV), the genus' prototype species that is responsible for the majority of human and animal rabies cases worldwide, has established independent transmission cycles in several mammal species (Rupprecht, et. al., 2008, Vallasco-villa, 2017, Marston et al., 2018).

According to the International Committee on Taxonomy of Viruses (ICTV), the genus lyssavirus comprises 17 species. Based on genetic distances within their G-protein ectodomains and serologic cross-reactivity, lyssaviruses have been split into two phylogroups on a global scale (Badrane et al., 2001). Rabies lyssavirus, Aravan lyssavirus, Australian bat lyssavirus, Bokeloh lyssavirus, Duvenhage lyssavirus, type 1 and type 2 European bat lyssaviruses, Khujand lyssavirus, and the newest Gannoruwa bat lyssavirus (Rupprecht et al., 2017) are all included in Phylogroup I. Lagos bat lyssavirus, Mokola lyssavirus, and Shimoni bat lyssavirus are all members of Phylogroup II (Rupprecht et al., 2017). The level of genetic divergence and lack of cross-neutralization prevent placement of West Caucasian bat lyssavirus, Ikoma lyssavirus, and Lleida bat lyssavirus in a single phylogroup based on the established demarcation criteria, despite the fact that phylogenetically they do appear related (Horton et al., 2014).

In terms of distribution, European bat 1 virus and European bat 2 colonize Europe while Aravan lyssavirus and Khujand lyssavirus are of Central Asian origin. Irkut lyssavirus is found in Eastern Siberia whereas West Caucasian bat lyssavirus is found in the Caucasian region. Lagos

bat virus, Mokola lyssavirus, Duvenhage lyssavirus, Ikoma lyssavirus and Shimoni bat lyssavirus are found in Sub-Saharan Africa, Southern Africa, and east Africa respectively. Rabies lyssavirus has worldwide distribution, except in few Islands (Rupprecht et al., 2017).

2.2 Rabies in Africa

In Africa, Lyssaviruses known to circulate include the Shimoni bat lyssavirus (SHBV), Duvenhage lyssavirus (DUVV), Mokola lyssavirus (MOKV) and Rabies lyssavirus (RABV). Of these, Rabies lyssavirus (RABV) is the most prevalent (Rupprecht et al, 2017) and is designated as Africa lineage 1, 2, 3 and 4. Africa1 and 2 are prominent in dogs and humans while Africa 3 is mainly associated with the mongoose (Kissi et al., 1995, Bourhy et al., 2008). Africa 1 is further divided into 1a restricted to North and West Africa, and Africa 1b limited to South East and parts of Central Africa while Africa 2, 3 and 4 occur in west and central Africa, southern Africa and Egypt, respectively (Kissi et al., 1995, Johnson et al., 2004, Cohen et al., 2007, Bourhy et al., 2008, Talbi et al., 2009).

Recent studies in Central African countries including Democratic Republic of the Congo, Cameroon, Central African Republic and Chad show high incidences of rabies (Mbilo et al., 2019, Kalthan et al., 2020, and Madjadinan et al., 2020). Generally availability of Post Exposure Prophylaxis (PEP) in Africa is low and very costly. A study revealed that five clinic doses of rabies vaccine costing an average of $40 are required for complete Post Exposure Prophylaxis (PEP) treatment in Africa (WHO, 2018). In West Africa, reports from Nigeria, Côte d'Ivoire, and Ghana also show high prevalence and disease burden amidst low dog vaccination coverage with rabies PEP occurring at irregular intervals or only in a few places (Lopes et al., 2018, Ukamaka et al., 2020, Ibrahim et al., 2022).

2.3 Rabies in Zambia

From the early 20th century, rabies has been present in Zambia and remains a weighty public health and animal welfare problem (Munang'andu et al., 2011). Dog rabies is endemic in Zambia and is a top 5 priority disease. According Mulipukwa et al. (2017), the country's relative rabies prevalence is 39.7%, with dog vaccine coverage at 20%, PEP treatment at 4,479 cases per year and an estimated annual cost of $5,539,741. The actual dog population is not well known but it is widely assumed that only a small percentage of the Zambian dog population is vaccinated against rabies (Mulipukwa et al., 2017).

In 2018 Zambia reported over 16,000 dog bites and 23 rabies deaths (Nakazwe and Gianetti, 2019). Since dogs are the main maintenance hosts for RABV in Zambia, control is mainly through mass dog vaccination (Muleya et al., 2012). RABV strains circulating in all regions of Zambia belong to the Africa 1b lineage and no evidence of clustering according to host or geographical origin has been observed so far (Muleya et al., 2019).

2.4 Laboratory diagnosis of Rabies

There are several methods used for the diagnosis of rabies virus in the laboratory. These methods include, virus isolation using cell cultures, rapid immuno-histochemical test, rapid immune-diagnostic test, reverse transcription polymerase chain reaction (RT-PCR), direct fluorescent antibody test (DFAT) and more recently reverse transcription loop mediated isothermal amplification test (RT-LAMP). The direct immunohistochemical and rapid immunodiagnostic test both employ the use of anti-rabies monoclonal antibodies specific for the nucleoprotein, a viral protein abundantly produced during productive infection (Boldbaatar, et al., 2009). Reports have however shown that these tests have lower sensitivity as compared to DFAT (Banyard et al., 2018). Direct fluorescent antibody Test, also known as Fluorecis the most frequently used test and the gold standard approved by both WHO and World Organization for Animal Health (WOAH) for rabies diagnosis (WHO, 2018). The test employs polyclonal or monoclonal antibodies, specific to rabies nucleocapsid protein conjugated to fluorescein isothiocyanate (FITC). DFAT provides 98-100% reliable diagnosis for all rabies virus strains (WHO, 2018).

Generally, molecular diagnostic methods, such as polymerase chain reaction (PCR) for amplification of viral RNA, are easier and more reliable. The principle of RT-PCR is the reverse transcription of the target RNA into complementary DNA (cDNA) which is then amplified by PCR (Coertse et al., 2019). RT-PCR has the ability to establish a definitive diagnosis even in decomposed samples (Fooks et al., 2009). One of the main advantages of RT-LAMP is its simplicity and ease of use, which make it a valuable diagnostic tool in resource-limited settings where specialized equipment and technical expertise are often lacking. In addition, RT-LAMP is highly specific and sensitive, with detection limits similar to those of RT-PCR and DFAT (Banyard et al., 2018). RT-LAMP rapidly amplifies DNA under isothermal conditions using specific primer sets and a DNA polymerase with high strand displacement activity (Notomi et al., 2000, Mori et al., 2001).

