Thesis Ref. No._____

DEVELOPMENT OF RAPID, SPECIFIC, AND FIELD-DEPLOYABLE DIAGNOSTIC ASSAYS FOR SHEEPOX AND GOATPOX VIRUSES USING CRISPR-CAS TECHNOLOGY

MSc. THESIS

BY:

BIRTUKAN ZENEBE

ADDIS ABABA UNIVERSITY, COLLEGE OF VETERINARY MEDICINE AND AGRICULTURE, DEPARTMENT OF VETERINARY MICROBIOLOGY, IMMUNOLOGY AND PUBLIC HEALTH

MSc PROGRAM IN VETERINARY MICROBIOLOGY

JUNE, 2024

BISHOFTU, ETHIOPIA

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A Thesis submitted to the College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements

BY:

BIRTUKAN ZENEBE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

MSC IN VETERINARY MICROBIOLOGY

ADVISORS

OLANA MERERA (DVM, MSc, ASSIS. PROFESSOR)

SAMSON LETA (DVM, MSc, ASSO. PROFESSOR)

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Submitted by:

Birtukan Zenebe _____ _____

Name of Student **Signature** **Date**

Approved for submittal to dissertation assessment committee

1. Dr. Olana Merera _____ _____

Major Advisor **Signature** **Date**

1. Dr. Samson Leta _____ _____

Co-advisor **Signature** **Date**

2. Professor Bekele Megersa _____ _____

Department chairperson **Signature** **Date**

ADDIS ABABA UNIVERSITY

COLLEGE OF VETERINARY MEDICINE

DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINAEY PUBLIC HEALTH

As members of the Examining Board of the final MSc/PhD open defense, we certify that we have read and evaluated this Thesis prepared by **BIRTUKAN ZENEBE ZEMMA**.

Entitled: **DEVELOPMENT OF RAPID, SPECIFIC, AND FIELD-DEPLOYABLE DIAGNOSTIC ASSAYS FOR SHEEPOX AND GOATPOX VIRUSES USING CRISPR-CAS TECHNOLOGY** and recommend that it be accepted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology

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TABLE OF CONTENTS **PAGES**

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College of Veterinary Medicine and Agriculture, Bishoftu

AKNOWLEDGEMENTS

First of all, I would like to thanks God for his almighty and generosity in every moment in general and in the preparation of this research paper.

My deepest and heartfelt gratitude goes for my advisor Dr. Olana Merera for his guidance, comment and material support necessary for preparation.

I would like to extend my special thanks to Dr. Samson Leta for his help in joining his research project, overall guidance and advice in the preparation of this research.

I would especially like to thank Dr. Dereje Shegu of the Animal Health Institute (AHI) for his prompt assistance in gathering the data needed to isolate and characterize the pox virus.

I would like to express great thanks to Meskerm Mulisa for her technical support during my work in the molecular laboratory of college of veterinary medicine and agriculture.

Furthermore, I want to express my pleasure to thank my family for inseparable love and financial support.

Finally, I would like to thank all staff members of the veterinary medicine and my classmates for their friendship, support, advice and encouragement during the course of the study.

LIST OF ABBREVIATIONS

AGID	Agar Gel Immunodiffusion test
ASFV	African Swine Fever Virus
CaPV	Capripoxvirus
Cas12a	Clustered regularly interspaced short palindromic repeat associated nuclease 12
Cpf1	Clustered regularly interspaced short palindromic repeats from Prevotella and Francisella
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CrRNA	Clustered regularly interspaced short palindromic repeats Ribonucleic Acid
DETECTR	Deoxyribonucleic acid Endonuclease Targeted Clustered Regularly Interspaced Short Palindromic Repeats Trans Reporter
ELISA	Enzyme Linked Immunosorbent Assay
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
GPCR	G-Protein Coupled Receptor
GTPV	Goat Pox Virus
HDA	Helicase-Dependent Amplification
ITR	Inverted Terminal Repeats
KSGPV	Kenyan Sheep and Goat Pox Virus
LFS	Lateral Flow Strips
LSDV	Lumpy Skin Disease Virus
LAMP	Loop Mediated Isothermal Amplification
LK	Lamb kidney cells
LT	Lamb Testis cells
Mabs	Monoclonal Antibodies
NASBA	Nucleic Acid Sequence-Based Amplification
PAM	Protospacer Adjacent Motif
PBS	Phosphate-Buffered Saline

LIST OF ABBREVIATIONS (continued)

PCR	Polymerase Chain Amplification
RCA	Rolling Circle Amplification
REA	Restriction Enzyme Analysis
RFLP	Restriction Fragment Length Polymorphism
RPA	Recombinase Polymerase Amplification
RSV	Respiratory Syncytial Virus
SGPV	Sheep And Goatpox Virus
TracrRNAs	Transactivating Ribonucleic Acid
VNT	Virus Neutralization Test
WOAH	World Organization for Animal Health

LIST OF TABLES

LIST OF FIGURES

LIST OF ANNEXES

ABSTRACT

Ethiopia is home to a large population of sheep and goats, which are raised for their meat, hides, and skins. Even with this huge financial resource, animal diseases are still the main factor that limits their productivity. sheeppox and goatpox is one of the most prevalent diseases caused by a genus of *Capripoxvirus* that have a negative impact on sheep and goats throughout the world. One of the main obstacles to the quick diagnosis of the sheeppox and goatpox viruses is the lack of rapid, specific, and point-of-care diagnostic tests. Thus, the aim of this study is to develop a rapid, specific and field deployable diagnostic assay to detect sheeppox and goatpox viruses based on recombinase polymerase amplification (RPA) and trans-cleavage activity of clustered regularly interspaced short palindromic repeats (CRISPR/Cas12a) and to validate its diagnostic specificity. Thus, a lab-based experimental study was employed from November 2023 to April 2024 to develop this assay. RPA-CRISPR/Cas12a-lateral flow assay was developed to detect the entry fusion complex component gene of the sheeppox and goatpox viruses' genomes. The assay was optimized to be completed within one hour, including nucleic acid extraction for 25 minutes, RPA reaction at 39°C for 15-20 minutes, CRISPR/Cas12a reaction at 37°C for 20 minutes, and visual detection through lateral flow (3 minutes). The primers for RPA, CRISPR RNA (crRNA), and ssDNA reporter for lateral flow detection (LFD) were synthesized and optimized. In this study, quick, specific, and versatile diagnostic method for goatpox and sheeppox virus was successfully developed. This innovative approach harnesses recombinase-polymerase amplification in conjunction with the CRISPR/Cas12a system, offering a cost-effective and efficient solution for identifying these viral infections. This diagnostic technique shows no cross-reactivity with Peste des petits ruminants (PPR). In conclusion, RPA-CRISPR/Cas12a is a potentially useful method for quickly and accurately diagnosing goatpox and sheeppox, especially in environments with limited resources. Therefore, Further research is needed to ensure the assay's reliability and robustness across diverse sample populations and varying environmental conditions.

Key words: *Cas12a*, *CRISPR*, *crRNA*, *LFD*, *RPA, Sheep and goatpox*

1. INTRODUCTION

Ethiopia is home to one of the large populations of small ruminants in the world, with 38 million sheep and 46 million goats (CSA, 2022). This represents approximately 10% of Africa's livestock population and 4% of all small ruminant populations worldwide (FAO, 2019). Small ruminants are very popular because they can produce multiple offspring annually, have low capital costs, and have short fattening and breeding cycles. This can contribute to short-term cash flows, helps herds recover from droughts more quickly than cattle, and provides a quicker return on investment. Small ruminants provide approximately 25% of Ethiopia's meat production (Shapiro *et al*., 2017). In addition, they contribute significantly to the national economy through their export trade. For example, in 2018–19, the country's revenues from live animal exports (US$45 million) and meat (US$93 million) were approximately 86% and 86%, respectively, from small ruminants (Mamo, 2019).

Despite having a large impact on Ethiopia's economy, small ruminants are unable to make a meaningful contribution because of numerous impediments. Foremost among these challenges is the prevalence of small ruminant diseases, which have hampered livestock productivity and impeded trade operations (Jilo *et al*., 2016; Yune and Abdela, 2017). Infectious diseases like *Capripoxviruses*, which are common throughout Ethiopia, are of special concern (Hurisa *et al*., 2018). The highly contagious Sheeppox and Goatpox viruses are among the viruses that cause diseases in shoats and belong to the genus *Capripoxvirus* (CaPV), subfamily Chordopoxvirinae, and family Poxviridae. Studies show that 96% of the nucleotides and amino acids shared by the lumpy skin disease virus (LSDV), goatpox virus (GTPV), and sheeppox virus (SPPV) are the same (Yan *et al*., 2012).

Sheeppox and goatpox, according to the World Organization for Animal Health (WOAH), are systemic infectious disease that can cause serious illness and even death in small ruminants. It is also a significant transboundary and notifiable disease (Madhavan *et al*., 2016). Clinical manifestations of the disease often include skin lesions, fever, conjunctivitis with oculonasal discharge, internal organ pox lesions, and increased salivation. *Capripoxviruses* are quickly and readily transmissible.

Transmission occurs when a diseased animal comes into small-scale contact with an uninfected animal. In addition, direct or indirect contact with lesions, aerosolized respiratory droplets and oro-nasal secretions can all transmit the shoatpox and goatpox virus between animals (Balinsky *et al*., 2008).

Given the highly contagious nature of sheeppox and goatpox, prompt and precise laboratory confirmation is imperative. Goatpox and sheeppox antigens could be identified by ELISA, agar-gel immunodiffusion (AGID) using lymph node biopsies and specific immune sera, and direct fluorescent antibody testing using edema fluid. In addition to those methods, a range of laboratory diagnostic techniques are utilized for detecting sheeppox and goat pox. These include electron microscopic observation and virus isolation, as well as indirect detection of fluorescent antibodies through virus neutralization tests. Additionally, characteristic histopathological lesions may be to identify sheeppox and goatpox (Davies, 1981). Additionally, the "gold standard" in molecular diagnostics for the quick diagnosis of goatpox and sheeppox is PCR amplification of genetic material. There are many PCR-based tests available for the quick diagnosis of sheeppox and goatpox viruses, including qPCR, conventional and real-time PCR, and others (Bowden *et al*., 2008; Balinsky *et al*., 2008).

Isothermal amplification, an alternative to qPCR, has the benefit of allowing nucleic acid amplification to be carried out at a constant temperature without the use of a thermal cycler. Common methods of isothermal amplification include loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), nucleic acid sequence-based amplification (NASBA), and helicase-dependent amplification (HDA) (Pumford *et al*., 2020). Particularly, RPA stands out as a one-tube, one-temperature amplification method, typically conducted at temperatures ranging from 37 to 42°C, with a reaction time of approximately 15-20 minutes. Numerous variables, including temperature, reaction duration, probe and primer design, and amplicon size, affect the outcome of an RPA reaction (Sun *et al*., 2016). RPA exhibits exceptional selectivity in amplifying both DNA and RNA. Despite having a high specificity (usually 100%) in non-closely related species, RPA tolerates mismatches, which makes multiplex detection less successful in closely related species (Mondal *et al*., 2016).

A type of RNA-mediated adaptive immune defense mechanism that protects bacteria and archaea from plasmid invasions and bacteriophages is known as clustered regularly interspaced short palindromic repeats (CRISPR) (Jinek *et al*., 2012). It was originally identified in 1987 as repeated 29-nt fragments separated by 32-nt fragments in the genome of Escherichia coli. (Mojica *et al*., 2005). Among the bacterial adaptive immune system, CRISPR-Cas12/Cpf1, a type V CRISPR, operates by binding to and cleaving DNA (Zetsche *et al*., 2015). CRISPR-Cas12 recognizes single-stranded DNA or double-stranded DNA with a protospacer adjacent motif (PAM) that is rich in T (thymine) and activate its trans-cleavage activity, shearing the surrounding single-stranded DNA as needed. By combining the trans-cleavage activity of CRISPR-Cas12 with RPA, the DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR) nucleic acid detection technique was created (East-Seletsky *et al*., 2016).

Statement of the problem

Diagnosis plays a pivotal role in the control of sheeppox and goatpox, prompting the development of several molecular detection assays targeting the viral genome. Techniques such as PCR, real-time PCR (RT-PCR), and loop-mediated isothermal amplification (LAMP) have been deployed for this purpose (Haegeman *et al*., 2020). On the other hand, a lot of these techniques call for specialized tools and personnel. A new and promising tool in molecular diagnostics is the CRISPR/Cas endonuclease detection system. It has a wide range of applications in disease detection and is well-known for its increased sensitivity, specificity, and affordability (Zetsche *et al*., 2015).

This study aimed to develop a novel nucleic acid diagnostic tool by combining the CRISPR/Cas12a system with recombinase polymerase amplification (RPA) in order to address the challenges posed by sheeppox and goatpox viruses in Ethiopia. This novel method, which is predicated on the DETECTER platform, enables on-site testing with immunochromatographic strips or portable instruments. The RPA-Cas12a system makes it possible to quickly identify sheeppox and goat pox viruses without the use of specialized tools, which is essential for the diagnosis and treatment of these illnesses in environments with limited resources like Ethiopia.

