MOLECULAR BASED DETECTION, VALIDATION OF LAMP ASSAY AND PHYLOGENETIC ANALYSIS OF CAPRIPOXVIRUS IN KENYA

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2017

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A Thesis submitted in partial fulfillment for the Degree of Master of Science in Bioinformatics and Molecular Biology in the Jomo Kenyatta University of Science and Technology

2017

DECLARATION

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

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DEDICATION

This thesis is dedicated to my parents, Mr. and Mrs. Omoga for their love, support and sacrifices without which I wouldn't have been able to get to this stage.

ACKNOWLEDGEMENT

I would like to express my gratitude to the Almighty God for the gift of life and his favour and grace upon me.

A very special thanks goes to my mentor and role model, Dr. Jacqueline Kasiiti Lichoti whose expertise, understanding, and patience has transformed me wholly. I appreciate her vast knowledge, skills, and her trust, which has molded me to who I am today and made a difference in my life. I would like to thank my supervisors, Dr. Timothy Holton, Prof. Magiri and Dr. Kinyua for their assistance all through the levels of this research project.

My heartfelt appreciation goes to my dear family for the unending support through my entire life. I must acknowledge my dear husband, Sam for being by my side throughout this study and without whose love, encouragement and patience, I would not have finished this thesis. Appreciation also goes to my loving children, Andy, Darlene, Wayne and Casey for their patience and trust.

In conclusion, I recognize that this research would not have been a success without the financial assistance of ABCF/ BecA- ILRI Hub, and their donors. Thanks to BecA research fellows Mercy, Solomon, Joyce and Moses for their technical support, and the Department of Veterinary Services-Central Veterinary Laboratories, Kabete for the provision of samples and the opportunity to enable me undertake the study.

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LIST OF ABBREVIATIONS AND ACRONYMS

ABCF:	Africa Biosciences Challenge fund
AU-IBAR	African Union - Interafrican Bureau for Animal Resources.
BecA:	Biosciences Eastern and Central Africa
CaPVs:	Capripoxviruses
CPE:	Cytopathic Effect.
CVL:	Central Veterinary Laboratories
DNA:	Deoxyribonucleic Acid
DVS:	Department of Veterinary Services
FMD:	Foot and Mouth Disease.
GMEM:	Glasgow Modified Essential Medium.
GP:	Goat Pox
GPV:	Goatpox Virus
KALRO:	Kenya Agriculture and Livestock Research Organization.
LAMP:	Loop Mediated Isothermal Amplification
LSD:	Lumpy Skin Disease
LSDV:	Lumpy Skin Disease Virus
NGS:	Next Generation Sequencing.
OIE:	World Organization for Animal Health
ORF:	Open Reading Frame
PBS:	Phosphate Buffered Saline.

PCR: Polymerase Chain Reaction

PPR: Pestes des Petit Ruminants

SP: Sheep Pox

SPV: Sheeppox Virus

TBE: Tris Boric EDTA.

ABSTRACT

The genus Capripoxvirus (CaPVs) comprises of sheeppox virus (SPV), goatpox virus (GPV) and Lumpy skin disease virus (LSDV) that cause sheep pox (SP), goat pox (GP) and Lumpy skin disease (LSD) in sheep, goats and cattle respectively. They are serious poxviruses of ruminants and are of significant economic impact for the livestock industry and food security. These diseases are contagious and are endemic in parts of Africa and the Middle East. Being viral diseases, the mainly employed methods of management and control of spread is by vaccination and quarantine which are only effective with rapid detection of the disease. These diseases are transboundary and notifiable. There has always been challenges in diagnosis when using traditional methods like ELISA and Cell cultures due to time factor and cross reactivity of the Pox virus antigen with other closely related viruses like Orf virus. Therefore, a need to employ more rapid, simple, specific and sensitive detection methods for diagnosis which will ensure early detection of the disease and therefore effective implementation of control measures. The objective of this study was to compare the available molecular techniques, validate LAMP assay and evaluate CaPVs genome variability, diversity and evolutionary relationships. A cross-sectional study of 130 samples that included skin scrapings, whole blood and cell cultures from sheep, goats and cattle were analyzed by LAMP assay, real time PCR as the gold standard method and Conventional PCR and the results compared. Genomic DNA libraries were prepared from extracted DNA, deep sequenced and the obtained data analyzed. The sensitivity of LAMP assay was 97% and a proportion of detection of 61% was realized in the studied population. The specificity of the LAMP assay was at 100% and successfully detected CaPVs DNA extracted from all samples without cross reacting with closely related virus like Orf virus and Pestes des Petit Ruminants. The deep sequenced LAMP positive samples confirmed the presence of the detected CaPVs after blast analysis against the NCBI database. The phylogenetic analysis confirmed the 3 distinct capripoxviruses lineages: LSDV, GPV and SPV lineages. The Multiple sequence analysis of the P32, GPCR and RP030 genes revealed the virus specific signature found in specific viruses and therefore used to differentiate them. The study therefore supports the adoption of LAMP assays for rapid CaPVs diagnosis and also provides a simpler, effective and rapid method of detection, monitoring and controlling outbreaks

and the spread of disease. This study also forms a basis for molecular epidemiological studies and a reference in CaPVs genomic research in Kenya as a result of determined diversity and evolutionary relationships.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