2.5 Principle of LAMP assay

Notomi and colleagues published the first description of the loop-mediated isothermal amplification (LAMP) assay (Notomi et al., 2000). The assay's basic working principle relies on the high strand displacement action of a polymerase such as Bst and proceeds by forming stem-loop DNA with a dumb-bell-shaped structure using four specific primers (FIP, BIP, F3 and B3) that may recognize six different places on the target gene sequence and amplify at the same temperature. F3 and B3 are used in the first step to create dumbbell-shaped DNA that serves as the starting material during the first step referred to as the non-cyclic step while FIP and BIP are used in the secondary step for strand displacement DNA synthesis (Figure 1). The cyclic step involves the exponential amplification of the dumbbell-shaped DNA, producing a mixture of stem-loop DNA with various lengths and cauliflower-like structures with numerous loops (Notomi, al., 2000).

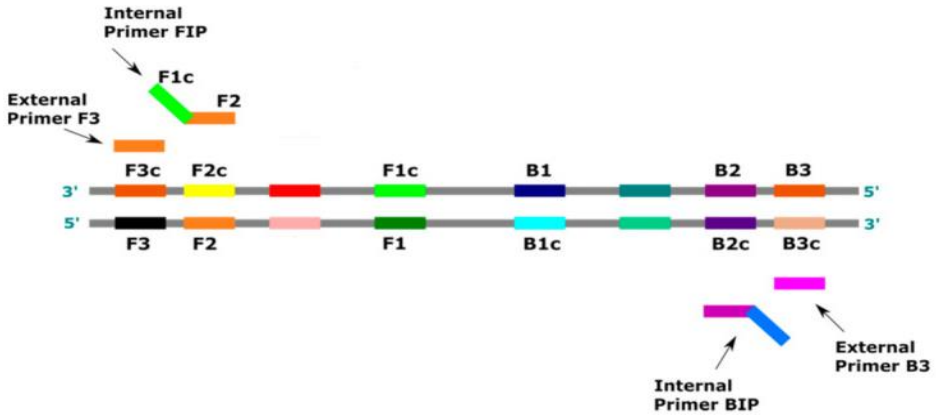

Figure 1: RT-LAMP Primer locations. Primers used in the LAMP reaction. FIP (forward internal primer) contains a region F2 complementary to F2c of the matrix, and a free region F1c complementary to F1 on the newly-formed strand; BIP (backward internal primer) contains a region B2 complementary to B2c of the template, and a free region B1c complementary to B1 on the newly-formed strand; F3 (forward external primer) contains a region F3 complementary to F3c of the template; B3 (backward external primer) contains the region B3 complementary to B3c of the template; (Based on http://loopamp.eiken.co.jp/e/lamp/primer.html (accessed on 9 March 2023)

In a LAMP assay, reaction fidelity is greatly influenced by factors like target sequence specificity, guanine-cytosine content and primer length. Improved primer extension reaction fidelity comes from the inclusion of 5'-tails in primer design, which is crucial for LAMP assays. Additionally, it is crucial to consider the annealing process' stringency, which is based on the temperature and ionic strength of the reaction mixture (Notomi et al., 2000). While it is difficult to modify the ionic strength of the LAMP assays due to the Bst enzyme requirements, the

stringency of the assays can be increased by raising the reaction temperature (Notomi et al., 2000). The design of LAMP primers with high sensitivity and specificity is essential for the success of RT-LAMP analysis because LAMP reactions also have a high level of specificity.

2.6 LAMP assay and Diagnosis of disease

Because it doesn't require expensive equipment like thermo cyclers, RT-LAMP is a low-cost alternative diagnostic approach. Instead, a straightforward heat block or water bath can be utilized (Notomi et al., 2000). Application of LAMP for detection and clinical diagnosis of pathogens such as West Nile virus, *Plasmodium*, Japanese Encephalitis virus, *Mycobacterium* tuberculosis, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), Foot and Mouth Disease Virus (FMDV) has been reported (Chen et al., 2011, Madhanmohan et al., 2013, Kemleu et al., 2016, Kumar et al.,. 2018, Wu et al., 2018, Kundrod et al., 2022, Hu et al., 2020). Over the last two decades, several studies have evaluated the use of RT-LAMP for rabies virus detection as an alternative to traditional methods such as Direct Fluorescent Antibody Test (DFAT) and RT-PCR. A study by Tao et al. (2017) compared the performance of RT-LAMP with that of RT-PCR and DFAT for rabies virus detection in clinical samples. The study found that RT-LAMP had a sensitivity and specificity of 97.5% and 100%, respectively, which were comparable to those of RT-PCR and DFAT. The authors concluded that RT-LAMP is a reliable and practical alternative to traditional methods for rabies virus detection. Another study by Adu-Gyamfi et al. (2021) evaluated the performance of RT-LAMP for rabies virus detection in clinical samples from dogs in Ghana. The study found that RT-LAMP had a sensitivity and specificity of 98.1% and 100%, respectively, which were comparable to those of RT-PCR and DFAT. The authors concluded that RT-LAMP is a reliable and cost-effective alternative to traditional methods for rabies virus detection in resource-limited settings. A study by Lu et al. (2017) also evaluated the performance of RT-LAMP for rabies virus detection using clinical samples. The study found that RT-LAMP had a sensitivity and specificity of 100% and 97.1%, respectively, which were comparable to those of RT-PCR and DFAT. The authors concluded that RT-LAMP is a reliable and rapid alternative to traditional methods for rabies virus detection. Overall, these studies suggest that RT-LAMP is a reliable and cost-effective alternative to traditional methods such as DFAT and RT-PCR for rabies virus detection in clinical samples. The sensitivity of RT-LAMP assay has been reported to compare very favorably with that of a RT-PCR by Hayman et al. (2011) and Reddy et al. (2016). The detection of several lineages of African RABV as well as ante-mortem human rabies in cerebral spinal fluid (CSF), saliva, and post-mortem applications using RT-LAMP has been

documented (Wacharapluesadee and Hemachudha, 2001, Binladen 2007, Fooks et al., 2009). According to a study by Wakeley and colleagues, RT-LAMP can amplify both cosmopolitan and arctic-like strains of the classical rabies virus, hence it is expected to be effective universally regardless of where the virus is isolated in the world (Wakeley et al., 2011). These results are consistent with those of Reddy et al. (2016), who found that the RT-LAMP test had a high degree of specificity for detecting the Arctic-like 1, sub-continental, and cosmopolitan lineages.