Therefore, the objectives of this study are:

General objective: -

- To develop rapid, specific and field deployable diagnostic assay to detect sheeppox and goatpox virus based on RPA and trans-cleavage activity of CRISPR/Cas12a.

Specific objective: -

- To develop rapid, specific, and field-deployable diagnostic assays for the detection of Sheeppox and Goatpox viruses.
- To validate the analytical specificity of the developed diagnostic assays.

2. LITERATURE REVIEW

2.1. Characteristics and Taxonomy of Sheeppox and goatpox viruses

Goatpox and sheeppox are two economically significant infectious diseases that affect goats and sheep, respectively. They belong to the genus *Capripoxvirus* (CaPV) and the Poxviridae family, specifically the Chordopoxvirinae subfamily (King *et al*., 2011). The viruses that cause sheeppox and goat pox, which most commonly affect sheep and goats, respectively, are closely related to the lumpy skin disease virus (LSDV) that affects cattle. Certain viral isolates, on the other hand, usually have transitional host specificity because they can cause mild to severe disease in both species (Babiuk *et al*., 2008; Tuppurainen *et al*., 2014). The genome and a multitude of viral proteins are housed in the central core of the virion, which is surrounded by the two lateral bodies and the capsid (Moss *et al*. 2006). The genome of *Capripoxvirus* is approximately 150 kbp in size. It also includes large, brick-shaped, complex, double-stranded DNA and enveloped viruses (Figure 1) (Biswas *et al*., 2020).

Collectively, conserved genes related to virulence and host ranges, replicative and structural genes, and conserved genes amount to about 150 putative genes. Most of the genes in the core genomic region of CaPV are involved in replicative mechanisms, while the genes in the terminal area have an impact on pathogenesis and host range functions (Zeng *et al*., 2014). The CaPV genome is protected from various acids and disinfectants by a fake lipid sheath (Hosamani *et al*., 2004). *Capripoxvirus* replicates in the cytoplasm of infected cells rather than the nucleus, in contrast to other viruses with double-stranded DNA genomes (Schramm and Locker, 2005).

There is only one CaPV serotype, making it challenging to distinguish between SPPV, GTPV, and LSDV using serological techniques (Babiuk *et al*., 2008; Bowden *et al*., 2008) and antigenic approaches (Babiuk *et al*., 2008). However, genetic sequencing and phylogenetic analysis of the GPCR (G-protein coupled receptor) and RPO30 component genes encoding the 30 kD genes have been produced in order to distinguish between LSDV, GTPV, and SPPV (Lamien *et al*., 2011). Among *Capripoxviruses*, P32,

GPCR, and RPO30 genes are highly conserved (Venkatesan *et al*., 2012; Mahmoud and Khafagi, 2016).

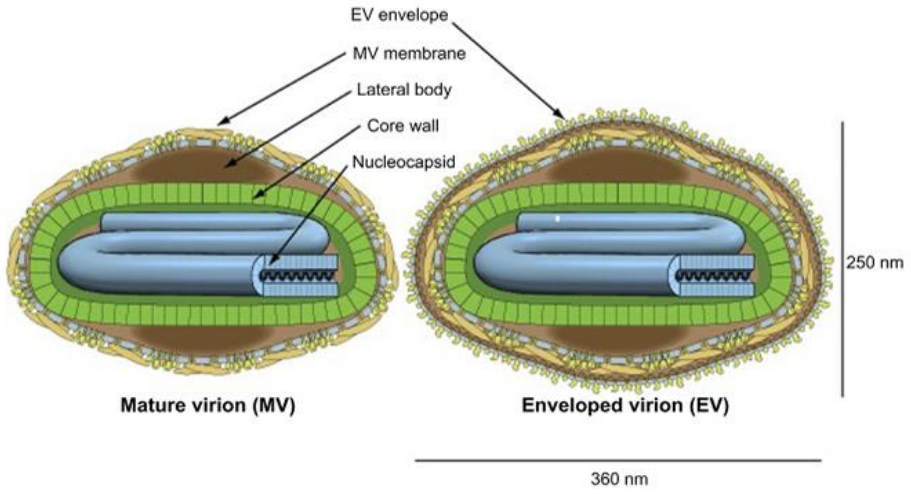


Figure 1: Genomic structure of pox virus (Hurisa *et al*., 2018)

2.2. Epidemiological distribution of sheeppox and goatpox virus in Ethiopia

The diseases outbreaks occurred in all months of the year, but were most common from November to May, with March marking the peak of outbreaks. Sheep are exposed to low temperatures in winter, which can stress them out and weaken their immune systems, making them more susceptible to infections. The observed seasonality of sheeppox and goatpox may be explained by the viruses' ability to survive for several months in cold and wet weather, their correlation with the lambing season, or the low physiological status of the flocks (Bhanuprakash *et al*., 2005). The diseases are widespread throughout Ethiopia (Table 1). The Animal and Plant Health Regulatory Directorate received 893 reports of sheeppox and goatpox outbreaks in 2007–2008, with the exception of Gambela, Harari, and Dire Dawa, 6,401 (11.1%) of the 57,638 sick sheep and goats died. Only about 35–40% of diseases are reported in Ethiopia (Sileshi, 2009).

Table 1: Goatpox and sheeppox viral prevalence in various regions of Ethiopia

No.	Study area	Prevalence	Diagnostic test	Reference
1	Amhara region (NG, SG, WG)	15.5%	virus neutralization test (VNT)	Fentie et al. (2017)
2	Central Ethiopia (West Shewa, East and west Arsi.)	4.022%	PCR	Kenubih et al. (2021)
3	Ada berga district	33.3%for sheep, 27% for goats	Field clinical examination	Abdi (2017)
4	Gonder	5.94	Clinical examination	Teshome (2016)

2.3. Diagnostic testing approaches for sheeppox and goat pox viruses

2.3.1. Molecular tests

Molecular-based assays are powerful and accurate diagnostic instruments for identifying infectious agents that are clinically significant. Compared to conventional culture-based methods, these innovative technologies have shown a variety of benefits, such as improved sensitivity and specificity, quick turnaround times, multiplexing, repeatability, and the capacity to identify fastidious and unculturable species (Yimer, 2021). Viral diseases, such as the sheeppox and goatpox virus, can now be quickly detected and diagnosed using molecular diagnosis, which amplifies the nucleic acid, either DNA or RNA (Das *et al*., 2012).

Polymerase chain reaction (PCR)

The polymerase chain reaction method works better for diagnosing GPV and SPV from field samples when it is combined with restriction enzyme analysis (REA) of PCR amplicons (Hosamani *et al*., 2004). Using REA of PCR-amplified P32 gene products, skin biopsies and infected cell culture supernatants demonstrated a strong distinction

between SPV and GPV. Additionally, whether combined with restriction fragment length polymorphism (RFLP) or by focusing on a particular species, gel-based PCRs are capable of differentiating between the three Capripox species. Using the HinfI enzyme, the P32 gene has been employed in several PCR-RFLP tests to differentiate between SPPV and GTPV (Yan *et al*., 2012).

Molecular assays for the differentiation of virulent and vaccine strains are necessary for epidemiological field research. Gel-based assay and RT-PCR techniques have been developed to differentiate between virulent and attenuated SPPV (Haegeman *et al*., 2016; Chibssa *et al*., 2018). Another technique for identifying field and vaccine viruses is to sequence the GPCR gene (Gelaye *et al*., 2015).

Loop-Mediated Isothermal Amplification (LAMP)

The revolutionary loop-mediated isothermal amplification (LAMP) method uses auto-cycling strand displacement DNA synthesis to amplify DNA. It takes place for 45–60 minutes at 60–65 degrees Celsius in an isothermal setting. The *Bacillus stearothermophilus* DNA polymerase, along with four to six specifically designed primers, hybridizes six or eight different regions of the target DNA sequence. Three steps make up the mechanism: production of the starting material, cycling amplification, and elongation and recycling. The LAMP reaction, which doesn't require expensive equipment or specialized molecular procedures, is actually one of the fastest amplification approaches for molecular DNA detection (Fakruddin, 2011; Li *et al*., 2016).

Simple detection techniques, like the use of magnesium pyrophosphate, a byproduct of DNA synthesis, as an indicator or fluorescence detection using fluorescent chelating reagents, can be employed because of the high specificity and high yield of the amplification products. Both techniques allow for visual evaluation and real-time detection. Furthermore, it is feasible to detect with traditional DNA probes (Notomi *et al*., 2015). Sheeppox and goatpox are identical in terms of clinical manifestations and serological similarity. A LAMP method for the specific differential detection of GTPV

and SPPV was developed using three sets of LAMP primers that were designed based on ITR sequences (Zhao, *et al*. 2014).

LAMP assay that targets the highly conserved DNA polymerase gene of the *Capripoxvirus* genome was developed and tested in order to quickly identify the viruses that cause goatpox and sheeppox with a diagnostic sensitivity and specificity of 96.6 percent and 100%, respectively. The optimized LAMP assay is found to be more sensitive and specific for target DNA amplification than quantitative PCR (Venkatesan *et al*., 2016).

Recombinase polymerase amplification (RPA)

The isothermal nucleic acid amplification method known as recombinase polymerase amplification (RPA) was initially reported in 2006 and has since been used successfully in a number of areas, including transgenic ingredient identification, food authentication, and pathogen detection in humans, animals, and plants. Due to its benefits, which include simplicity of use, quick amplification, reaction at 37–42 °C, and more, it was predicted to replace PCR (Li *et al*. 2019). According to Liu's research, the RPA can quickly identify sheeppox and goatpox virus when combined with real-time fluorescence and naked-eye lateral flow strip. The tests detect specifically and work well at 39°C for 20 minutes. They demonstrated exceptional specificity and sensitivity with a limit of detection of 1.0×10^2 for the real-time RPA assay and 1.0×10^1 copies for the lateral flow strip-RPA assay, respectively (Liu *et al*. 2023).

2.3.2. Serological tests

Virus Neutralization Test

The virus neutralization test (VNT), which only detects neutralizing antibodies, is still one of the most widely used methods for determining the existence of sheeppox virus antibodies (Boshra, *et al*., 2015). A neutralization assay can have a sensitivity of 70% to 96% and a specificity of 100%. However, the assay's sensitivity might vary between laboratories, and its standardization is a challenge. Only biosafety level 3 laboratories

are permitted to conduct the test, which necessitates the use of live viruses (Lafar *et al*., 2020). Compared to LT or LK cells, Vero cells have been shown to produce more reliable neutralization outcomes. Fetal calf muscle cells are less sensitive than LT or LK cells, So the virus breakthrough can be avoided by using a constant virus-variable serum method with serum dilutions between 1/5 and 1/500. (Abdi, 2017).

According to Fentie, the VNT is intended to determine whether sheeppox and goatpox antibodies are present in the serum samples. Since virus neutralization test (VNT) cannot distinguish between sheeppox and GP antibodies, the serological result is referred to as "sheeppox and goatpox antibodies" (Fentie *et al*. 2017). Sheeppox vaccination is the best approach to prevent the disease from spreading, and the virus neutralization test is the most reliable way to monitor the effectiveness of vaccinations. ELISA and VNT are used to quantify the humoral response to SPV vaccination. The fact that the ELISA was unable to identify 22% of sera that tested positive and that weakly positive VNT sera were either doubtful or negative in the ELISA suggests that VNT is more sensitive, according to the results of (Rhazi *et al*., 2022).

Fluorescent antibody tests

Fluorescence antibody tests can also be used to detect the sheeppox and goatpox virus antigen on infected cover-slips or tissue culture slides. Slides or cover slips should be cleaned, allowed to air dry, and then fixed in cold acetone for ten minutes. There is a risk of nonspecific reactions and high background color in the indirect test conducted with immune cattle sera. However, a direct conjugate can be produced using sera from sheep or goats recovering from capripox, convalescing cattle, or rabbits hyperimmunized with purified *Capripoxvirus*. Uninfected tissue culture should be used as a negative control because cross-reactions from antibodies against cell culture can lead to problems (OIE, 2008).

Agar Gel Immunodiffusion (AGI)

The Agar Gel Immunodiffusion test has been utilized to identify sheeppox and goatpox virus precipitating antigen; however, this test has the drawback of sharing this antigen with the *parapoxvirus* and having lower sensitivity (OIE, 2008). The AGI test can be used to identify the goatpox virus from serum samples that are taken during a disease outbreak. Only six of the fourteen serum samples that were collected were found to be AGID positive, according to study by (Abhilash *et al*. 2017).

Enzyme linked immune sorbent assay

The enzyme-linked immunosorbent assay (ELISA) is a potent immunochemical method that can be used to identify and measure a particular protein, antigen or antibody within a given sample. Its specificity varies from 84% to 100%, and its sensitivity varies from 70% to 100% (Lafar *et al*., 2020). Ever since the highly antigenic structural protein P32 of the shoatpox virus was successfully cloned, diagnostic reagents such as P32 mono-specific polyclonal antiserum and monoclonal antibodies (MAbs) have been made using expressed recombinant antigen (OIE, 2010).