The *Capripoxvirus* (CaPVs) genus of the family *Poxviridae* consists of lumpy skin disease virus (LSDV), sheeppox virus (SPV) and goatpox virus (GPV) of cattle, sheep and goats respectively. They cause Sheep pox (SP) in sheep, Goat Pox (GP) in goats and Lumpy Skin Disease (LSD) in cattle. These are well-known economically significant animal diseases and are classified as notifiable animal diseases by the World Organization for Animal Health (OIE) (Babiuk *et al.,* 2008). Capripoxviruses are important etiological agents of LSD, SP and GP in ruminants and have up to 96% - 97% similarity in their genomes. They are large, complex ovoid virions with a linear double stranded DNA and a genome size of about 151kb (Tulman *et al.,* 2001).

LSD was first reported in Zambia in 1929, spreading to Botswana by 1943 (Kitching, 1986), and then to South Africa, where it affected over eight million cattle causing a major economic loss (Kitching, 1986). It was first encountered in Kenya in 1957 associated with an outbreak of sheep pox (Babiuk *et al*., 2008).

Capripox viruses are named according to the animal infected or where the virus is isolated from. In sheep, the virus is known as the sheeppox virus, in goats the goatpox virus and in cattle the lumpy skin virus (Bowden *et al.,* 2008). These viruses are closely related genetically, and the little difference in their nucleotide sequence is mostly host based. In indigenous animals, generalized disease and mortality are less common, although they are seen where disease has been absent from an area or village for a long period of time, when intensive husbandry methods are introduced, or in association with other disease agents such as Pestes des Petit Ruminants virus (PPR) or Foot and Mouth Disease virus (FMD). In cases of outbreaks, there are high morbidity and mortality up to around 50% (Beard *et al.*, 2010).

Capripoxviruses diseases (Capripox diseases) are common in both dry and rainy seasons, but due to high number of vectors and spread of the disease, the mortality is high in the rainy seasons (Mellor *et al.*, 1987). This is also because it is suitable for intermediate hosts, mechanical carriers (arthropods) to reproduce and to survive longer under moist and warm conditions (Kitching, 1986). The incubation period is about 5-14 days. The transmission of the virus mostly occurs by direct contact. However, indirectly, insects can also cause mechanical transmission of the disease. Shedding of the virus from the infected animals occurs at each stage of the disease. Mainly, the disease spreads with the movement of infected animals to new areas. The virus remains alive for around three months in wool, and up to six months in the hair and scabs. Fomites and insects play a vital role in the rapid spread of capripox over a large area (Bhanuprkarash *et al*. 2006).

Symptoms of the Capripox.

These diseases are highly contagious and are characterized by an initial rise in temperature of 40-42ºC, increased pulse and respiratory rates, salivation, edema of eyelids, hypersensitivity, arched back, in-appetence, lacrimation, coughing, nasal discharge leading to crust formation scanty urine, pneumonia and constipation (Bhanuprkarash *et al*., 2006). The characteristic pox lesions usually become visible on the skin, mucosa, gastrointestinal and respiratory tract. After infection, the skin lesions usually erupt within 24-48 hours (Das *et al*., 2012). The skin lesions spread mostly in areas of little or no hair, like the groin, face, under the tail, ears, and maxillae. These lesions also occur on mucous membranes of the vulva, nostrils and mouth. The development of the lesions occurs by passing through the typical pox lesions developmental stages including; skin erythema, papule and vesicle with clear fluid which convert to pustule containing pus and on rupture form crusts on the lesion, and finally scabs (Diallo, 2007). The skin lesions usually heal within 5-6 weeks which is very slow. Mostly, the mortality occurs within two weeks after infection but the animal may die at any stage of the disease (Gelaye *et al.*, 2013).

Diagnosis of Capripoxvirus.