Effectiveness of a diagnostic technique is highly influences by the cost per test. The cost may vary depending on several factors such as the manufacturer, the equipment used, the location, and the volume of samples being processed. It is also important to note that while RT-LAMP is generally considered a cost-effective alternative to traditional methods such as RT-PCR and direct fluorescent antibody test (DFAT), the cost may vary depending on the specific implementation and requirements of the laboratory. According to Iqbal et al., (2022), the average cost per test for COVID-19 for RT-PCR was $14.75 while that of RT-LAMP was $8.45. Another study on detection of Peste des Petits Ruminant Virus (PPRV) by RT-LAMP, estimated the cost per reaction to be £2.4 for RT-LAMP and £4.8 for real time RT-PCR (Mahapatra et al., 2019). Everything being equal, LAMP equipment such as the Gene III (OptiGene Ltd., UK), is significantly more affordable (£6,750) and less complex than real-time PCR equipment i.e. the Quant Studio 5 real-time PCR system (Thermo Fisher) which costs £25,000.

2.7 Determining test sensitivity and specificity

Determination and evaluation an assay's sensitivity and specificity is an important aspect in the development and evaluation of diagnostic assays and tools. The terms "sensitivity" and "specificity" should be used with the requisite adjectives because the "diagnostic" and the "analytical" meanings of these terms are very different. Analytical sensitivity, is associated with limit of detection (LOD), and it is a metric of the assay's accuracy. It represents the smallest amount or concentration of substance in a sample that can accurately be measured by an assay (Huttinga, 1996). On the other hand, diagnostic sensitivity of a test is the percentage of truly infected samples that are found to be infected by the test. In other words, diagnostic sensitivity is the percentage of persons who have a given disorder who are identified by the assay as positive for the disorder (Huttinga 1996). Analytical specificity refers to the ability of a diagnostic test to detect only the target analyte or pathogen, without detecting other similar

analytes or pathogens. In other words, it measures the ability of a test to avoid false positives by detecting only the intended target. Analytical specificity is usually determined by testing the diagnostic assay against a panel of related organisms or analytes, and assessing the level of cross-reactivity (Saah and Hoover, 1997). Diagnostic specificity of a test equals the percentage of non-infected samples that are found negative by the test. Put differently, diagnostic specificity is the percentage of persons who do not have a given condition who are identified by the assay as negative for the condition (Huttinga 1996, Saah and Hoover, 1997).

Positive Predictive Values (PPV) and Negative Predictive Values (NPV) are determinants of the feasible and usefulness of a test in the diagnosis of an infection or a disease. They indicate the relative likelihood that a predictor will be accurate or that an exposure will cause an infection (Maro, 2014). The PPV calculates the likelihood that a disease is present when a test is positive by taking into account both true positive (TP) and false positive (FP) results (Maro et al, 2014). PPV is expressed as a percentage and is determined by the ratio of TP/ (TP + FP). Consequently, PPV is the percentage of TP positive test results (correct diagnoses).

In summary, analytical specificity measures the ability of a diagnostic test to detect only the intended target, while diagnostic specificity measures the ability of the test to correctly identify individuals without the disease or condition being tested for.

2.8 Rabies diagnosis and surveillance

In Zambia, rabies diagnosis is mainly done by DFAT and RT-PCR in specialised laboratories at the central veterinary research institute and the University of Zambia, School of Veterinary medicine, respectively. It is has been difficult to adopt these methods in other parts of the country because they both require the use of expensive equipment such as fluorescent microscopes and PCR machines and as such has been a major constraint to the assessment of the true burden of Rabies in Zambia. A number of other factors have been cited as major constraints to assessment of the true burden of rabies in Zambia and these include the lack of inexpensive, rapid and simple diagnostic tools, poor surveillance and public awareness. Furthermore, lack of surveillance data has resulted in the underreporting of cases thus limiting opportunities for the implementation of effective control interventions (Saitou et al., 2010). Therefore, in order to address these gaps, rapid, cheap and easy to apply diagnostic tests are greatly needed in Zambia and RT-LAMP is a good candidate in this regard. The first RT-LAMP assay for rabies in Zambia was established by Muleya et al., 2012. The RT-LAMP assay reported by Muleya et al., (2012) was established using a thermal cycler with 12 archived

Zambian samples and relative fluorescent intensity was measured using VersaDoc Mp 400 imaging system (Bio-rad Laboratories, USA) to determine positivity. The intensity of each sample signal was divided by the negative control result to give the relative intensity of the sample (Muleya et al, 2012). Muleya et al. (2012) also reported an incubation time of 63◦C for 60minutes. However, the study did not establish the accuracy indices (diagnostic sensitivity and specificity,) and cost analysis of the RT-LAMP for rabies. In view of this, this study set out to evaluate the RT-LAMP assay as an alternative technique for rabies detection and surveillance in resource limited settings.

The findings and information produced from this study has potential for improving the diagnostic capabilities of resource poor laboratories nationwide and improve the surveillance of rabies in Zambia which will positively contribute to the WHO/WOAH goal of eliminating dog mediated human rabies cases by 2030.

CHAPTER THREE

3. MATERIALS AND METHODS

3.1 Ethical consideration

Ethical approval for use of archived samples for the study was obtained from the University of Zambia Biomedical Ethics Review Committee (UNZABREC), study approval number: Ref. No. 3240-2022 (see appendix II).

3.1 Research Design

This research was a cross-sectional study employing both qualitative and quantitative data.

3.2 Study Site

The study was conducted at the University of Zambia, School of veterinary medicine, Lusaka Zambia.

3.3 Samples

This study utilized purposive sampling such that no statistical calculations were used to determine sample size. The study was carried out on archived brain samples (n=84) obtained from three geographical regions (Zambia, Malawi and Zimbabwe), and stored at -80°C at the School of Veterinary Medicine, University of Zambia repository.