The creation of an ELISA with high specificity has been made easier by these reagents. The purified sheeppox and goatpox virus can be injected into rabbits to produce hyperimmune rabbit antiserum, which can be used to trap sheeppox and goatpox virus antigen from tissue culture supernatant or biopsy suspensions on an ELISA plate. The presence of the trapped antigen can then be determined using commercial horseradish-peroxidase-conjugated rabbit anti-guinea pig immunoglobulin, a chromogen or substrate solution, and guinea-pig serum raised against the group-specific structural protein P32 (OIE, 2008).

Western blotting

Protein analysis and detection are commonly accomplished through the use of the Western blotting test. The process involves binding particular antibodies to proteins that have been immobilized on a membrane to form an "antibody protein" complex, and

then using one of several detection techniques to find the bound antibody. It is not possible to use the test as a primary assay; it is laborious to conduct, and it requires purified antigen (Lafar *et al*., 2020).

2.4. Clustered regularly interspaced short palindromic repeats (CRISPR)

CRISPR-Cas systems, which are collections of DNA sequences present in the genomes of prokaryotic organisms, offer adaptive immunity against bacteriophages, viruses, and foreign genetic elements like plasmids (Barrangou *et al*., 2007).

2.4.1. Background of the CRISPR-Cas system.

The gene in Escherichia coli that converts alkaline phosphatase isozyme was first discovered using the CRISPR-Cas system in 1987 (Ishino *et al*., 1987). This gene contains 24 nucleotide repetitive sequences and has five direct repeats. The second instance was discovered in Haloferax mediterranei, where the direct repeats were located 35 bps apart. The former sequence's short, inverted repeats were also present in the direct repeats. The variable sequences that the CRISPR-Cas system used as spacers or between direct repeats captivated scientists between 1987 and 1993 (Mojica *et al*., 1993).

Subsequent studies on bacteria and archaea also discovered mysterious arrays of repetitive sequences spaced regularly, which over time demonstrated their biological significance (Jore *et al*., 2012). In 2002, Ruud Jansen and associates called the repetitive DNA sequences in the genome near the "DNA repair system" Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) (Makarova *et al*., 2002). The years 1993–2005 were covered in this work. Since they were mostly found close to CRISPR genes, these nearby genes—which were previously believed to be parts of the DNA repair mechanism—were named CRISPR-associated (Cas) genes. Two separate investigations from 2005 connected bacteriophages to the origin of the spacer (Mojica *et al*., 2005; Pourcel *et al*., 2005).

Eugene and colleagues discovered associations between the CRISPR and Cas genes as a system and a bacterial RNA interference immune system through their computational research (Makarova *et al*., 2006). After a virus infection, the CRISPR-Cas System (CCS) provides antiviral defense. The host genome retains the phage genomic sequences as spacers. A revised analysis of the evolutionary connections between CRISPR and Cas proteins was presented in 2011 by Kira S. Makarova and associates (Barrangou *et a*l., 2007; Makarova *et al*., 2011; Jore *et al*., 2012).

After that, CRISPR research increased annually in thousands of labs worldwide; a few articles illustrate the success tales of this ground-breaking technology. The CRISPR array and its associates were found to be efficient survival and defense mechanisms against viral invasion in the genomes of bacteria and archaea. These findings were repurposed as a gene-editing technology to modify the genomes of eukaryotic organisms, leading to enormous applications in numerous biological fields, ranging from agriculture to medicine. The CRISPR-Cas system experienced significant transfers and reprogramming throughout this time. The transfer of the CRISPR-Cas9 system from Streptococcus thermophilus to Escherichia coli has been shown in a study to provide protection against phage and plasmid invasion (Sapranauskas *et al*., 2011). In order to inhibit RNA polymerase (RNAP) from attaching to promoter regions or to inhibit RNAP, David Bikard and colleagues developed Cas9 as a transcriptional repressor (Bikard *et al*., 2013).

2.4.2. Classification and types of the CRISPR Cas system

Rapid adaptive evolution in Cas systems is facilitated by the co-evaluation of anti-CRISPR proteins produced by invasive viruses, which enhances innate immunity in archaeal and bacterial species (Koonin *et al*., 2017). The architecture of effector modules, Cas1 phylogeny, the existence of exclusive signature genes in the CRISPR-Cas loci, and clustering and phylogenetic algorithms are all used to classify CRISPR-Cas systems based on evolutionary relationships. For the purpose of naming CRISPR-Cas systems and figuring out their molecular mechanisms, comparative genomic analysis and experimental data are crucial (Ahmadian, 2020).

Cas genes are primarily referred to by four designations, Cas1, Cas2, Cas3, and Cas4, depending on their highest homology and frequency (Ahmadian, 2020). Since 2020, there has been a substantial development in the categorization of CRISPR-Cas systems. According to the published taxonomy, there are two main classes of CRISPR-Cas systems: Class 1 is more commonly found in the genomes of fungi, while Class 2 is more commonly found in bacteria. While Class 2 effector proteins are more prevalent in bacteria and comprise single multidomain effector proteins, Class 1 effector complexes are characterized by heteromeric multiprotein effecter complexes (Shmakov *et al*., 2017; Ahmadian, 2020).

Class 1 systems

CRISPR-Cas class 1 systems come in three different varieties: type I, type III, and type IV. Class I systems are defined by a wide variety of effector proteins. All CRISPR-Cas type I systems share the effector module Cascade, which consists of Cas proteins combined with a crRNA molecule (Brouns *et al*., 2008). When the Cascade complex identifies protospacer adjacent motif (PAM) sequences, it unwound the target DNA, enabling crRNA to bind to the corresponding DNA strand. Once the target location is identified, the Cas3 protein needs to be brought in order to cut the DNA strand that is not bound by the Cascade complex (Fig. 2) (Hayes *et al*., 2016).

The multi-subunit complex that governs CRISPR-Cas type III is made up of crRNA, Csm complexes in subtype III-A systems, and Cmr complexes in subtype III-B systems. The Cas10 protein is able to identify these systems. Recent research has revealed that Cas10 aids in the activation of the non-specific RNases Csm6 and Csx1. After the Cas10 protein synthesizes cyclic oligo-(A)-nucleotides (cOA) and CRISPR-Cas type III systems recognize the target site, the Cas10 protein starts to function as a polymerase. Thus, upon binding cOA, Csm6's RNase domain is activated, leading to the RNA target's destruction as well as any RNA molecules in the vicinity (Kazlauskiene *et al*., 2017; Niewoehner *et al*., 2017). RNA molecules are the target of many CRISPR-Cas type III systems as opposed to DNA molecules. Despite the fact that plasmids typically contain CRISPR-Cas type IV systems, little is known about their functionality (Koonin & Makarova, 2019).

Class 2 systems

CRISPR-Cas systems of type II and the less common types V and VI are included in class 2 (Makarova *et al*., 2015; Shmakov *et al*., 2017). Each of these systems has a unique collection of effector proteins. Their less complex organizational structure sets them apart, as the effector module consists of only one large, multidomain, multifunctional protein. Class 2 CRISPR-Cas systems are currently the most widely used in gene engineering because of their high level of success with gene editing and ease of usage. The CRISPR-Cas type II Cas9 protein targets the targeted DNA region by means of trans-activating crRNA (tracrRNA) and crRNA (Cong *et al*., 2013).

The three-component system (Cas9 protein, crRNA, and tracrRNA) was reduced to two components (Cas9 protein and sgRNA) by creating a chimeric RNA molecule (single guide RNA) that incorporates crRNA and tracrRNA. This simplifies CRISPR-Cas genome editing. When the Cas9 protein reaches the targeted DNA spot, SgRNA brings it to the region and causes blunt-ended double-strand breaks (Jinek *et al*., 2012). The Cas9 protein from Streptococcus pyogenes (SpCas9) is the most often used gene editing option because of its superior on target gene editing capabilities. On the other hand, SpCas9 often participates in off-target activity, defined as cleaving non-specific genomic regions of DNA that resemble the target site (Jiang *et al*., 2013).

There are several subtypes of CRISPR-Cas type V systems (V-A, V-B, etc.), and the proteins which distinguish these systems are called Cas12 proteins (Cas12a, Cas12b, etc.) (Makarova *et al*., 2020). The Cas12a and Cas12b proteins cleave the DNA and then cut the target sequences, leaving sticky ends behind. Cas12a, or Cpf1, is a commonly used tool in gene engineering. In CRISPR-Cas type V systems, the target site can be altered with just the Cas protein and a crRNA. Compared to Cas9, Cas12 is more advantageous for genetic engineering due to its smaller size and decreased tolerance for nucleotide mismatches between the target DNA and crRNA (Kim *et al*., 2016).

As a form of low-error repair, homologous recombination fixes sticky ends left over from Cas12-mediated DNA cleavage, improving the precision of gene editing (Bothmer

et al., 2017). Recently, CRISPR-Cas type V systems have yielded miniature Cas proteins (400–700 aa) known as Cas14 proteins (Harrington *et al*., 2018). Single-stranded DNA can be broken down by Cas14 without the need for PAM, as demonstrated. Certain Cas12 and Cas14 proteins exhibit what is known as collateral activity, which is the non-specific destruction of any nearby DNA the proteins bind to upon binding the target DNA (Makarova *et al*., 2020),

Subtypes VI-A, VI-B, VI-C, and VI-D (also called C2c2) are included in CRISPR-Cas type VI systems. Cas13 is the distinctive protein of CRISPR-Cas type VI. These systems are special because they can identify single-stranded RNA molecules. Type VI Cas proteins indiscriminately degrade any nearby single-stranded RNA after binding target RNA using a guiding crRNA (no tracrRNA) (Abudayyeh *et al*., 2017),

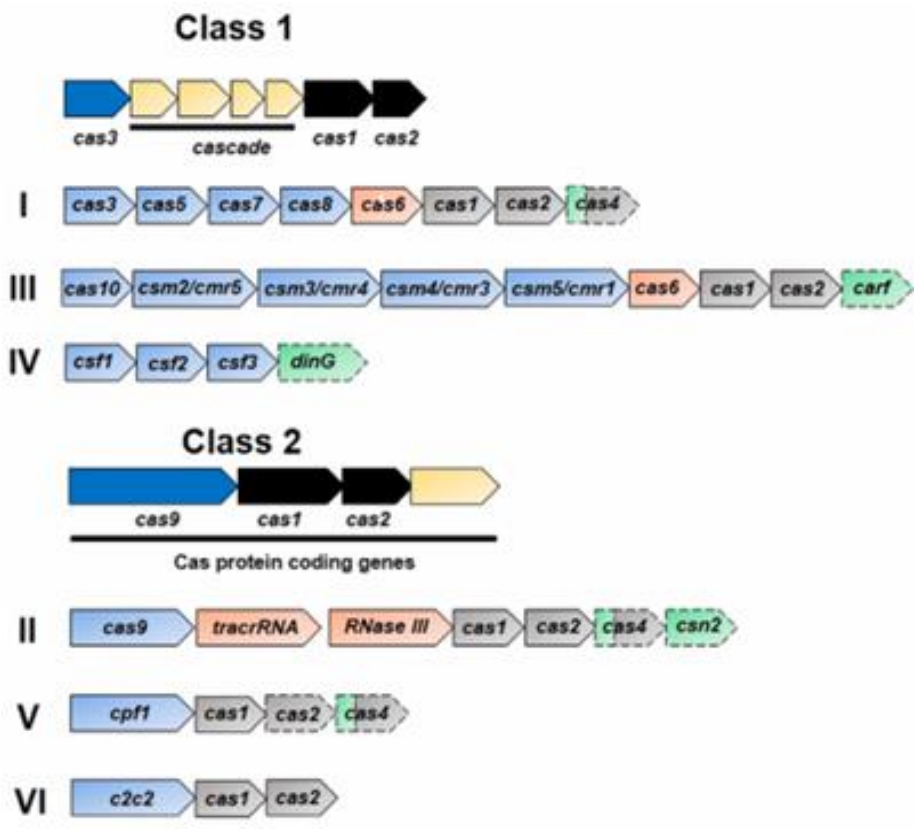


Figure 2: A schematic illustration of the CRISPR system and associated nuclease (Javaid and Choi, 2021)

2.4.3. CRISPR Cas12a.

The RNA-guided DNase enzyme CRISPR-associated protein (Cas) 12 serves as a prokaryotic defense mechanism and is made up of many family members (Zhang, 2019). Classified as a class 2 type V-A CRISPR/Cas system, the RNA-guided monomeric endonuclease protein Cas12a, also known as Cpf1, is found in the bacterial genera *Prevotella* and *Francisella*. Its length is between 1200 and 1500 amino acids (Li *et al*., 2018a). The Cas12a CRISPR array is made up of 36 nucleotide-long repetitive repeats that separate 9 spacer sequences. Cas12a recognizes a 5′-TTTV-3′ PAM within a DNA target, allowing base pairing of the crRNA spacer segment with the complementary target DNA. In addition, Cas12a's simultaneous RNase and DNase activity results in an independent crRNA synthesis that doesn't require tracrRNAs. Thus, Cas12a identifies the pre-crRNA and cleaves it to create a pseudoknot structure (Li *et al*., 2019a).