Diagnosis of the Pox diseases is based on clinical signs and laboratory confirmation either by way of serological tests, virus isolation, neutralization tests and also molecular based methods (Balinsky *et al*., 2008). Molecular diagnostic tests play an important role in monitoring the spread of these viruses and controlling outbreaks in susceptible livestock (Das *et al*., 2012). This is because they provide a rapid, robust and sensitive method of detection, therefore enabling early detection which leads to the timely implementation of control measures. Agarose-gel based polymerase chain reaction (PCR) assays, or more recently developed real-time PCR assays are rapid and highly sensitive tests widely used in diagnostic laboratories (Hosamani *et al*. 2008). Real-time PCR assays can also be used to differentiate LSDV, SPPV, and GTPV from each other. However, poorly equipped and field laboratories face difficulties accessing these molecular techniques that require expensive and relatively fragile equipment (Lamien *et al.,* 2011). A new group of nucleic acid detection assays that exploit isothermal amplification mechanisms has been developed as potential diagnostic tools for use in either the field or low cost laboratory settings (Das *et al*., 2012). These assay formats include loop-mediated isothermal amplification (LAMP) which is a DNA-dependent amplification method that uses a combination of four to six primers targeting six to eight genomic regions, whilst utilizing the activity of a strand displacing DNA polymerase (Notomi *et al.,* 2000).

The isothermal nature of LAMP potentially allows reactions to be performed simply in a water bath or using a heat pack, usually at a temperature between 60-65°C (Notomi *et al.*, 2000). These factors make LAMP an ideal candidate for use as the basis of an inexpensive test for use in the field. LAMP assays have been developed for the detection of a wide range of viruses, parasites, and bacteria including foot-and-mouth disease virus, human immunodeficiency virus (HIV-1), malaria protozoan *Plasmodium* in blood samples and *Escherichia coli* (Notomi *et al.,* 2000).

1.2 Problem Statement

CaPVs diseases cause a permanent threat to livestock population and industry. Therefore, there is a dire need to rapidly and specifically diagnose the causative agent of this disease in order to develop and apply new ways and means for efficient and effective control. According to the World Bank report in 2011, about 70% of the poor people live in rural areas and most of them keep livestock which places livestock matters in the center of policies for poverty alleviation, especially in sub-Saharan Africa where nearly 73% of the world's poor people are living (Muthami, 2011). Urbanization and a rise in income and population are driving a shift in diet and food consumption patterns towards livestock products in developing countries including Kenya. The diagnosis of Capripoxvirus diseases at the Central Veterinary Laboratories (CVL) – Kabete in Kenya is based on, clinical signs and symptoms like gross lesions, and serological techniques. However, clinical signs can be easily confused with other diseases like contagious pustular dermatitis and Orf virus infections. Similarly, the isolation and serological techniques have limited specificity and accuracy due to antigenic and biochemical similarities among closely related viruses. These techniques are also time consuming, laborious, have low specificity, and require sterile conditions (Santhamani *et al.*, 2015). Therefore, the control and eradication of these diseases constitute a never ending challenge due to lack of effective detection methods. This study therefore, aimed at determining the specificity and sensitivity of the available molecular techniques for recommendation and adoption for use by the agencies concerned with livestock diseases diagnosis including the Department of Veterinary Services (DVS).

Generally, all viruses continue to pose threats to both humans, animals and plants and their detection and identification is mostly a challenge. Next generation sequencing has been recommended as a novel technique in viral diagnostic.

The 3 Capripoxviruses (LSDV, SPV and GPV) are genetically related and the only method that has been used to differentiate the viruses is Kenya is by the host of origin which may be misleading. This is because there is lack of genetic information on the circulating

capripoxvirus that can be used to specifically identify the virus, develop very specific molecular detection methods based on the sequences and also used to produce alternative forms of vaccines like recombinant vaccines and understand their molecular levels of attenuation. The currently available vaccines for capripox management in Kenya are live attenuated vaccines. These vaccines sometimes pose a danger to the livestock sector when right levels of passages are not adhered to during production, resulting to a virus that is not completely weakened.

1.3 Justification

Considering the threat caused by CaPVs of food security in many developing countries, there is need to develop strategies for early diagnosis, control and eradication of these viruses as part of efforts towards poverty alleviation and food security. It is possible to make a rapid and specific diagnosis based on Polymerase chain reaction (PCR) and recently developed Loop Mediated isothermal amplification (LAMP) assay which are more specific and sensitive tools for rapid and accurate diagnosis. LAMP assay is a robust, rapid and specific method that can be carried out in resource poor laboratories even without electricity. Such a technique is what is required in certain parts of Kenya like in the North Eastern and Upper Eastern regions where livestock farming is very vital yet the resources are inadequate. This CaPVs LAMP assay has been validated and adopted for usage in Uganda and Tanzania. Generally, the LAMP assay has been used in diagnosis of human pathogens like Mycobacteria tuberculosis, Influenza and Herpes viruses among others and therefore is a reliable molecular technique.

Effective development of these molecular diagnostic techniques requires proper understanding of the genome sequences of these viruses. Sequence analysis is important because viruses are always prone to mutations like deletions, transversions and insertions which may lead to changes in their genomes that can result to more serious diseases due to lack of response to vaccination and challenges in molecular detection (Rao & Bandyopadhyay, 2000). Phylogenetic analysis is important in demonstrating virus diversity in the population.