3.4 RNA extraction and RT-PCR

Total RNA was extracted directly from brain tissue using RNeasy (Qiagen Inc., USA), according to the manufacturer's instructions. Briefly the brain tissue was dissolved in Dulbecco's Modified Eagle Medium (DMEM), lysed and homogenized at 14000rpm for 2 minutes. After discarding the supernatant, 140µl of sample and 560µl absolute ethanol was mixed for 15 seconds and incubated at room temperature for 10 minutes. Thereafter, RNA was precipitated using 560µl of 100% ethanol and vortexed for 15 seconds. Then 630µl of sample was transferred to the spin column and centrifuged at 6000g for 60 seconds. The column was washed with 500µl AW1 at 6000g for 60 seconds. The spin column was then washed with 500µl AW2, centrifuged at 14000 rpm for 3 minutes and transferred to new Eppendorf where it was rinsed with 40µl AVE. The sample was incubated at room temperature for 60 seconds and then centrifuged for 60 seconds after which the Eppendorf tube containing RNA was stored at -30°C. Determination of RABV RNA concentration was determined by measuring the

absorbance using a spectrophotometer (NANODROP 1000, Thermo Fisher Scientific, USA) after which it was used for downstream processing or stored at -80 °C.

The RT-PCR protocol was carried out using the outer primers RN1 (5-CTACAATGGATGCCGAC-3) and RN2 (5-GAGTCACTCGAATATTGC-3) and the inner primers RN3 (5-GACATGTCCGGAAGACTGG-3) and RN4 (5-GTATTGCCTCTCTAGCGGTG-3) (Kamolvarin et al., 1993) in a 15µl tube containing 7.5µl of 2X One step buffer, 0.8µl of 10µM Reverse primer, and 0.8µl of 10µM Forward primer, 0.6µl Primescript mix enzyme, 4.3µl nuclease free water and 1.0µl template. The primers The reaction cycles were: 50°C for 30 minutes, 94°C for 2 minutes, 43 cycles at 94°C, 52°C and 72°C for 30 seconds each, 72°C for 5 minutes and 4°C for infinity. The amplified products were visualized on 1.5% agarose gel coated with ethidium bromide. To prevent contamination, separate rooms with restricted access and unidirectional workflow were used for reagent preparation, RNA and master mix addition, and amplification. Each PCR run included a non-Template Control (NTC) to screen for contamination during optimization and validation.

3.5 RT-LAMP primer design, assay and detection of amplification

Two sets of primers targeting the N gene for use in this study were designed using Primer Explorer Ver. 4 (Eiken Chemical, Japan [http://primerexplorer.jp/ lamp4.0.0/index.html]) for the RT-LAMP assay**.** The primers were designed based on the consensus sequence of the N gene fragment from reference sequences from GenBank (RVU22647_Tanzania, RVU22638_Democratic Republic of Congo, AB572951_Zambia, KF831554_Zimbabwe, and RVU22484_Mozambique). The F3 (forward outer primer), B3 (backward outer primer), FIP (forward inner primer) and BIP (backward in primer) that met the specifications for RT-LAMP assay were selected (Table 4.1). The RT-LAMP assay was performed in a total reaction volume of 20 µL using the Loopamp RNA Amplification Kit (Eiken Chemical, Japan) with a yellow fluorescent detection reagent (Eiken Chemical). The reaction mixture was incubated at 58°C, 59°C, 60°C, 62°C, 64°C, and 65°C for 30min, 33min, 35min, 38min, 40mins, 45mins, 50mins, 55mins, and 60 minutes followed by an incubation at 95 °C for 2 minutes to stop the reaction. A greenish color was indicative of a positive RT-LAMP reaction while an orange color was indicative of a negative reaction. In addition, success of amplification was assessed by visualizing RT-LAMP products on 1.5% agarose gel stained with ethidium bromide.

3.6 Analytical sensitivity, limit of detection, cost analysis and turnaround time of RT-LAMP and RT-PCR assays

To determine the analytical sensitivity of the two assays, serial 10-fold dilutions of rabies field sample RNA were tested up to last positive reaction on agarose gel electrophoresis and visualized under UV. The limit of detection was determined using a ten-fold serial dilution of working standard positive RNA starting from a concentration of $2.2x10^2$ ng/µl. In order to ensure reproducibility and precision of results, each serial dilution was run in triplicate for each assay. In order to determine the assay turnaround time (TAT) for RT-LAMP and RT-PCR, the time interval from specimen sample pretreatment up to reporting of results for both assays was measured.

3.7 Specificity of RT-LAMP and RT-PCR assays

Evaluation of the specificity of the RT-LAMP assay was achieved by testing for cross-reactivity with the nucleic acids from ticks, SARS-CoV-2 and *Rickettsia.*

3.8 Data Analysis

Data was entered in Microsoft Excel contingency (2 x 2 matrix) tables and after which formulae were used to determine the analytical specificity, sensitivity, negative predictive values and the positive predictive value of RT-LAMP assay using RT-PCR as reference method. The cost of the RT-LAMP and RT-PCR was calculated.

CHAPTER FOUR

4. RESULTS

4.1 Reverse Transcription Loop Mediated isothermal Amplification Assay (RT-LAMP)

4.1.1 Primer set, reaction and temperature assessment

The two designed primer sets (Table 1) were assessed on their ability to amplify rabies nucleoprotein gene using a temperature range of 59℃ to 64℃ on the Veriti® 96-well thermal cycler (Applied Biosystems Inc., California, USA). Green fluorescence, indicative of positive amplification was only observed in tube 1 containing the LA_CVS primer set (Figure 3) while no such fluorescence was observed in tube 2 containing the GS CVS (Fig. 3a and b). Further, gel electrophoresis revealed the presence of RT-LAMP amplification products in tube 1 and none in tube 2 (Figure 3c). In addition, an attempt to optimize the RT-LAMP assay on heat block for temperature optimization was made using a further 10 samples on the Double Alumi Bath ALB-301 (Scinic, Japan) at 59°C, 60°C, 61°C, 62°C, 63°C, and 64°C and reaction times of 30, 45 and 60 minutes. Following this, a temperature and time of 60°C and 45 minutes was determined as the most appropriate for amplification.