The two components that make up the structure of the CRISPR-Cas12 system, specifically Cas12a, are a single RuvC endonuclease domain for dsDNA cleavage and CRISPR-RNA (crRNA) with T-rich protospacer adjacent motif (PAM) sequences that guide the dsDNA to cleave at the catalytic domain's active site. (Li *et al*., 2018). Cas12a also cleaves non-specific single-stranded DNA in close proximity to the target due to its collateral cleavage activity. A sensitive CRISPR-Cas12a platform for DNA detection is called DETECTR, or DNA endonuclease-targeted CRISPR trans-reporter. The single-pot DNA detection method DETECTR uses Cas12-based detection and isothermal amplification to enhance sensitivity (Chen *et al*., 2018).

The RPA assay was utilized to theoretically amplify the target DNA at a constant temperature, and the resulting products were then utilized in the Cas12a reaction. Using gRNAs with complementary sequences, Cas12a can bind to the target DNA more directly than Cas13a can. Next, target DNA cleavage is triggered by Cas12a endonuclease and collateral activity as a result of the formation of a complex of target DNA, gRNA and Cas12a. High specificity and sensitivity dsDNA detection in HPV was accomplished using DETECTR. This method has good sensitivity at attomolar levels and can detect and separate HPV types 16 and 18 in patient samples in less than

an hour (Chen *et al*., 2018). Furthermore, HPV DNA has been directly detected in liquid samples without DNA separation using the DETECTR approach in conjunction with a fluorescent-biotin reporter. Additionally, the lateral flow method was coupled with the CRISPR-Cas12a detection platform. Compared to detection by PCR-based assay, the limit of detection (LOD) for this approach was 0.24 fM (Tsou *et al*., 2019).

2.4.4. Mechanism of action of cas12 protein

The ssDNA and dsDNA can be efficiently cut by the Cas12 protein with just the crRNAs. The Cas12 protein possesses the RuvC and nuclease lobe (NUC) domains for cleavage action. (Fonfara *et al*., 2016). Similar to Cas9, Cas12 comes across a possible target site next to a PAM sequence. Once Cas12 encounters a target DNA strand, it starts the R-loop process, which creates base-pair hybridization between the target DNA strand and the crRNA. In this stage, Cas12 binds to the target sequence's less than 17 bp, which causes the formation of an R-loop (Anders *et al*., 2014). The PAM sequence assists the Cas12 protein's active RuvC domain in cleaving the non-target DNA strand once R-loop has been established (Fonfara *et al*., 2016) (Fig.2).

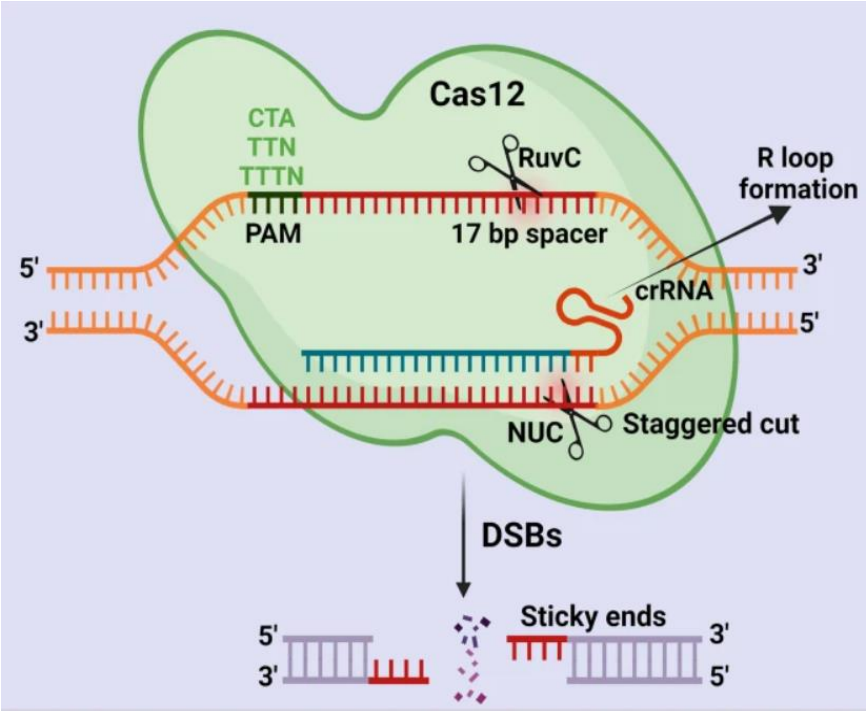


Figure 3: Schematic diagram of CRISPR/Cas12 mechanism (Anders *et al*., 2014)

2.5. The application of RPA in virus detection

In order to detect viruses, RPA and the CRISPR system were frequently utilized. To determine if an individual is infected with respiratory syncytial virus A (RSV A) or respiratory syncytial virus B (RSV B), an integrated trinity test utilizing the RPA-CRISPR/Cas12a-fluorescence assay was created. In just 37 minutes, this approach was able to identify between the respiratory syncytial virus A or respiratory syncytial virus B infection and detect the target sequence of 1.38×10^{1} copies/μl (Gong *et al*., 2022). The RPA-CRISPR/Cas12a system was created to detect human noroviruses quickly. In this technique, RPA products were found using CRISPR/Cas12a in conjunction with fluorescence or lateral flow strips. The qRT-PCR detection coincidence rate was 98.3%, and the minimal detection threshold was 9.65×10^{2} copies/ml (Qian *et al*., 2021b). Human metapneumovirus could also be identified using RPA-CRISPR/Cas12a (Qian *et al*., 2021a).

Additionally, CRISPR/Cas13a was a protein that could be utilized for nucleic acid identification. It was successfully utilized in conjunction with RPA to identify the rabies and African swine fever viruses. (Ren *et al*., 2021a, 2021b). By using the orthogonal collateral cleavage activity of the CRISPR/Cas12a and CRISPR/Cas13a systems, a dual-gene diagnostic approach for SARS-CoV-2 and African swine fever virus (ASFV) that demonstrated 100% sensitivity and specificity in the examination of clinical samples was created by (Tian *et al*., 2022).

3. MATERIALS AND METHODS

3.1. Materials and Laboratory set up

An experimental lab-based study was conducted at the Bishoftu Molecular Biology Laboratory of the College of Veterinary Medicine and Agriculture from November 2023 to April 2024. The main activities included: DNA extraction, optimization and screening of primers for PCR and RPA, analysis of the products using gel electrophoresis and final development of the kit using CRISPR Cas12a reaction. However, virus culture was performed in cell culture laboratory of the Animal Health Institute (AHI), Sebeta.

All primers, ssDNAs reporter, and CrRNA was synthesized by Integrated DNA Technologies (IDT). In addition to this, lateral flow assay kit and an RPA assay kit was also purchased from milenia biotec and TwistDX company respectively. The NucleoSpin® RNA Virus Kit was purchased from (Macherey-Nagel, Germany) and QIAquick® PCR & Gel Clean up kit (100) was synthesized by Qiagen (Germany). Vaccine strain of sheeppox and goatpox virus was used for virus isolation.

3.2. Designing RPA primers and crRNA

To design crRNA and RPA primers, all the available complete genome sequences of sheeppox and goatpox viruses was downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/ accessed on August 15, 2023) as aligned sequences. Conserved regions were identified using BIOEDIT software (https://bioedit.software.informer.com/7.2/) and DNA-dependent RNA polymerase and entry-fusion complex component genes were selected as target genes due to their conserved nature. The primers were designed to amplify a fragment of (139bp and 125bp) within the DNA dependent RNA polymerase gene and a fragment of (138bp and 145bp) within the Entry-fusion complex component gene of sheeppox and goatpox virus (Table 1). The online programmed OligoEvaluator was used to analyze the primer hairpin, and dimer (http://biotools.nubic.northwestern.edu/).

The crRNA was also designed for Cas12a to recognize a 21-bp on the opposite strand of the PAM site (TTTV, V = A, C or G) in the nucleotide sequence between RPA primer pairs. The sequence of Sheeppox virus 10700-99 strain TU-V02127 (GenBank accession no. AY077832.1) and the sequence of Goatpox virus Pellor (GenBank accession no. AY077835.1) was used as the reference sequence to check whether or not all of the primers bind or amplify both of the sheeppox and goatpox virus genome using SnapGene viewer (https://www.filehorse.com/download-snapgene-viewer/). The specificity of the crRNA and RPA primers was analyzed using the NCBI primer BLAST. (https://www.ncbi.nlm.nih.gov/tools/primer-blast).

DNA-dependent RNA polymerase (target gene 1)

5'-AAAATAACTTCATTAAAGGGGAAACATTTTATATACTTTTCTAACTCAA

CATATGGTGGATTAGTAATAAAATATATAATGCTTAGTAATG**GATACTCA**

Forward primer (F1)

GAATATAATGGGTCACAAGGTACAAATCCAAAACTTATAAATGGAAAA

PAM

CCTAAAATG**TTTA**CAATAGTAACTAGTAAAATGAAGTCATCATTAGAAG

crRNA sequence

ATT**TATTATCTGTATACAACTCACCAACAAATGA**TGATGG **-3'**

Reverse primer(R1)

Entry-fusion complex component gene (targe gene 2)

Forward primer(F3)

5'-AATTCTATACTAACTAGAT**TTTTAACATTTTATCCACCAAAAATATGC**

PAM

TATTTTTTAACAGCTACTCCTAGAAAATATAACAGGC**TTTA**TTGTAATG

crRNA sequence

Reverse primer(R3)

AAATTGTTAACATTTTAAAAATTACTGATA**TGGTAAAGACAATTAAAGTT**

GTTGAATACTTTTTTGAAAATTATTCGTCTGTATCTATACGTAAAATGTTA

AA**-3'**

Table 2: List of Primers and crRNA designed in this study

Target gene	Primer and crRNA	Sequence	Product length	Reference
DNA-dependant RNA polymerase	RNA poly Fwd.1	GATACTCAGAATATAATGGGTCACAAGGTA	139bp	This study
	RNA poly Rev.1	TCATTTGTTGGTGAGTTGTATACAGATAATA		
	RNA poly Fwd.2	CAGAATATAATGGGTCACAAGGTACAAATC	125bp	
	RNA poly Rev.2	TGGTGAGTTGTATACAGATAATAAATCTTC		
	CrRNA 1	rUrArArUrUrUrCrUrArCrUrArArGrUrGrUrArGrArU rUrArArUrUrUrCrUrArCrUrCrUrUrG		
Entry-fusion complex componenet	EFC Fwd.3	TTTTAACATTTTATCCACCAAAAATATGCT	138bp	This study
	EFC Rev 3	AGTATTCAACAACTTTAATTGTCTTTACCA		
	EFC Fwd.4	ACTAACTAGATTTTTAACATTTTATCCACC	145bp	
	EFC Rev 4	TTCAACAACTTTAATTGTCTTTACCATATCA		
	CrRNA.2	rUrArArUrUrUrCrUrArCrUrArArGrUrGrUrArGrArU rUrArArUrUrUrCrUrArCrUrCrUrCrUrUrG		
	ssDNA reporter	(5′FITC-TTATT-CCCCC-Biotin3′; TTATT-5C)		Lee et al (2022)
	PAM	TTTA		This study

3.3. Virus isolation

The sheeppox and goatpox vaccine strain (KSGP O-180) from national veterinary institute (NVI) was inoculated when the Vero cell monolayer reached 80% confluence two days after subculturing. Before inoculation, the processed supernatants, which were stored at −80 °C, were thawed. After discarding the exhausted maintenance medium from the flask containing the confluent monolayer, the monolayer was cleaned with sterile PBS. They used three flasks. Using two flasks per sample, a monolayer of Vero cells grown in 25 cm2 tissue culture flasks was inoculated with 1 ml of sample supernatant. After the inoculum was discarded, the flasks were washed three times in the medium, and the maintenance medium contained 2% calf serum, 10,000 UI/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml kanamycin, and 2.5 µg/ml amphotericin B added. The flasks were then incubated at 37°C for two hours to allow adsorption of the virus. The third flask, containing confluent monolayer cells, was reserved as a control flask and filled with medium only (WOAH, 2012).

Then the samples were incubated in three flasks at 37ºC for 7-10 days, and the development of the cytopathic effect (CPE) was checked on a daily basis in each flask. Every 48 hours, the medium was replaced, and the flasks were frozen at -20°C when 80% CPE was noted. Following two freeze-thaw cycles, the virus was harvested. The culture was then freeze-thawed three times and inoculated onto a fresh Vero cell culture when no CPE was visible until day 14. Chromatin fragmentation, detachment, ballooning, high refractility, rounding, intracytoplasmic inclusion bodies, and plaque formation are among the cytopathic effects that are produced by both SPPV and GTPV (WOAH, 2012).

3.4. DNA extraction

DNA extraction was carried out in the Molecular Biology Laboratory of College of Veterinary Medicine and Agriculture. Extraction of DNA from cell-free biological fluids was carried out using the NucleoSpin® RNA Virus Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. Accordingly, 150 µl of sheepox and goatpox viral isolates was transferred into a labelled 1.5 ml Eppendorf tube. Then,

20 µl proteinase K and 600 µL buffer RAV1 containing carrier RNA were added and mixed by vortexing, and incubated at 70ºC for 5 minutes. After that, 600 µl of 96% ethanol was added and mixed thoroughly and gently by vortexing. Then 700 µl of the lysed sample was transferred to a labelled NucleoSpin® RNA Virus Column placed in a 2 ml collection tube and centrifuged for 1 minute at 8000 rpm. The collection tube was replaced with a new one, and the residual lysis sample was added to the spin column and centrifuged for 1 minute at 8000 rpm.