As there is no approved treatment for Capripox, the diseases can only be managed through effective control measures like vaccination and quarantine. To ensure the development of much more reliable and effective vaccines other than the live attenuated forms, then proper understanding of the genomic basis of the virus is necessary, therefore, availing some genetic data through this study will contribute to that.

1.4 Research Questions

1. What is the sensitivity and specificity of LAMP assay compared to that of real time and conventional PCR in CaPVs detection?

2. What is the sensitivity of Next Generation sequencing in Capripoxvirus diagnosis?

3. What are the evolutionary relationships and diversity of the CaPVs strains found in Kenya?

1.5 Objectives

1.5.1 Main Objective

To detect Capripoxvirus using molecular based techniques and analyze its genetic variability, diversity and evolutionary relationships in cattle, sheep and goats in Kenya.

1.5.2 Specific objectives.

1. To validate LAMP assay and compare its sensitivity and specificity to real time and conventional PCR in detection of Capripoxvirus.
2. To determine the sensitivity of Next generation sequencing (NGS) in diagnostic virology using capripoxvirus
3. To evaluate Capripoxvirus genetic diversity, variability and evolutionary relationships.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

Kenya has a large population of livestock well adapted to the environmental condition of the country. Cattle, sheep, and goats forms a major part of the livestock population. They produce milk, meat, skin and hides, wool and play a major role in contributing to the country's economy and food security. A number of health hazards including disease and natural disasters like floods, drought endanger their population (Muthami, 2011).

The losses caused by Capripoxvirus diseases are usually reflected in the form of poor leather, low quality wool and reduced productivity (Rao and Bandyopadhyay, 2000). These cause a lot of economic losses to farmers, and they have negative impacts on trade across the border in livestock and their products (Babiuk *et al*., 2008). Cattle, sheep, and goats have tremendous roles in the life of shepherds in terms of wool, skin and meat. Capripoxes, therefore, compromises the productivity of animals and also affect the international trade of their products (Gari *et al.*,2011).

Lumpy Skin disease (LSD) originated from Zambia from where it spread to Zimbabwe, Mozambique, and South Africa. The transmissible character of LSD quickly became apparent after its spread to South Africa, where it assumed epizootic proportions and in 2-3 years extended over most of the country. More than 8 million cattle were affected in the outbreak and the economic losses sustained were considerable, according to the 2013 AU-IBAR report. In 1957, LSD was first reported in Rift Valley, Kenya in a fairly localized outbreak (Davies, 1976).

The Capripox diseases are transboundary and therefore a significant impediment to the economic wellbeing of farmers in developing countries and would have substantial economic impacts on industrialized countries, should the disease spread there (Panel, 2014). Capripoxviruses are expanding their territories and have the potential to become

emerging disease threats because of global climate change and changes in patterns of trade in animals and their products. They could also be used as an economic bioterrorism agent (Coetzer, 2004)

2.2 Etiology of the Capripox diseases

Capripox diseases are caused by Capripoxviruses which belong to the family Poxviridae, sub family Chordopoxvirinae and genus Capripoxvirus and they are highly contagious (Le Goff *et al.,* 2009). They have brick shaped morphology of 170 to 260 by 300 to 450-nm in diameter (Gershon & Black, 1988). A false lipid envelope surrounds the genome. The virus is endemic in Africa, Middle East, India, and Asia (Hunter & Wallace, 2001).

2.3 Life cycle of Capripoxvirus

Replication of the capripoxvirus occurs in the cytoplasm of infected cells. The virus enters the host cell through the attachment of the viral proteins to host glycosaminoglycans (GAGs) which mediates endocytosis of the virus into the host cell. Fusion with the plasma membrane releases the core into the host cytoplasm (Tulman *et al.,* 2001). This occurs in phases, in the early phase, early genes are transcribed in the cytoplasm by viral RNA polymerase. Early expression begins at 30 minutes post-infection and the core is completely uncoated as early expression ends, the viral genome is now free in the cytoplasm (Santhamani *et al.,* 2014). In the intermediate phase, the intermediate genes are expressed, triggering genomic DNA replication at approximately 100 minutes post-infection and the late genes expressed during the late phase, from 140 min to 48 hours post-infection, produced all structural proteins (Bowden *et al*., 2008). Assembly of progeny virions starts in cytoplasmic viral factories, producing a spherical immature particle. This virus particle matures into brick-shaped intracellular mature virions (IMV). IMV virion can be released upon cell lysis, or can acquire a second double membrane from trans-Golgi and bud as external enveloped virion (EEV) host receptors, which mediates endocytosis. Replication follows the DNA strand displacement model. The virus exits the host cell by existing in occlusion bodies after cell death and remaining infectious

until finding another host (Tulman *et al.,* 2001). Sheep, goats, and cattle serve as the natural host. Transmission routes are fomite, mechanical, and contact (*Poxviruses*, 2007).