Table 1: RT-LAMP primers designed for amplification of target N gene sequences for rabies

Primer pair	Primer name	5' position	3' position	5'-3' Sequence
1	*LA_CVS_F3*	363	382	GAAAAGGAGACAAGATCACC
	LA_CVS_B3	528	545	CCGGTGTTTTGTCCTGAT
	LA_CVS_FIP	383	460	CCTTGTCAGCTCCATGCCTC-CCGGACTCTCTAGTGGAAAT
	LA_CVS_BIP	461	524	ACCCCACTGTCTCTGAGCAT-TGCTCAACCTATACAGACTCA
2	*GS-CVS_F3*	750	769	TGACAACTCACAAAATGTGT
	GS-CVS_B3	927	946	CCTTTTCTCGCAATCAGGAT
	GS-CVS_FIP	770	841	TTCAATCCGGGAGAAAAACATG TC-GCTAACTGGAGTACTATACCAA
	GS-CVS_BIP	842	924	CATCTGTATTCAGCAATCAGAGT GG-ATGAACCCAGTAAACGATACC

Note**:** FIP (Forward inner primer), B3 (Backward outer primer), BIP (Backward inner primer) and F3 (Forward outer primer) are the primer names. The expected band size is 150 – 300bp

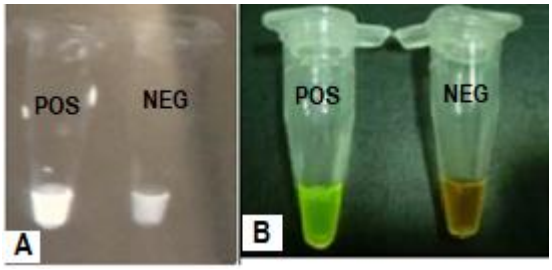

Figure 2: RT-LAMP primer optimization on heat-block. (A): RT-LAMP products with fluorescent dye visualized under UV light. (B): RT-LAMP products with fluorescent dye visualized under visible light.

4.2 Assay Performance Characteristics

4.2.1 Diagnostic specificity and diagnostic sensitivity

A total of 84 samples were tested using both RT-LAMP and RT-PCR, with 25 samples coming from Zimbabwe, 40 samples coming from Zambia, and 19 samples coming from Malawi. Three (3) negatives that were actually positive on RT-PCR were reported by the RT-LAMP (one from Zimbabwe and two from Zambia). The remaining results from Malawi, Zambia, and Zimbabwe were in agreement (Table 2).

Table 2**:** Comparison of RT-LAMP and RT-PCR assays for detection of rabies RNA virus from different geographical locations

Geographical Area	Number of samples tested	RT LAMP		RT PCR	
		Positive	Negative	Positive	Negative
Malawi	19	19	0	19	0
Zambia	40	30	10	32	8
Zimbabwe	25	24	1	25	0
Total	84	73	11	76	8

In this study, the RT-PCR is considered the reference method for diagnosing rabies virus. Using the data given, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the RT-LAMP were calculated as follows:

- True positive (TP): samples which were positive for both RT-PCR and RT-LAMP = 73
- False negative (FN): samples which were positive for RT-PCR but negative for RT-LAMP = 3 (76 - 73)

- False positive (FP): samples which were negative for RT-PCR but positive for RT-LAMP = 0
- True negative (TN): samples which were negative for both RT-PCR and RT-LAMP = 8

Sensitivity = TP / (TP + FN) = 73 / (73 + 3) = 0.961 or 96.1%

Specificity = TN / (FP + TN) = 8 / (0 + 8) = 1 or 100%

PPV = TP / (TP + FP) = 73 / (73 + 0) = 1 or 100%

NPV = TN / (FN + TN) = 8 / (3 + 8) = 0.727 or 72.7%

Therefore, the RT-LAMP test has a sensitivity of 96.1%, specificity of 100%, PPV of 100% and NPV of 72.7% for diagnosing rabies virus, compared to the RT-PCR (Table 3).

Table 3: RT-LAMP performance data compared with RT-PCR

		RT-LAMP		
		+	-	Total results
RT-PCR	+	73 (True Positive)	3 (False Negative)	76
	-	0 (False Positive)	8 (True Negative)	8
Sensitivity		96.1%		
Specificity		100%		
Positive predictive value		100%		
Negative predictive value		72.7%		

4.2.2 Analytical sensitivity and the limit of detection (LOD) for RT-LAMP and RT-PCR

Following a ten-fold serial dilution of a positive sample, RT-LAMP assay showed positive amplification from tube 1 – 8 while RT-PCR showed positive reaction from lanes 1 – 6 (Figure 3A, B and C).