The collection tube was again replaced with a new tube and 500 µL of buffer RAW was added and centrifuged for 1 minute at 8000 rpm. The flowthrough was discarded, and 600 µL of Buffer RAV3 was added and centrifuged for 1 minute at 8000 rpm. The collection tube was changed, and again, 200 µL of Buffer RAV3 was added and centrifuged for 5 min at 11,000 rpm. Finally, the spin column was transferred into a labelled 1.5ml Eppendorf tube and 30 µL Buffer RE (elution buffer) was added to the center of bottom of the column and the content was incubated for 2 minutes at room temperature and centrifuged for 1 minute at 11,000 rpm to elute the DNA into the Eppendorf tube. The nucleic acid bound to the silica membrane was eluted and the tube was labeled properly and kept at -20ºC until analysis.

3.5. Conventional PCR detection of sheeppox and goatpox virus

PCR was done in a total volume of 25µL containing 19.95 nuclease free water, 0.5µL forward primer (10 µM), 0.5µL reverse primer (10 µM), 0.05µL Taq polymerase (VWR, Leuven, Belgium), 2.5µL reaction buffer (10mM) (VWR, Leuven, Belgium), 0.5µL dNTP(10Mm) and 1µL template DNA sample. The PCR was consisted of an initial denaturation process for 1 minutes at 94°C, then it was followed by 35 cycles of denaturation process for 30 seconds at 94°C, the annealing process for 30 seconds at (51°C for F1R1, 51°C for F2R2, 52°C for F1R2, 48°C for F3R3, 49° C for F4R4 and 50 °C for F3R4) and extension for 30 seconds at 72 °C and then a final extension for 2 minutes at 72 °C (Fig.4).

In this study, the annealing temperature for each primer pair (Table 2) was optimized using gradient PCR. For each temperature step in the gradient, a reaction tube number

is assigned to determine the position of the tube. A pop-up text file provides information about the locations of these PCR tubes. Reactions are prepared for gradient amplification after mixing and the gradient was used to select tube positions. Then the temperature at which the primer works best or amplifies the given target gene was selected as the annealing temperature of that primers.

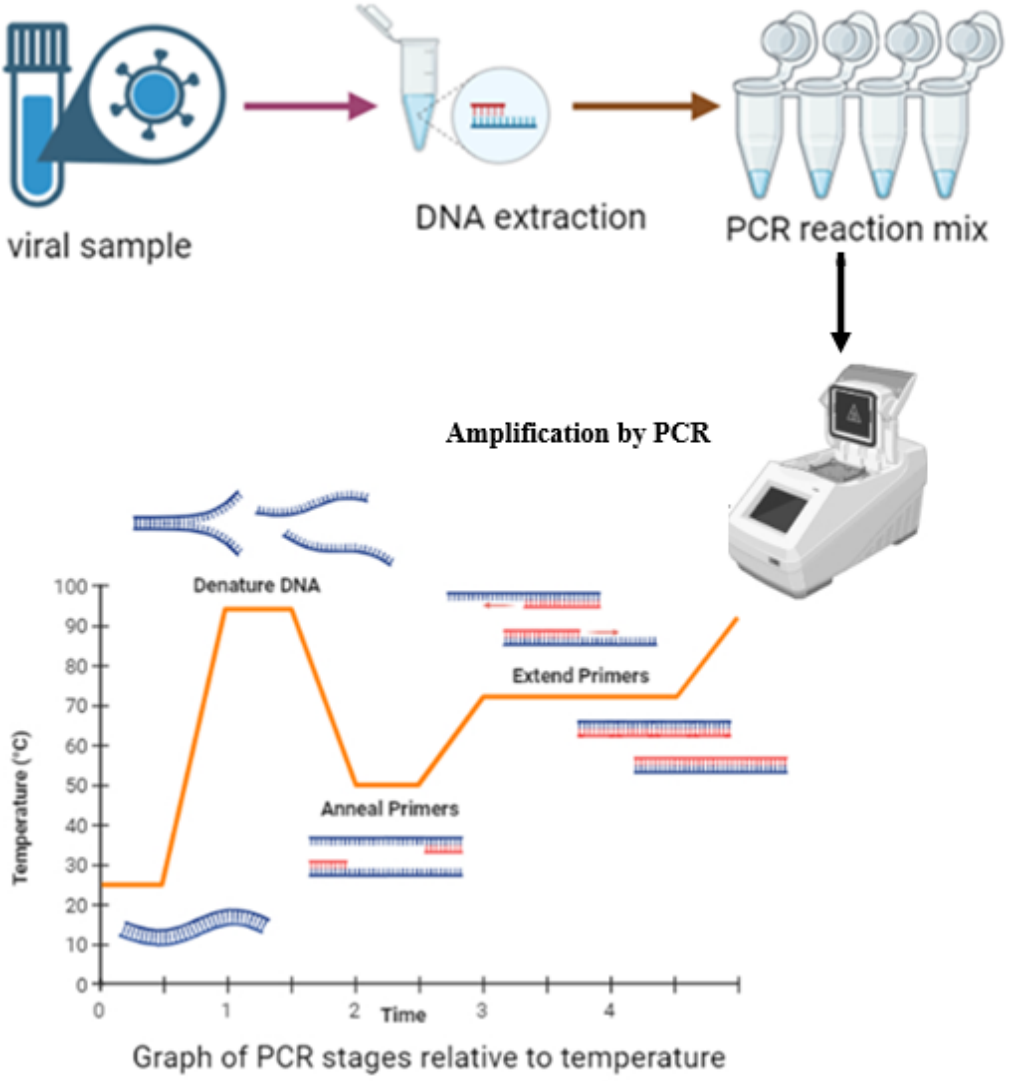


Figure 4: Schematic description of sheeppox and goatpox virus detection using polymerase chain reaction (PCR) **(**Source: Own Sketch using Biorender)

3.6. Analysis of PCR product

PCR products were analyzed using 2% agarose gel electrophoresis. Briefly, 1 gm Agarose was added into a flask containing 50 ml of 1X TAE (*Tris-acetate-EDTA*) buffer. The mixture was boiled to dissolve and cooled to 55ºC.Then, 0.5 µl Ethidium bromide(10mg/ml) nucleic acid stain was added. The gel was poured on gel caster placed horizontally and the comb was placed in the caster. When the gel was completely solidified after 25 minutes, the comb was removed carefully and the gel was placed in the electrophoresis tank containing 1X TAE running buffer. In the first lane 5 µl 100bp plus DNA ladder was loaded, while in remaining lanes 5 µl PCR products mixed with 1 µl DNA 6x loading dye were loaded in each well by using micropipettes. Micropipette tips were changed for each sample. The gel-running tank was connected to the power supply. The voltage was adjusted to 50 volt and run for 45 minutes. The gel was then observed under the UV trans-illuminator gel documentation system and gel picture was captured using UVP PhotoDoc-It™ Imaging system (UVP, Montpelier, MD, USA). Virus genotyping was determined and recorded based on the band size of the PCR product.

3.7. Isothermal amplification

The isothermal Recombinase Polymerase Amplification (RPA) was performed using a commercial TwistAmp™ Basic Kit (TwistDX, #TABAS03KIT). A comprehensive evaluation was conducted to determine the specificity of various candidate RPA primer pairs in distinguishing sheeppox and goatpox viruses. For this purpose, four different primer pairs (listed in Table 2) were employed to amplify sequences specific to sheeppox and goatpox virus strains. The selection of the optimal primer pair was based on a thorough analysis of nucleic acid amplification and gel electrophoresis result.

The RPA reaction was performed in 25 µL volumes using a commercial RPA kit. Briefly, the reaction mixture consisting of 12.5 µL of primer free rehydration buffer, 1µL of forward primer (10 µM), 1 µL of reverse primer (10µM),1 µL of template DNA and 8 µL of nuclease free water was added to a tube containing lyophilized RPA enzyme mixture and vortex o dissolve the content completely. Then, 1.5 µL of 280 mM MgOAc

was added before incubation at 39 °C for 20 min as previously done by (Li *et al*., 2022). The RPA products was purified by QIAquick® PCR & Gel Clean up kit (100) (Qiagen, Germany) and verified by electrophoresis on a 2% agarose gel. Images were taken using UVP PhotoDoc-It™ Imaging system (UVP, Montpelier, MD, USA).

3.8. CRISPR-Cas12a detection assay for lateral flow readout

The RPA-CRISPR-Cas12a process was combined with lateral flow strips to develop an RPA-CRISPR-Cas12a lateral flow strip assay (RPA-Cas12a-LFS). The ssDNA probe reporter was labeled with fluorescein isothiocyanate (FITC) at the 5′ end and biotin at the 3′ end (5′-FITC/FAM-TTATT-CCCCC-Biotin-3′). To create the crRNA-Cas12a complex, an 18 µl reaction mixture consisting of 1 µl Cas12a nuclease (1 µM), 2 µl NEBuffer r2.1 (10x), 1.2 µl crRNA (1 µM), 13.5 µl nuclease-free water, and 0.5 µl RNAse inhibitor was incubated at 25°C for 10 minutes. Subsequently, the reaction mixture containing the crRNA-Cas12a complex was incubated at 37°C for 20 minutes after the addition of 1 µL of RPA products and 1 µL of lateral flow ssDNA reporter (0.2 µM).

In addition, after CRISPR/Cas12a cleavage at 37 °C, the CRISPR/Cas12a reaction product was diluted 1:3 in Hybrid Detect Assay Buffer to meet the solution volume requirements for LFD. Then, 10 µL of CRISPR/Cas12a reaction solution was applied to the conjugate pad of the test strips. The assays were then added to the tube containing 60 µL of Hybridetect assay buffer and incubated at room temperature for at least 3 minutes before being removed and photographed. If the sample is positive, the C line and T line appear, while if the sample is strongly positive, the C line does not appear and only the T line is present (Figure 5). The Milenia Hybri Detect Kit contains the Hybri Detect Assay Buffer and Lateral Flow Strips (Milenia-Biotec, Gießen Germany).

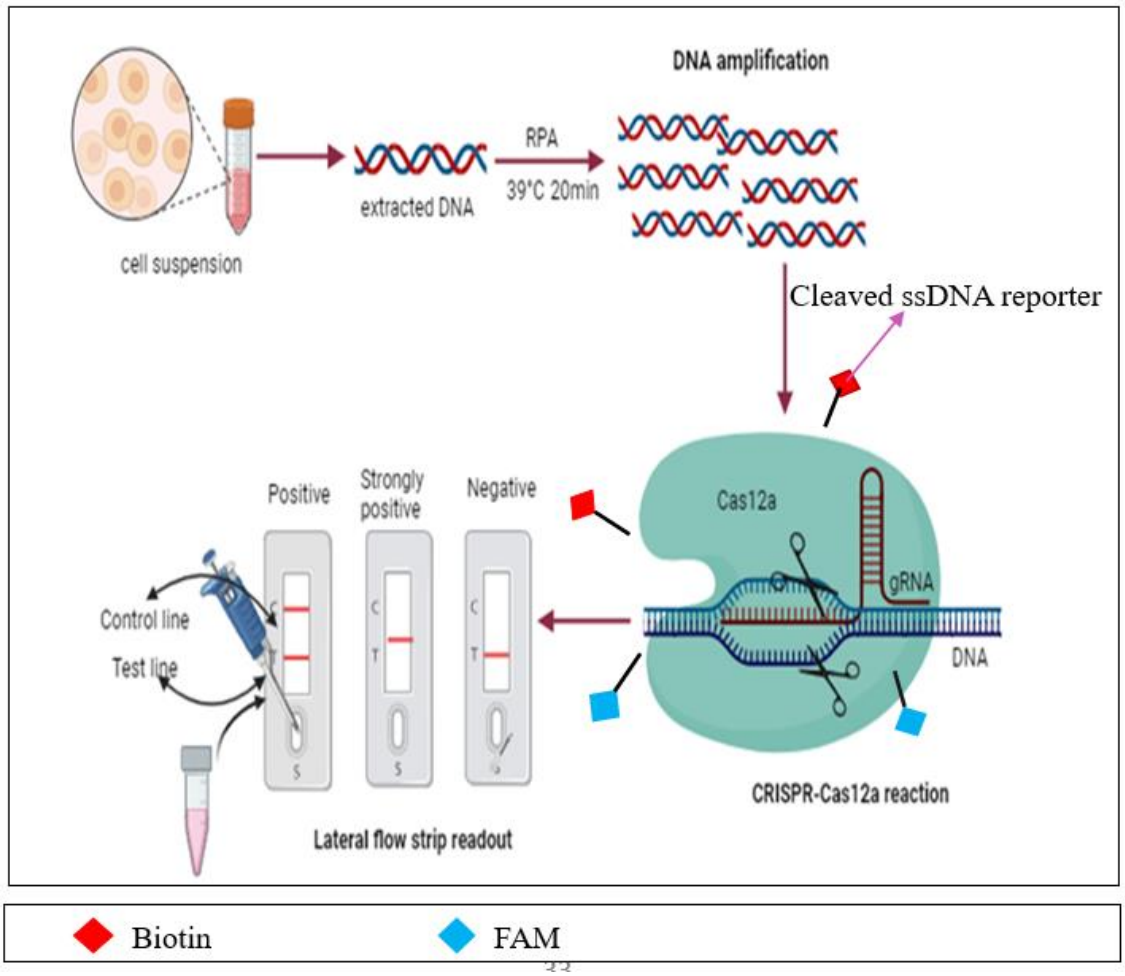


Figure 5: Schematic illustration of sheeppox and goatpox virus detection using CRISPR Cas12a combined with lateral flow strips **(**Source: Own Sketch using Biorender).