2.4 Pathogenesis of Capripox

Following inoculation into the skin by insects, the virus multiplies locally to the dermis producing a primary nodule. Viraemia follows, accompanied by fever, and the appearance of the nodules is widespread (Tulman *et al.,* 2001). Skin nodules involve both the dermis and epidermis. The nodules arise from the damage to endothelial cells, vasculitis, thrombosis and infarction leading to coagulative necrosis (Stram *et al.,* 2008). Nodules on the mucous membranes of the mouth and nose quickly ulcerate (Babiuk *et al*., 2008). Some skin lesions may develop into a "sit-fasts" where a central plug of necrotic tissue develops over a number of weeks before sloughing off to reveal a deep granulating rock involving all layers of the skin (Diallo *et al*., 2007).

2.5 Transmission of Capripoxvirus diseases.

Transmission is usually by aerosol after close contact with severely affected animals containing ulcerated papules on the mucous membranes (Babiuk *et al.,* 2008). There is reduced transmission once papules have become necrotic and the neutralizing antibody is produced (Zro *et al*., 2014). Animals with mild localized infections may also occur through other mucous membranes or abraded the skin. Indirect transmission by contaminated equipment, vehicles or products occurs. Indirect transmissions by insects (mechanical vectors) mainly *Stomoxys calcitrans* have also been established (World Organization for Animal Health [OIE] Manual of Diagnostics Tests and Vaccines for Terrestrial Animals, 2010). Recently, new evidence has been published reporting a possible role for hard ticks in the transmission of LSDV (Tuppurainen *et al*., 2014). The study showed molecular evidence of transstadial and transovarial transmission of LSDV by *Rhipicephalus (Boophilus) decoloratus* ticks, and mechanical or intrastadial transmission by *Rhipicephalus appendiculatus* and *Amblyomma hebraeum* ticks.

The Capripoxvirus prevalence is expanding, which is evident from outbreaks. Poor quarantine measures and trade of live animals across the border may lead to further spread of the disease (Babiuk *et al.,* 2008). The grazing and migration patterns indicate that the disease was probably introduced into Kenya through transboundary movement of cattle (Rao and Bandyopadhyay, 2000).

The occurrence and spread of this skin disease is associated with poor management, climatic factors, feed scarcity and inadequate veterinary services (Tuppurainen *et al.,* 2014). The increasing threat of the disease to the development of livestock production warranting an urgent control intervention is needed.

Secondary infection and myiasis are common complications in untreated cases. Recovery is slow and animals are frequently debilitated for several months. Lesions may occur in the rumen, abomasum and trachea and lungs (Bowden *et al*., 2008).

The sheep pox and goatpox viruses replicate locally in the skin or lungs depending on the route of infection. Spread to the regional lymph nodes is followed by a primary viraemia and replication in various internal organs (Mellor *et al.,* 1987). Skin lesions appear at about seven days post infection as macules. Within 24 hours, the macules become papules which persist for about one week before becoming necrotic. Scabs form during the following few days. The scabs fall off leaving a permanently depressed scar (Scott, 2003).

2.6 Hosts of Capripoxviruses

The host range of Capripoxviruses is limited to ruminants and they primarily infect sheep, goats, and cattle which serves as their natural hosts. Although it has been noted that the pox disease can also infect other breeds like wild goats, camels and even horses; however some strains are restricted to one species and some are difficult to distinguish (Carn, 1993). The classification of pox viruses was made on the basis of the host from which these viruses were isolated. Serologically, all viruses (sheeppox virus, goatpox virus and

lumpy skin disease virus) are identical and usually cross react, but can be differentiated by using molecular techniques (Christian *et al*., 2009).

Native breeds in endemic areas are far less susceptible than introduced breeds of European or Australian origin (OIE Terrestrial Manual, 2008). Some studies have shown a possibility of giraffes and impala being susceptible to lumpy skin disease virus. Sheep pox is a highly contagious, host specific, viral infection, and causes a high rate of mortality and morbidity in sheep, irrespective of age, sex and breed (Singh *et al*., 2007). Natural infections with LSD have only been described in cattle in sub-Saharan Africa, and both *Bos taurus* and *Bos indicus* breeds, are susceptible. *Bos taurus* animals exotic to Africa are generally more susceptible than the zebu-type cattle, which are indigenous to sub-Saharan Africa. A single clinical case of a Capripox infection, probably LSD, was described in an Arabian oryx in a zoo in Saudi Arabia. Many wild ruminant species, which share grasslands with cattle in many parts of Africa, are not naturally infected with LSD as a clinical syndrome (African Union – Interafrican Bureau for Animal Resources [AU - IBAR], 2013). The first LSD outbreak in Kenya was at a farm where there was a concurrent outbreak of sheep pox (Burdin, 1959). The possibility was raised that there may have been a change in the host affinity of the virus and that it may have crossed from sheep and goats to infect the cattle (AU - IBAR , 2013).