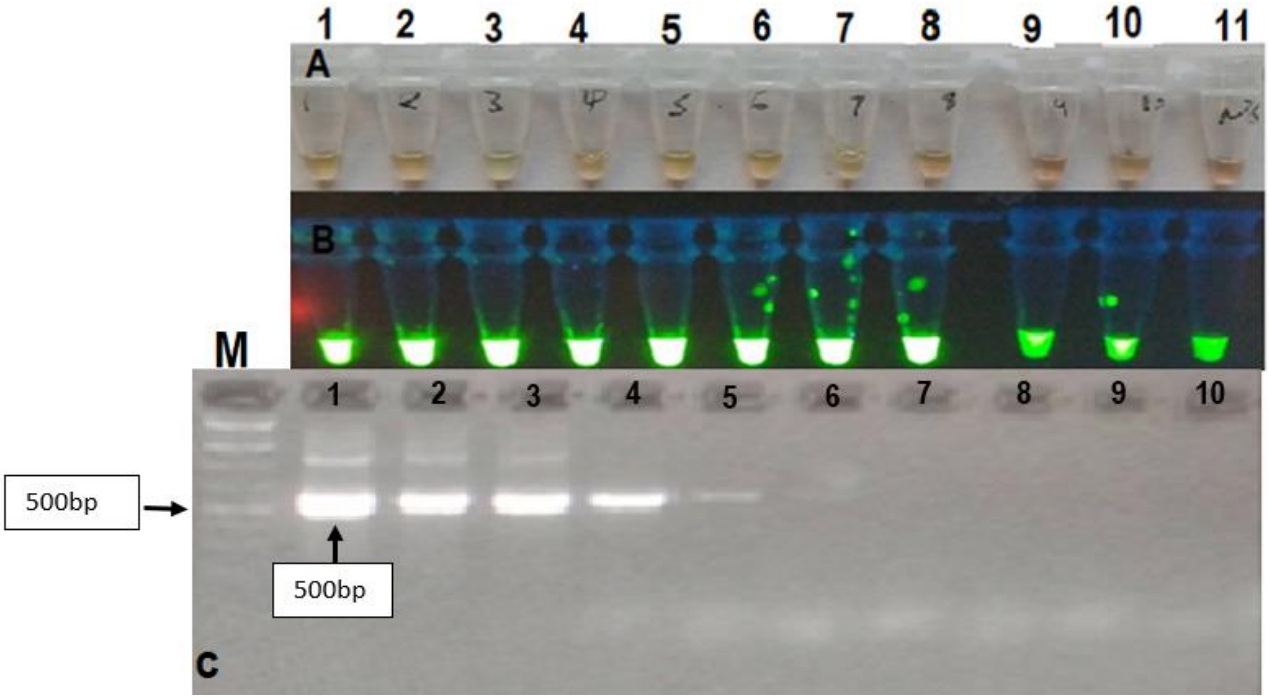

Figure 3: Visual inspection of fluorescent dye after RT-LAMP from ten-fold serial dilutions. The panels A and B indicate the results under visible and UV lights, respectively. Tubes 1- 8 indicated positive amplification for RT-LAMP while tubes 9 and 10 showed negative amplification with tube 11 (No template control) also being negative. Panel C indicates results for 10-folpd serial dilution results for RT-PCR on 1.5% agarose gel electrophoresis. Lanes 1 - 6 indicative of positive amplification with expected band size of 500bp. Lanes 7-10 had no amplification. Lane is 100 bp ladder.

For the limit of detection of RT-LAMP was able to detect as low as $2.2x10^{-5}$ ng/µl of RNA whereas RT-PCR had a detection limit of $2.2x10^{-3}$ng/µl of RNA (Table 4). These findings suggest that RT-LAMP assay was more sensitive than RT-PCR by a 100-fold.

Table 4: Limit of RT-LAMP and RT-PCR for a positive clinical sample

Assay		Concentrations of Rabies RNA (ng/µl)									
		$2.2x10^{2}$	$2.2x10^{1}$	$2.2x10^{0}$	$2.2x10^{-1}$	$2.2x10^{-2}$	$2.2x10^{-3}$	$2.2x10^{-4}$	$2.2x10^{-5}$	$2.2x10^{-6}$	$2.2x10^{-7}$
RT-PCR		+	+	+	+	+	+	-	-	-	-
RT-LAMP	Naked eye detection	+	+	+	+	+	+	+	+	-	-
	UV light detection	+	+	+	+	+	+	+	+	-	-

4.2.3 Analytical specificity of RT-LAMP assay

When tested on nucleic acids obtained from ticks, SARS-CoV-2 and *Rickettsia*, the RT-LAMP assay only amplified rabies RNA with no cross-reactivity (Figure 6).

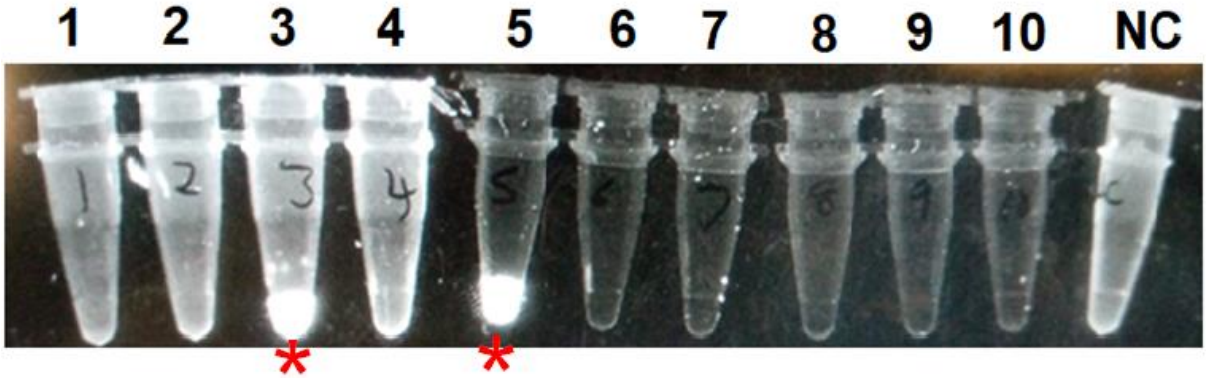

Figure 4: Visual inspection of results for RT-LAMP products with fluorescent dye under UV light. Tubes 1,6,7,9 and 10 had nucleic acids from ticks. Tubes 2 and 4 had RNA from SARS-CoV-2. Tube 8 had nucleic acid from *Rickettsia*. Tubes 3 and 5 had rabies positive RNA. Results show that only tubes containing rabies RNA indicative of positive amplification.

4.2.4 Cost analysis of RT-PCR and RT-LAMP assay

The RT-LAMP assay was found to be a low-cost alternative molecular test to RT-PCR for detection of rabies for screening in resource limited settings (see Table 5).

Table 5: Cost comparison between RT-PCR and RT-LAMP

Assay	Number of tests per run	Cost per run for RNA extraction ($)	Cost per run for test reagents, consumables, utilities, equipment, and primers ($)	Cost for labour per run ($)	Cost per test ($)
RT-PCR	96	150	1279	11	15
RT-LAMP	96	150	678	7	8.7

Cost per test for RT-PCR = Total cost / Number of tests

= [(RNA extraction) + (reagents, consumables, utilities, equipment, and primers) + (labour)]/Tests per run

= ($150+$1279+$11)/96

Therefore, cost per test of RT-PCR = $15

Cost per test for RT-LAMP = Total cost / Number of tests

= [(RNA extraction) + (reagents, consumables, primers, utilities, equipment) + (labour)]/Tests per run

= ($150+$678+$7)/96

Therefore, cost per test of RT-LAMP = $8.7

4.2.5 Assay Turnaround Time

From sample pretreatment, RNA extraction and master mix preparation, both RT-LAMP and RT-PCR assays took the same length of time (approximately 75 minutes). With regards to amplification, RT-PCR took 120 minutes while RT-LAMP took 45 minutes to complete. Thus, the total Turnaround Time (TAT) for RT-PCR was approximately 4 hours while that of RT-LAMP was 2 hours, indicating that RT-LAMP was twice as fast as RT-PCR.