3.9. Specificity of the RPA-Cas12a-Lateral flow assay

To evaluate the specificity of RPA/Cas12a-based assays, pathogens showing similar clinical manifestations to sheeppox and goatpox viruses were selected. Therefore, in this study, Peste des petits ruminants (PPR) were selected for specificity assessment. Therefore, DNA templates extracted from the viral isolates of sheep and goat pox virus and RNA extracts from Peste des petits ruminants (PPR) virus isolates were used as viral template sources. Both viral templates were extracted from positive samples using the NucleoSpin® RNA Virus Kit (Macherey-Nagel, Germany). However, in the case of plague of small ruminants (PPR), its complementary DNA (cDNA) was synthesized by reverse transcription (RT). For amplification of peste des ruminants VIRUS (PPRV), the RPA primers and PCR Primers used were (F-5'-CTCGTCCATCATTACCCGTTCAAGACTGCTC3', R-5'-GGACCTAGTACTTTGAACTACCTCAACAAGG-3) (Zhang *et al*., 2018) and (PPR1821Fwd 5'-AGCATACCATGTCAACAAGG-3', PPR2712Rev 5'-ATCCTCATACTCATACTCGGAG-3') (Li *et al*., 2015) respectively. While the primers for sheeppox and goatpox virus were listed in Table 2. Then, after a 20-minute RPA reaction of the shoatpox virus and PPR virus, the RPA products were added to the Cas12a-LFD system for detection to validate the assay with crRNA specific for sheeppox and goat pox viruses.

4. RESULTS

4.1 Viral isolation

With sheeppox and goatpox virus strains (KSGP O-180) infecting Vero cells visible CPE was observed. Thus, The CPE effect was demonstrated by the formation of small syncytia, rounding, aggregation, intracytoplasmic inclusion bodies, plaque formation, and detachment of the cell sheet (Figure 6). Early CPE was observed in the infected cell lines an average of 6 days after inoculation. Within 7-10 days of the inoculation, the virus was harvested for further diagnosis.

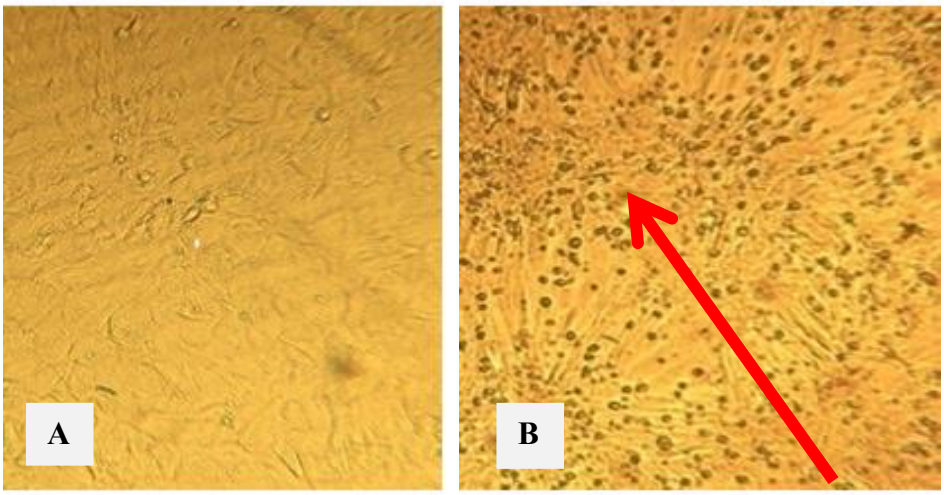


Figure 6: CPE image captured with an inverted microscope equipped with a camera. (A) Normal Vero cell monolayer, and B) The cells developed the CPE properties of the poxvirus, as shown by the arrow.

4.2. Detection of sheeppox and goatpox viruses using conventional PCR

PCR-based DNA amplification of the target DNA-dependent RNA polymerase gene using the first three sets of primers (F1R2, F2R2 and F1R2) shows negative results on agarose gel electrophoresis, whereas the amplification results for Entry-fusion complex component gene using F3R3, F4R4 and F3R4 shows positive results for sheeppox and

goatpox virus infection with a product length of 138, 145 and 134bp respectively (Figure 7).

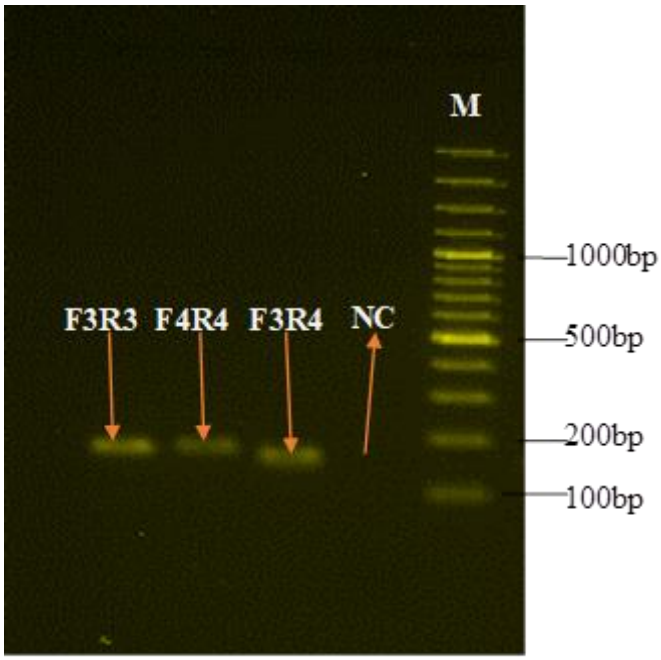


Figure 7: Detection of sheeppox and goatpox virus using conventional PCR (F: forward primer, R: reverse primer, NC: negative control and M: Ladder).

4.3. Detection of sheeppox and goatpox viruses using RPA

The sequences of forward primers and reverse primers for each of the two target genes were shown in Table 2, and the first three sets of primers (F1R1, F2R2, F1R2) were used for amplifying a (139bp, 125bp and 131bp) fragment of DNA-dependent RNA polymerase gene and the rest three (F3R3, F4R4, F3R4) were used for amplifying a (138bp, 145bp and 134bp) fragment of Entry-fusion complex component gene (Table 2).

The recombinase polymerase amplification (RPA) of the DNA dependent RNA polymerase gene shows negative results as in the case for polymerase chain amplification (PCR). The primer sets were evaluated by assessing the intensity and clarity of the band corresponding to the expected base pairs on agarose gel electrophoresis. Notably, among the second three sets of primers employed for

amplifying the Entry-fusion complex component gene, F4R4 exhibited a distinct band at a product length of 145bp (Figure 8).

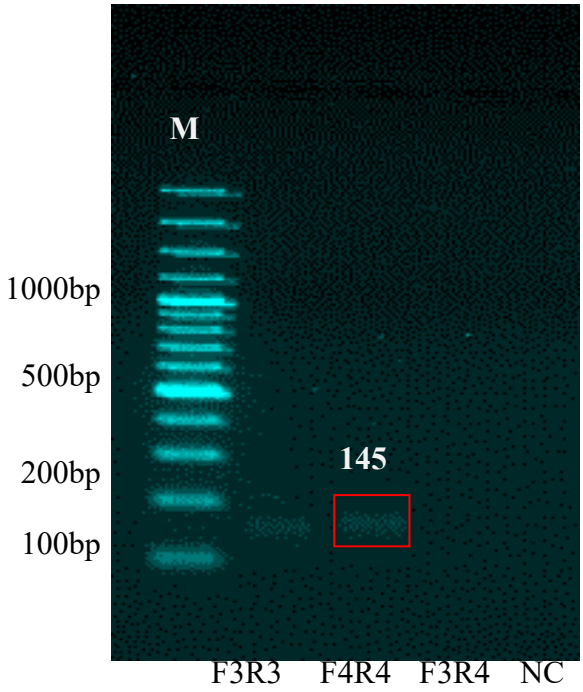


Figure 8: Visualization of RPA products by agarose gel electrophoresis (F: forward primer, R: reverse primer, NC: negative control and M: Ladder).

4.4. Development of the RPA-CRISPR/Cas12a assay through lateral flow readout

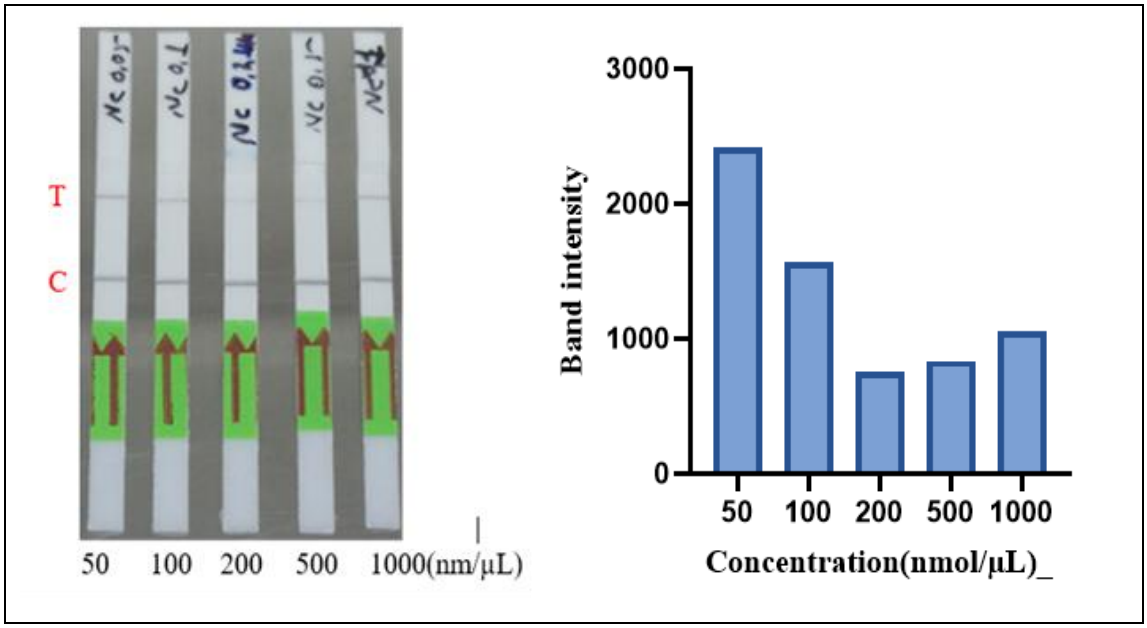
In this study, the RPA-Cas12a-LFD assay was developed to achieve the on-site diagnosis of sheeppox and goatpox virus, with the advantages of portability, affordability, and no need for specialized equipment. As a result, the Entry-fusion complex component gene was amplified using an RPA reaction, and the CRISPR/Cas12a reaction was then combined with the reaction product. Following crRNA binding, Cas12a could specifically identify the target gene sequence. This enabled CRISPR/Cas12a to cleave the double-labeled ssDNA reporter through the trans cleavage activity.

The cleavage of a dual-labeled reporter (FAM/FITC biotin) serves as the basis for the lateral flow readout; when multiple reporters are present, anti-FAM antibody–gold nanoparticle conjugates (Au-NP) aggregate at the strip's C (control) line. The ssDNA

FAM/FITC–biotin reporter was cleaved by the activated CRISPR/Cas12a in this experiment, resulting in a significant increase in band intensity on the T (test) line and a decrease on the C line. In the RPA-Cas12a-LFD assay, ssDNA reporter was diluted into various concentrations in order to decrease false-positive results. Quantification with ImageJ (https://imagej.en.download.it/) and visualization with GraphPad (https://www.graphpad.com/features) were used to examine the test line band intensities of the strips. Consequently, for the test line of negative control samples, 200nM ssDNA reporter produced the lowest band intensity (Figure 9a).

Thus, in this study after efficient amplification of the target Entry-fusion complex component gene using RPA for 20 min at the selected primer F4R4, the RPA products was added into the CRISPR Cas12a system in order to read the results using the lateral flow strips. Therefore, the assay shows positive results for sheeppox and goatpox virus (Figure 9b).