CaPVs are not zoonotic and therefore human beings cannot be infected by the virus (Babiuk *et al.*, 2008).

2.7 Diagnosis of Capripoxviruses

A definitive diagnosis that entails laboratory confirmation is required, although Capripoxviruses causes skin and visceral pox lesions that are indicative of the disease (Babiuk *et al*., 2008). Diagnosis can often be made based on clinical signs and postmortem appearance. Papules may be present at many internal sites including tongue, esophagus, rumen, abomasum, and large intestine (Stram *et al.*, 2008). Lesions in the lungs often coalesce into areas of consolidation and hemorrhage. Skin biopsies from live animals or

necropsy specimens can be submitted for laboratory confirmation. Material intended for virus isolation or antigen detection can be collected early in the clinical course prior to the development of neutralizing antibodies (El-Kenawy & El-Tholoth, 2010).

Histopathological examination of acute-stage skin lesions typically reveals a large cellular infiltration, vasculitis, edema, and the presence of eosinophilic intracytoplasmic inclusions in cells in the dermis (Binepal *et al*., 2001).

Electron microscopy can be used to rapidly identify poxvirus particles in the lesions material. The morphology of Capripox virus is easily distinguishable from Para poxviruses (Mori and Notomi, 2009).Virus isolation is possible in lamb testis or kidney cells and results in a characteristic cytopathic effect and intracytoplasmic inclusions bodies. An antigen trapping ELISA has been developed for the detection of the highly antigenic P32 Capripox virus structural protein (Carn, 1995). Capripoxviruses are serologically identical, their specific identification relies exclusively on the use of molecular tools (Le Goff *et al.*, 2009). Specific primers have been designed for the detection of viral of Capripoxvirus DNA in biopsy or tissue cultures samples using PCR and LAMP (Balinsky *et al*., 2008). It is possible to differentiate sheep pox and goat pox on the basis of restriction enzymes analysis of the PCR- amplicons of the P32 gene, which encodes a major antigen (Hosamani *et al*., 2004).

Western blot analysis and indirect ELISA are available for the detection of antibodies to Capripoxviruses (OIE, 2010).

2.7.1 Viral Isolation/ Cell cultures.

Virus isolation is considered the gold standard method for the diagnosis of viral diseases, but its application in the detection of CaPVs is limited due to the long incubation times typically needed to obtain results (Bowden *et al*., 2008).

Capripoxviruses grow in tissue culture of bovine, ovine or caprine origin, although primary or secondary cultures of lamb testis (LT) or Lamb kidney (LK) cells are

considered to be the most susceptible, particularly those derived from a wool sheep breed (OIE *Terrestrial Manual* 2010). The virus infected cells develop a characteristic cytopathic effect (CPE) consisting of retraction of the cell membrane from surrounding cells and eventually rounding of cells and margination of the nuclear chromatin, this is between 7-14 days. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size and surrounded by a clear halo are indicative of poxvirus infection (OIE *Terrestrial Manual* 2010). Some strains of Capripoxvirus have been adapted to grow in African green monkey kidney (Vero) cells, but these are not recommended for primary isolation. Fetal Bovine muscle (FBM) cells are an effective alternative to lamb testis cells (LT) for growing LSDDV for diagnostic and vaccine production purposes (Binepal *et al.*, 2001).

2.7.2 Enzyme Linked Immunosorbent Assay (ELISA).

This is done using highly specific monoclonal antibodies raised against the P32 coat protein (OIE, 2014). The presence of the trapped antigen can then be detected using guinea-pig serum raised against the group-specific structural protein P32, commercial horseradish-peroxidase-conjugated rabbit anti-guinea-pig immunoglobulin, and a chromogen/substrate solution. However the method is not very specific (OIE, 2010). There has been a lot of challenges when employing ELISA technique in Capripoxvirus diagnosis, the major one being the problem of cross reactivity with other viruses like Pestes des petit ruminants (PPR) and therefore nonspecificity. This can, therefore, lead to unreliable false positive results.

2.7.3 Virus Neutralization Test

It is the gold standard method for determining anti-capripoxvirus antibodies, though it is slow, labour intensive and requires live capripoxvirus which is not very possible in disease free countries (Babiuk *et al.*, 2008). Development and use of recombinant capripoxvirus would decrease the length and time required for detection of virus neutralization activity by several days.