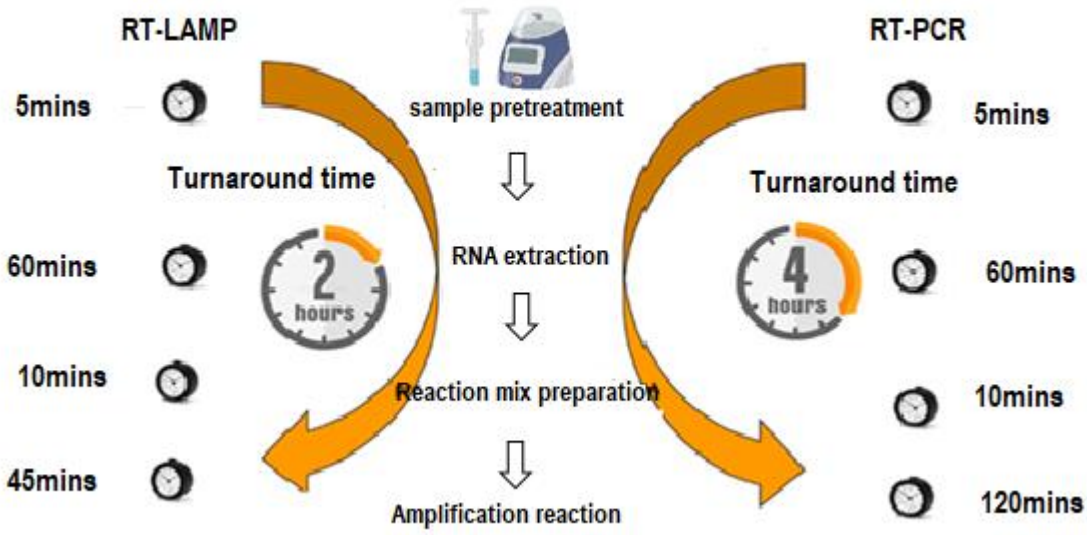

Figure 5: Illustration of workflow for assay turnaround time (TAT) from sample preparation up to nucleic acid amplification and read-out for RT-LAMP and RT-PCR.

The results presented thus demonstrate that this RT-LAMP is straightforward, less time-consuming, and cost-effective and has comparable specificity and sensitivity to RT-PCR.

CHAPTER FIVE

5. DISCUSSION

In this study, the evaluation of RT-LAMP assay was performed to assess the viability of implementing the RT-LAMP assay as an alternative to the current rabies-diagnostic techniques in Zambia. To determine the optimal conditions for the assay, we designed two set of primer and altered incubation the temperature, time and primer concentration. The RT-LAMP assay was found to be optimal at temperature of 60°C and reaction time of 45 minutes with primer concentration of 10µM. It is important to note that the earliest positive reaction was detected at 38 minutes, however, the most reliable results were obtained at 45 minutes.

Relating to the limit of detection (LOD), the results on RT-LAMP detected positivity to as low as $2.2x10^{-5}$ng/µl of rabies RNA by both visual detection with fluorescent dye in visible light and also under UV light while RT-PCR could not detect beyond $2.2x10^{-3}$ng/µl of RNA (Table 4). To ensure reproducibility of the results, the serial dilution were run in triplicate. This is suggestive of the superiority of RT-LAMP sensitivity over that of RT-PCR. Contrary to the findings of Reddy and colleagues where the RT-LAMP was a log lesser in sensitivity to RT-PCR (Reddy et al., 2016), this study reports RT-LAMP assay as being about 100-fold sensitive than RT-PCR. Other studies have shown RT-LAMP sensitivities 10-1000 times greater than conventional RT-PCR and equivalent to real-time RT-PCR (Baldbaatar et al., 2009, Saitou et al., 2010, Hayman et al., 2011). The RT-LAMP assay also yielded no cross-reactivity with the nucleic acids from ticks, SARS-CoV-2 and *Rickettsia* (Figure 5) as only rabies containing RNA showed positive amplification. These findings imply that this this RT-LAMP assay has potential to detect rabies negative samples without producing false-positive results.

For the evaluation of the RT-LAMP assay's performance and accuracy indices, a total of 84 samples were examined using RT-LAMP and RT-PCR originating from Zimbabwe (n=25), Zambia (n=40) and Malawi (n=19). The RT-LAMP had diagnostic specificity of 100% as all the eight (8) negative samples yielded negative results on both RT-LAMP and RT-PCR. This implies that the RT-LAMP assay was able to detect negative samples without producing any false positives. The RT-LAMP assay reported three (3) false negatives, which were positive on RT-PCR (Table 3) leading to a diagnostic sensitivity of 96.1% and a negative predictive value of 72.7%. This may have been caused by degeneration of RNA due to several cycles of thaw and freeze. The degradation of rabies ribonucleic acid, regardless of cause, can significantly decrease the integrity and quality of the sample, which could lead to a false-negative results.

Additionally, seventy-three (73) samples were truly rabid out of the 73 positive tests denoting a PPV of 100% for RT-LAMP. Since PPV and NPV are not fixed parameters of a test, they cannot be generalized across populations with different prevalence rate of infections as they are dependent on the prevalence of disease in a population. If the prevalence of the disease is high in a given population, PPV increases while NPV decreases. By and large, predictive values are not frequently used in clinical settings as sensitivity, which is the rate at which we identify disease that is present, or specificity, which is the rate at which we exclude disease that is absent. But in the context of epidemiology, PPV and NPV are more valuable than sensitivity or specificity (Maro, 2014).

Concerning turnaround time, the current RT-LAMP assay took 2 hours from extraction to reporting whereas RT-PCR took up to 4 hours (Figure 5), implying that the RT-LAMP assay is two times faster than RT- PCR. A comparative study of RT-LAMP, RT-PCR and real-time PCR for Japanese encephalitis virus revealed that RT-LAMP took only 1 hour while RT-PCR took 2.5 hours to completion (Chen et al., 2011).

On top of that, the cost analysis backs up the viability of using RT-LAMP as a cheaper molecular technique to RT-PCR for the detection of RNA viruses. This study found that RT-LAMP cost $8.7 per test while RT-PCR cost per test was $15. These findings point to the cost-effectiveness of RT-LAMP over RT-PCR (Table 5). Several studies have compared the cost-effectiveness of RT-LAMP with RT-PCR and DFAT for the diagnosis of rabies. For example, a study conducted in Tanzania found that RT-LAMP was more cost-effective than RT-PCR and DFAT, with an overall cost per sample of $9.09 for RT-LAMP, $13.82 for RT-PCR, and $18.86 for DFAT (Mpolya et al., 2019). Similarly, a study conducted in India found that RT-LAMP was more cost-effective than RT-PCR and DFAT, with an overall cost per sample of $6.91 for RT-LAMP, $10.63 for RT-PCR, and $12.05 for DFAT (Hegde et al., 2017).