(a)



(b)

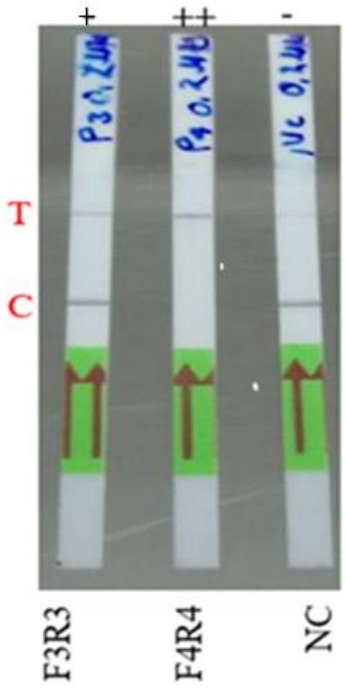


Figure 9: Detection of sheeppox and goatpox virus using RPA–CRISPR–Cas12a–LFD assay. (a) optimization of ssDNA reporter (50nm, 100nm, 200nm, 500nm and 100nm/µL). (b) RPA-CRISPR Cas12a-LFD based detection of sheep and goatpox virus (where T: test line, C: control line, NC: negative control, F: forward primer, R: reverse primer).

4.5. Specificity of the RPA-CRISPR/Cas12a-LFA Detection

The specificity of the RPA-CRISPR/Cas12a assay was evaluated by using templates extracted from sheeppox and goatpox viral isolates and other pathogens affects small ruminants such as Peste des Petits Ruminants (PPR). The results of Lateral flow detection showed positivity only for DNA of sheep and goatpox virus. It shows a clear band on the test line of the lateral flow dipsticks. Whereas, the detection for Peste des Petits Ruminants (PPR) Virus shows negative results like that of the negative control group which shows a clear line band on the control line (Figure 10C)). Therefore, sheeppox and goatpox virus RPA-CRISPR/Cas12a-LFD detection system showed specificity.

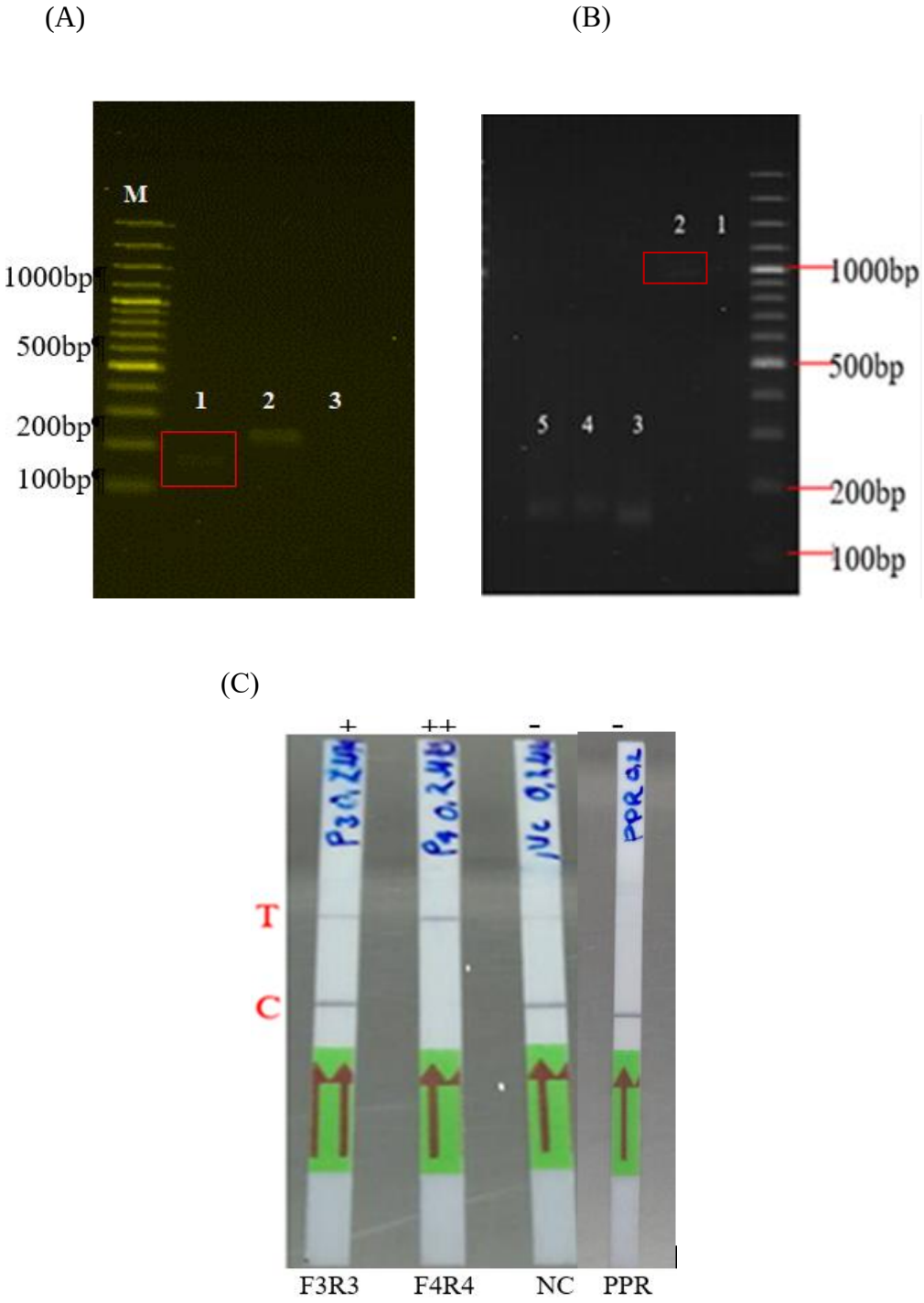


Figure 10: RPA-CRISPR/Cas12a assay's analytical specificity for detecting the sheeppox and goatpox virus.), (A) Amplification results of SGP virus and peste deste ruminantis (PPR) using RPA (1: F4R4, 2: PPR, 3: negative control, and M: Ladder.), (B) PCR amplification products of SGP virus and peste deste ruminantis (PPR) (1: negative control, 2: PPR, 3: F3R4, 4: F4R4, 5: F3R3, (C) The specific detection of sheep and goatpox virus using RPA-CRISPR/Cas12a -LFD assay.

5.DISCUSSION

Sheeppox and goat pox is one of the most common diseases of sheep and goats that is encountered in Ethiopia (Solomon *et al*., 2014). There is currently no effective therapeutic drug against these viral diseases. Therefore, development of a rapid and specific diagnostic method is crucial for disease control and surveillance of sheep and goatpox viruses. In this study, an efficient and practical sheep and goat pox virus detection method was developed by combining RPA and the CRISPR/Cas12a system for early sheeppox and goat pox virus infection. Thus, the entry- fusion complex component gene was detected from the sheep and goat pox virus genomes using the CRISPR/Cas12a method, and the CRISPR/Cas12a method was combined with immunochromatographic strips to simplify the field-testing equipment requirements. In small ruminants, sheeppox and goat pox (SGP) is a systemic infectious disease that can cause serious illness and even death (Madhavan *et al*., 2016).

Vaccination remains the primary shield against the pox virus, however, immunity lasts just one year (Bhanuprakash *et al*., 2006). Thus, precise and timely diagnosis of the virus is vital, enabling effective treatment and minimizing long-term impacts. Currently, real-time PCR, serological detection and virus isolation are the primary methods for detecting sheep and goatpox pox virus. Although virus isolation and identification are excellent methods for identifying the virus, the testing phase can take up to two weeks. Real-time PCR is an extremely sensitive detection technique, but it requires sophisticated laboratory methods and expensive equipment. The sensitivity and specificity of serological tests often do not meet our requirements. Therefore, none of the above three widely used detection techniques can be used for quick and efficient on-site detection (Haegeman *et al*., 2020).

There are several isothermal amplification techniques which can replace PCR and PCR-based methods. among them, loop mediated isothermal amplification is one that amplifies the target material within 45-60min by utilizing four to six primers. Although LAMP is a constant temperature amplification method, its primer design is complex and the amplification reaction requires a 1-h incubation at 65 C (Slana *et al*., 2021). In contrast, the design of the RPA primer is simple and the amplification reaction can be

carried out at 39 C for only 20–30 min. To date, many new isothermal amplification methods have been used in combination with various visualization tools such as LAMP LFD (Murray *et al*., 2013) and real-time and LFD-RPA assay (Yang *et al*., 2017) for the detection of sheep and goatpox virus. RPA requires only a single pair of primers, while LAMP requires four to six primers. This shows that RPA is more practical and economical than LAMP.

The CRISPR system, which comprises numerous distinct CAS proteins with nuclease activity, is an acquired immune defense mechanism against nucleic acids in bacteria (Gootenberg *et al*. 2018). Cas12a exhibits nuclease activity against DNA among them. Because of its function in cleavage and collateral cleavage, Cas12a has the ability to edit genes (Nguyen *et al*. 2020). Therefore, it has the potential to be used for pathogenic microorganism nucleic acid detection (Wang *et al*., 2020). In contrast to Cas13a and Cas13b (Mustafa and Makhawi, 2021), which are other CAS proteins that recognize and cleave RNA sequences, Cas12a specifically recognizes and cleaves DNA, saving time by removing transcription during the construction of the nucleic acid detection system (Yin *et al*. 2020).

The Cas12a reaction system's components are also simpler and typically more accurate in the absence of transcription reagents. It is worth noting that the RPA-CRISPR/Cas12a assay can be performed at low temperature, even with closed hands or a hand warmer, and that the trans-cleavage system of CRISPR/Cas12a can be activated at 37°C (Lobato and O' Sullivan, 2018). Another advantage is the higher sensitivity of the RPA-CRISPR/Cas12a assay compared to the LAMP-LFB.

The ssDNA can be modified with small molecule groups such as Fam, Biotin, BHQ1, etc. so that the released Fam or biotin can be detected using a fluorimeter or lateral flow assay. In this study, 5'-Fam-TTATT-5C-biotin-3' was used as the ssDNA reporter of combined RPA amplification and Cas12a-based detection to establish a portable all-in-one chip flow lateral assay for sheeppox and goatpox viruses. Thus, this assay reduces the need for specialized equipment and special operational training. The minimum equipment required to perform the protocol includes only pipettes, test tubes, a portable thermo block, and lateral flow strips.

Combining RPA and lateral flow assay could replace the instrument-dependent PCR amplification technology and fluorescence detection method. One important issue in lateral flow assay is avoiding the false positive bands to improve the accuracy and specificity. A low concentration of LF reporter easily resulted in the false positive band, since a portion of nanoparticles did not bind to the FAM-Biotin ssDNA reporter, kept moving, and reached the test band to form the potential false-positive result (Breitbach, 2020). So, in this assay the concentration of ssDNA reporter was optimized and 200nm was found to be used in the lateral flow assay to prevent the false positive band (Figure 9a).

In recent advancements in molecular diagnostics, the utilization of CRISPR-Cas12a has emerged as a powerful tool for the detection of various pathogens. African swine fever virus (ASFV) has been detected using CRISPR-Cas12a in conjunction with either the RPA or LAMP assay (Tao *et al*. 2020), the SARS-CoV2 virus (Broughton *et al*. 2020), Mycoplasma pneumoniae (Li *et al*., 2022), Neisseria gonorrhoeae (Tu *et al*, 2023). Bovine Anaplasma marginale infection (Sutipatanasomboon *et al*., 2021) and Mycobacterium tuberculosis (Ai *et al*. 2019,). The LOD of the CRISPR-Cas12a + RPA/LAMP assay is less than 100 copies/L meaning that it is comparable to qPCR and much more sensitive than PCR. Furthermore, because of the unique crRNAs and special primer design principle, its specificity is higher than that of qPCR and traditional PCR.

Notably, our data indicated that sheep and goatpox virus Entry-fusion complement gene amplification by F3R4 was determined as positive by conventional PCR, but determined as negative by the RPA (Figure 7,8). The possible reason for such a difference lies in the Cycle Threshold (Ct) value of this sample representing a low virus loading, which was under the LOD of the RPA-Cas12a-fluorescence assay. In addition to this one of our candidate genes that was selected as the target for the amplification of sheep and goatpox virus was not amplified with both PCR and RPA. This may be due to degradation of primers.

RPA-Cas12a assay developed in this study had no cross reactivity with peste des petits ruminants (PPR) (Figure 10c). Thus, the results for PPR were negative with the assays showing high specificity for sheep and goatpox virus detection. Additionally, we

optimized the components of the reaction system including the concentration of RPA primers, the selection of crRNA, and the concentration of ssDNA reporter. All of the efforts ensured that the whole reaction process can be completed within 1h.

There were some limitations to our study, first CRISPR/Cas12a detection requires opening reaction tubes after the RPA reaction, therefore, there is a higher chance of cross-contamination by aerosols. Second, I could not get clinical cases of outbreaks of sheeppox and goatpox viruses during our working period. Therefore, I used the vaccine strain as a virus source. Third, we performed cross-reactivity testing with only one pathogen that showed similar clinical manifestations to sheep and goat pox viruses. As a result, in the next investigation, other researchers could include other pathogens that affect small ruminants and have the same clinical symptoms as SGP to confirm that there is no cross-reactivity between them.

6. CONCLUSION AND RECOMMENDATIONS

In conclusion, this study has presented an innovative and field-deployable detection method for diagnosing sheeppox and goatpox viruses, by combining CRISPR-Cas12a technology with Recombinase Polymerase Amplification (RPA). Selection of an appropriate target gene, designed RPA primers and crRNA was performed to successfully establish a reliable RPA-CRISPR/Cas12a assay for detection of sheeppox and goatpox viruses. This novel approach holds significant promise for rapid and accurate diagnosis of these viral infections, offering a valuable tool for disease surveillance and control. With its simplicity, speed, and portability, this method has the potential to revolutionize the detection landscape, particularly in resource-limited settings where timely diagnosis is critical.