2.7.4 Molecular Diagnostics Techniques.

Molecular diagnosis that involves the amplification of the nucleic acid that is DNA or RNA has become a gold standard for rapid detection and diagnosis of viral diseases including CaPVs.

These diagnostic techniques include LAMP assay, Real time PCR, and Conventional PCR. They are rapid sensitive and specific in detecting capripoxvirus genome compared to immunological and viral isolation methods. The strength of the real time PCR has been its speed, sensitivity, its quantitative nature, detection of results in real time and the ability to include controls for detection of reaction inhibitors. However, it requires expensive high-precision instruments and specialized training for operation and data analysis (Das *et al.,* 2012).

LAMP is a novel method of nucleic acid amplification that occurs under isothermal conditions at temperatures between 60 and 65˚C and is catalyzed by a DNA polymerase with strand displacement activity (Notomi *et al.,* 2009). It employs a minimum of four specifically designed primers including a forward inner primer (F3), a backward outer primer (B3), a forward inner primer (FIP) with two binding domains, F1c and F2c, and a backward inner primer (BIP) with two binding domains, B1 and B2c (Zhao *et al.,* 2014). This combination recognize six specific regions within the target locus. Additional primers that is forward loop primer (FLP) and backward loop primer (BLP), are typically optional and are used to enhance sensitivity of the LAMP assay. LAMP assays are highly specific as the amplification only occurs when the six specific regions of the target amplicons are recognized by the primers (Notomi *et al.,* 2009). The LAMP assay is simple and there are several ways of monitoring the reaction including agarose gel electrophoresis, measuring turbidity of magnesium pyrophosphate a by-product of LAMP or fluorescence using a DNA intercalating dye, such as SYBR green (Tomita *et al.,* 2008), or a metal binding flourophore such as calcein and by colour change using a metal ion-binding indicator dye such as hydroxynaphthol blue (HNB) (Goto *et al.,* 2009) and

therefore can be used in the fields and resource poor laboratories unlike the PCRs (Das *et al.,* 2012).

The Genie® II real time LAMP machine is a compact, lightweight and robust instrument suitable for use in the field or laboratory. It is specifically designed to run any isothermal amplification method that employs target detection by fluorescence measurement. The device has two heating blocks, each of which takes a single 8-microtube strip that was specially designed for the instrument. The tubes feature locking caps that do not open after a run, so preventing any contamination. The blocks can be controlled independently or run together for processing up to 16-samples. The instrument has low power requirements and includes a rechargeable Lithium-Polymer battery that can keep it running for up to 8 hours (Bekele *et al.,* 2011).

The Genie® II LAMP machine allows for real-time isothermal amplification of the DNA. It is possible to observe real time data from isothermal amplification reactions with a precision LED based optical detection system that monitor all dyes with excitation at 470nm and detection above 510 nm. The real-time LAMP use fluorescence detection and the closed-tube nature of detection greatly reduces the risk of carry-over contamination, and the melt/anneal analysis can be formed to increase the robustness of the results (Bekele *et al.,* 2011)

Despite the several benefits of molecular based detection methods, there should be an additional method used for confirmation and quality controls in diagnostic cases (Babiuk *et al.,* 2008). This will help put the methods on continuous check and cases of unreliable results ruled out. Different PCRs and LAMP assays have been developed to identify all the capripoxvirus isolates, and the assays could be refined to be specific only for vaccine isolates or specific for only sheep, goat and cattle isolates if distinct signatures are found (Orlova *et al*., 2006). The primers used in these assays are designed from common conserved regions of the genome of the three capripoxviruses so that they are able to detect all the isolates.

2.8 Gold Standard method/Test

The gold standard is the best single test (or a combination of tests) that is considered the current preferred method of diagnosing a particular disease. All other methods of diagnosing the disease, including any new test, need to be compared against this ′gold′ standard. The gold standard is different for different diseases (Parikh *et al*., 2008). The gold standard for a particular disease may be considered outdated or inadequate, but any new test designed to replace the gold standard has to be initially validated against the gold standard (Rutjes *et al.*, 2007). If the new test is indeed better, there are ways to prove that; following which the new test may become the gold standard. In this study, real time PCR was used as the gold standard method. Real time PCR is the approved gold standard method for capripoxvirus diagnosis recommended by the World Organization for Animal Health (OIE).

2.9 Diagnostic Test Validity

It is the extent to which a test measures what it is supposed to measure; in other words, it is the accuracy of the test. Validity is measured by sensitivity and specificity (Parikh *et al*., 2008). Test validity is the ability of a diagnostic test to accurately identify diseased and non-disease individuals. An ideal diagnostic test is exquisitely sensitive (high probability of detecting disease) and extremely specific (high probability that those without the disease will screen negative). However, there is rarely a clean distinction between "normal" and "abnormal."