Since the RT-LAMP assay is extremely vulnerable to contamination, caution must be exercised throughout the entire process, especially during master mix preparation. Furthermore, solution-based RT-LAMP assays require a strict maintenance of cold-chain of the reagents for reliable results to be generated. A rapid lyophilized RT-LAMP assay with long term storage fidelity and improved tolerance to a wider range of incubation temperatures plus a reduction in false positives is required as has been developed for the detection of SARS-CoV-2 (Song et al., 2022).

In summary, the current study reports on the improvement and use of the RT-LAMP assay for the detection of rabies RNA and the determination of the assay's sensitivity and specificity. The assay is rapid, easy, cheap, and sensitive, making it useful for making a preliminary diagnosis of rabies. The results obtained are inspiring for application in remote rural laboratories and by so doing, the costly trans-shipment of infectious material to advanced laboratories and the delay in result dissemination can be circumvented through use of this technique.

CHAPTER SIX

6. CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

This study reports evaluation of RT-LAMP assay for the purpose of detecting the rabies virus in clinical samples. The assay was optimized on heat block with temperature set at 60°C and time set at 45minutes. Positive amplification was detectable with fluorescent dye when visualized in visible as well as under UV light. The RT-LAMP assay yielded diagnostic specificity 100% and diagnostic sensitivity of 96.1%. The RT-LAMP assay has comparable diagnostic sensitivity and specificity to RT-PCR. The assay has greatly reduced turnaround times to availability of results and is much cheaper in terms of cost hence better suited for field detection of rabies. The detection of rabies in Zimbabwe and Malawi samples suggests applicability of LAMP to neighboring countries. In conclusion, the RT-LAMP is an excellent cost-effective and reliable tool with great potential for field diagnosis and epidemiological studies of rabies in Zambia

6.2 Recommendations

Implementing a field trial of the RT-LAMP assay on a large sample size of field samples to allow for validation using water baths, heat-blocks, and/or field adaptable platforms such as Genie III in field/remote labs. The objective of this trial is to determine the accuracy and feasibility of this assay for use in the field.

We have seen consistent results with the RT-LAMP assay across all samples tested. However, we have identified some limitations in the RNA extraction process, which is required for this assay. RNA extraction is time-consuming and requires specialized equipment, which may not be available in remote field labs. Therefore, further research to develop a dry RT-LAMP assay without the RNA extraction step is required enable the assay to be more easily and rapidly conducted in remote field labs.

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APPENDICES

Appendix I: Approval of study

UNIVERSITY OF ZAMBIA SCHOOL OF VETERINARY MEDICINE

OFFICE OF THE ASSISTANT DEAN (POSTGRADUATE)

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Vet. Clinic Telephone: 291515

P. O. Box 32379
Lusaka, Zambia

Your Ref:

Our Ref:

23rd June, 2022

Golden Seulu Sandala
Department of Paraclinical Studies
School of Veterinary Medicine
P. O. Box 32379
LUSAKA

Dear Mr. Sandala

RE: APPROVAL OF RESEARCH PROPOSAL

At the meeting of the School Board of Graduate Studies held on Monday, 20th June, 2022, your research proposal entitled*: "**An Evaluation of Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) for Diagnosis of Rabies In Zambia***" was tabled and discussed. I am therefore, pleased to inform you that the research proposal was subsequently approved by the Board.

On behalf of the Board, I wish you success as you apply for ethical approval and carry on with your research activities.

Yours sincerely

Dr. Chisoni Mumba
ASSISTANT DEAN (PG), SCHOOL OF VETERINARY MEDICINE

Director, DRGS
Dean, School of Veterinary Medicine
Head, Paraclinical Studies
File

Appendix II: LETTER OF APPROVAL FROM UNZABREC

UNIVERSITY OF ZAMBIA
BIOMEDICAL RESEARCH ETHICS COMMITTEE

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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Federal Assurance No. FWA00000338 IRB00001131 of IORG0000774 NHRAR-REC No 2021-05-0002

22nd November, 2022

Your REF. No. 3240-2022

Mr. Golden Seulu Sandala,
University of Zambia,
School of Veterinary
Medicine, P.O Box 32379,
Lusaka.

Dear Mr. Sandala,

RE: AN EVALUATION OF REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP) FOR DIAGNOSIS OF RABIES IN ZAMBIA (REF. NO. 3240-2022)

The above-mentioned research proposal was presented to the Biomedical Research Ethics Committee on 21st November, 2022. The proposal is **approved**. The approval is based on the following documents that were submitted for review:

a) Study proposal
b) Questionnaires
c) Participant Consent Form

APPROVAL NUMBER : REF. No. 3240-2022

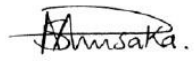
This number should be used on all correspondence, consent forms and documents as appropriate.

- **APPROVAL DATE : 22nd November 2022**
- **TYPE OF APPROVAL : Standard**
- **EXPIRATION DATE OF APPROVAL : 21st November 2023**

After this date, this project may only continue upon renewal. For purposes of renewal, a progress report on a standard form obtainable from the UNZABREC Offices should be submitted one month before the expiration date for continuing review.

- **SERIOUS ADVERSE EVENT REPORTING**: All SAEs and any other serious challenges/problems having to do with participant welfare, participant safety and study integrity must be reported to UNZABREC within 3 working days using standard forms obtainable from UNZABREC.
- **MODIFICATIONS**: Prior UNZABREC approval using standard forms obtainable from the UNZABREC Offices is required before implementing any changes in the Protocol (including changes in the consent documents).
- **TERMINATION OF STUDY**: On termination of a study, a report has to be submitted to the UNZABREC using standard forms obtainable from the UNZABREC Offices.
- **NHRA:** You are advised to obtain final study clearance and approval to conduct research in Zambia from the National Health Research Authority (NHRA) before commencing the research project.
- **QUESTIONS**: Please contact the UNZABREC on Telephone No. +260977925304 or by e-mail on unzarec@unza.zm.
- **OTHER**: Please be reminded to send in copies of your research findings/results for our records. You are also required to submit electronic copies of your publications in peer-reviewed journals that may
  emanate from this study. Use the online portal: unza.rhinno.net for further submissions.

Yours sincerely,



Sody Mweetwa Munsaka, BSc., MSc., PhD

CHAIRPERSON

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