- Therefore, based on the above study findings the following recommendations are forwarded.
  - Further research is needed to comprehensively assess the sensitivity and specificity of the CRISPR-Cas12a and RPA-based detection method for sheep pox and goat pox viruses at field level.
  - Conducting additional validation studies across diverse sample populations and under varying environmental conditions will help confirm the robustness and reliability of the assay.
  - Furthermore, conducting a thorough cost-benefit analysis of implementing this detection method in comparison to existing diagnostic techniques would be valuable.
  - Training human power to utilize this recent technology along laboratory fulfillment of material and reagents should be implemented at national level.
  - Widely utilizing this CRISPR Cas technology for other veterinary diseases diagnoses after optimization and validation is highly recommended.

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8.ANNEXES

Annex 1: NucleoSpin® DNA extraction procedure

Preparation of Lysis Buffer RAV1 and Wash Buffer RAV3

- To prepare lysis buffer RAV1, add 1 mL Lysis Buffer RAV1 to the Carrier RNA tube. Dissolve the RNA and transfer it back to the RAV1 bottle.
- Add the indicated volume of ethanol (96–100 %) to Wash Buffer RAV3 Concentrate. Mark the label of the bottle to indicate that the ethanol is added.

procedure

- Add 600 µL Buffer RAV1 containing Carrier RNA to 150 µL of the sample. Add 20 µL Proteinase K (20 mg/mL stock solution), to the lysis mixture. Pipette mixture up and down and vortex for 10–15 s. Incubate for 5 min at 70 °C.
- Add 600 µL ethanol (96–100 %) to the clear lysis solution and mix by vortexing (10–15 s).
- Place NucleoSpin® RNA Virus Columns in Collection Tubes (2 mL) and load 700 µL lysed sample. Centrifuge for 1 min at 8,000 x g. then, Load the residual lysis solution onto the NucleoSpin® RNA Virus Column and Centrifuge for 1 min at 8,000 x g.
- Add 500 µL Buffer RAW to the NucleoSpin® RNA Virus Column. Centrifuge for 1 min at 8,000 x g. Discard flowthrough.
- Add 600 µL Buffer RAV3 to the NucleoSpin® RNA Virus Column. Centrifuge for 1 min at 8,000 x g. Discard flowthrough with Collection Tube.
- Place the NucleoSpin® RNA Virus Column in a new Collection Tube (2 mL) and add 200 µL Buffer RAV3. Centrifuge for 2–5 min at 11,000 x g to remove ethanolic Buffer RAV3 completely.
- Place the NucleoSpin® RNA Virus Column into a new, sterile 1.5 mL microcentrifuge tube (not provided). Add 50 µL Buffer RE (preheated to 70 °C) and incubate for 1–2 min. Centrifuge for 1 min at 11,000 x g.

Annex 2: Recombinase polymerase amplification (RPA) master mix

RPA master mix

- Reaction components
  - Forward Primer(10µM) 1 µl
  - Reverse primer(10µM) 1 µl
  - Primer Free Rehydration buffer 12.5 µl
  - Template 1 µl
  - Water 8 µl
  - Total volume 23.5µl
- Vortex the above reaction mixes and add it to an RPA pellete.
- Add 1.5 µl of 280mM Magnesium Acetate (MgOAc) (supplied) and mix well to start reaction.
- Incubate at 39˚C for 20 minutes. For low template copy number, remove strip after 4 minutes, vortex and spin briefly, replace in heating device.
- After 20 minutes, clean an RPA product using QIAquick® PCR & Gel Clean up kit (100) before running on agarose gels.

Annex 3: QIAquick PCR purification procedure and gel electrophoresis

QIAquick PCR purification procedure

- Add 5 µL of buffer PB to 1 µL of RPA product and mix thoroughly
- Check the colour of the mixture to be turned to yellow, if it is not, add 10 µl of 3 M sodium acetate, pH 5.0, and mix to turn the colour to yellow.
- Then after to bind DNA, apply the sample to the QIAquick spin column and centrifuge the mixture at 13000rpm for 1 minute and discard the flowthrough and return the column back into the same tube.
- Add 750 µL of buffer PE into the center of QIAquick spin column and centrifuge at 13000rpm for 1 min.
- Discard the flowthrough and centrifuge once more for 1min.
- Then place QIAquick spin column into 1.5ml microcentrifuge tube.

- Add 50 µl buffer EB to center of the QIAquick membrane and centrifuge the column for 1 min to elute the DNA.
- To increase DNA concentration, add additional 30µ buffer EB into the center of QIAquick membrane and let it to stand for 1min and then centrifuge at 13000rpm for 1min.

Gel electrophoresis

Making the gel (for a 2% gel, 50mL volume)

- Weigh out 0.5g of agarose into a 250mL conical flask. Add 50mL of 1xTAE, swirl to mix.
- Microwave for about 1 minute to dissolve the agarose.
- Leave it to cool on the bench for 5 minutes down to about 60°C (just too hot to keep holding in bare hands).
- Add 2µL of ethidium bromide (10mg/mL) and swirl to mix
- Pour the gel slowly into the tank. Push any bubbles away to the side using a disposable tip. Insert the comb and double check that it is correctly positioned.
- Leave to set for at least 30 minutes, with the lid on if possible.
- Pour 1x TAE buffer into the gel tank to submerge the gel to 2–5mm depth.This is the running buffer.
- In the first lane load 5 µl 1kbp DNA ladder, while in remaining lanes load 5 µl sample after mixing with 2 µl DNA 6x loading dye in each well by using micropipettes.
- Connect the gel-running tank to the power supply.
- Observe the gel image under the UV trans-illuminator gel documentation system

Annex 4: Preparation of TAE buffer

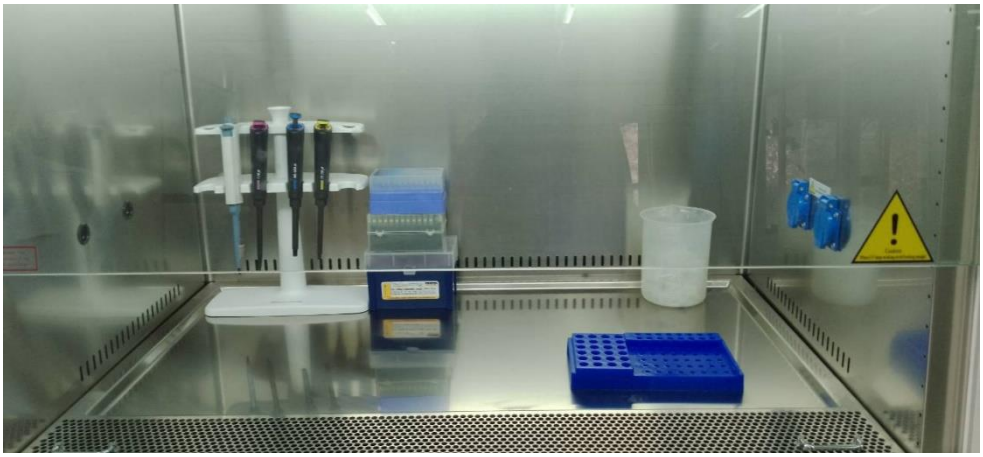
Reagents (per liter of 50 x solution)

- 242 g of Tris base
- 57.1 ml of Glacial acetic acid
- 0.5 M of 100ml EDTA

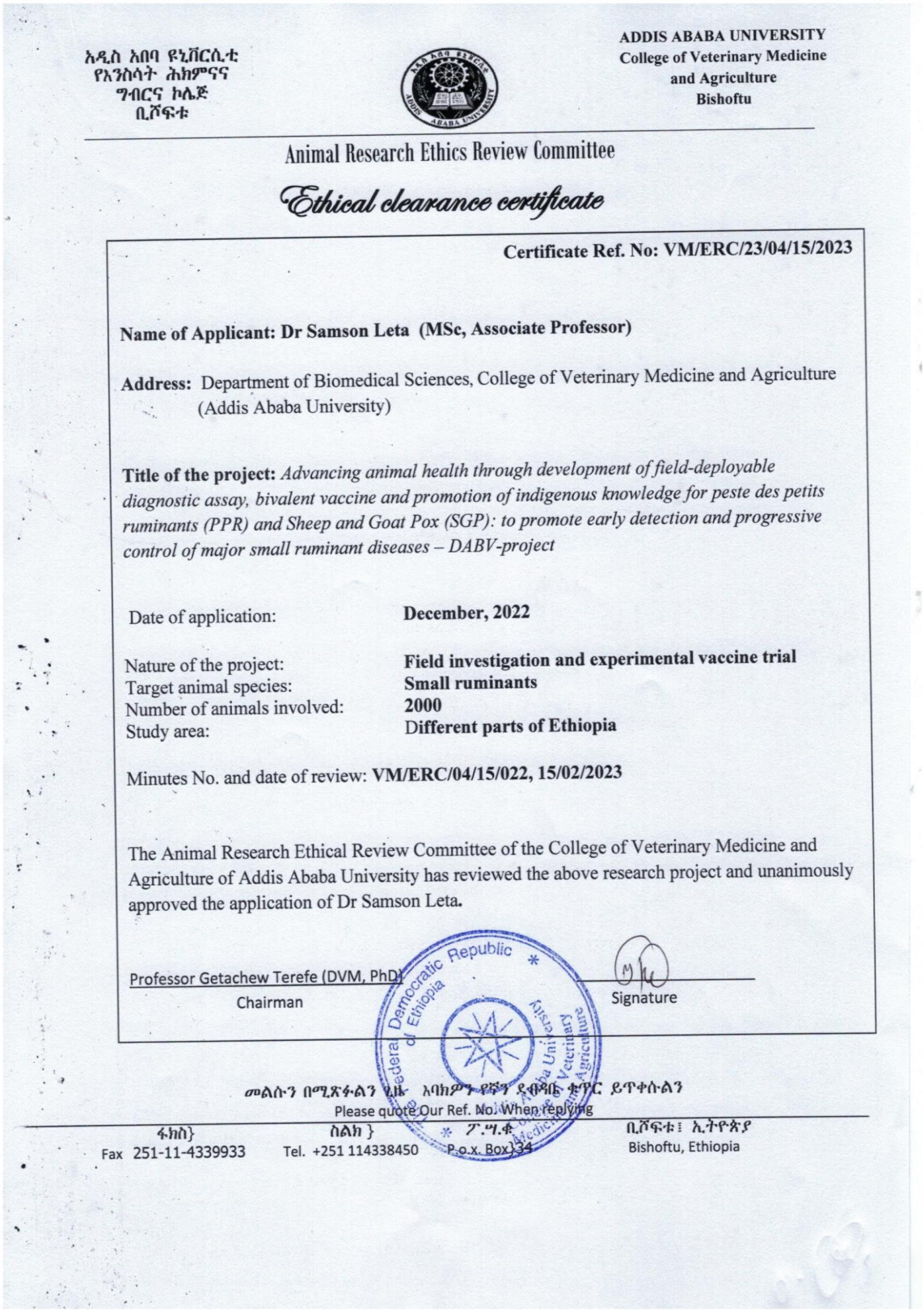
Procedure

1. Weigh out 242 grams of Tris-base (MW = 121.14 g/mol) and dissolve in approximately 700 milliliters of distlled water
2. Carefully add 57.1 milliliters of 100 % glacial acid (or acetic acid) and 100 milliliters of 0.5 M EDTA (pH 8.0)
3. Allow to stir until the tris goes into solution
4. Adjust the solution to a final volume of 1 liter
5. The pH of this buffer is not adjusted and should be about 8.5
6. Store stock solution at room temperature

Annex 5: Pictures taken during laboratory work.



Annex 6: Ethical clearance certificate.



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ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
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Animal Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/23/04/15/2023

Name of Applicant: Dr Samson Leta (MSc, Associate Professor)

Address: Department of Biomedical Sciences, College of Veterinary Medicine and Agriculture (Addis Ababa University)

Title of the project: *Advancing animal health through development of field-deployable diagnostic assay, bivalent vaccine and promotion of indigenous knowledge for peste des petits ruminants (PPR) and Sheep and Goat Pox (SGP): to promote early detection and progressive control of major small ruminant diseases – DABV-project*

Date of application:	**December, 2022**
Nature of the project:	**Field investigation and experimental vaccine trial**
Target animal species:	**Small ruminants**
Number of animals involved:	**2000**
Study area:	**Different parts of Ethiopia**

Minutes No. and date of review: **VM/ERC/04/15/022, 15/02/2023**

The Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture of Addis Ababa University has reviewed the above research project and unanimously approved the application of Dr Samson Leta.

Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

Federal Democratic Republic of Ethiopia * Addis Ababa University College of Veterinary Medicine and Agriculture

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ፋክስ} Fax 251-11-4339933
ስልክ } Tel. +251 114338450
ፖ.ሣ.ቁ P.o.x. Box 34
ቢሾፍቱ፤ ኢትዮጵያ Bishoftu, Ethiopia