The validity of a diagnostic test is based on its accuracy in identifying diseased and non-diseased persons, and this can only be determined if the accuracy of the screening test can be compared to some "gold standard" that establishes the true disease status (Rutjes *et al.*, 2007). The gold standard might be a very accurate, but more expensive diagnostic test. Alternatively, it might be the final diagnosis based on a series of diagnostic tests

2.10 Epidemiology of Capripoxes.

Capripox is endemic in parts of Asia, Africa including Kenya and recently Southern Europe has also been invaded with the disease (OIE Terrestrial Manual, 2010). The most recent outbreaks of sheep pox and goat pox in Europe occurred in Greece and Bulgaria in 2013-2014 (Panel, 2014). Lumpy skin disease, however, has been more confined to Africa (Abutarbush *et al.,* 2014). In Kenya, the most recent outbreaks were experienced in West and North Pokot, and parts of Garisa and Isiolo in the last quarter of 2014 and in parts of Western Kenya bordering Uganda in early 2015. Epidemiologically, the disease is very important and causes a huge morbidity and mortality in the endemic areas, ranging from 40-60%. The mortality may reach 100% in imported animals due to environmental changes and adaptation. Although capripox is endemic in Africa, the reported presence of evidence of disease in different countries varry (AU - IBAR, 2013 report) as indicated in Figure 2.1. The disease is present and widespread in many countries and completely absent or not reported in a few North Africa countries like Algeria and Libya.

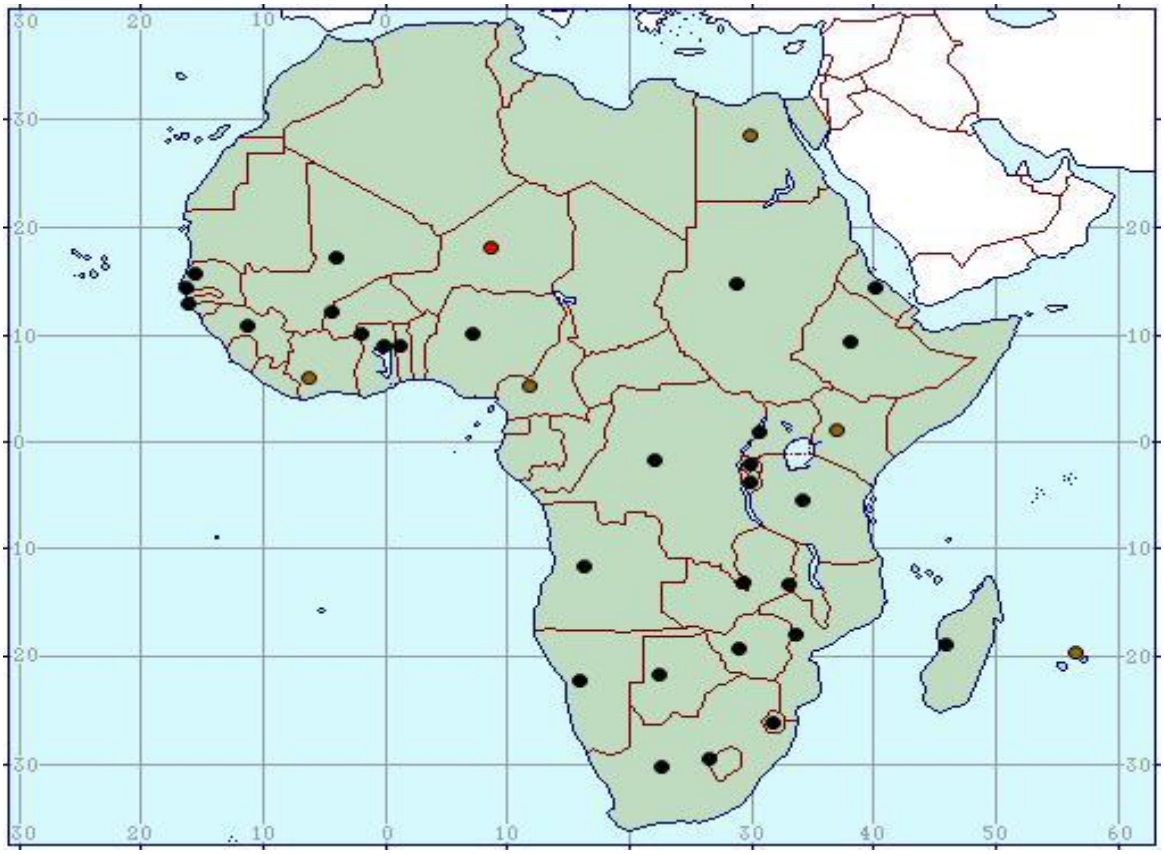

Figure 0.1: LSD distribution map for Africa (AU IBAR, 2013 report)

Key:

- Disease Present
- Widespread
- Localized
- Confined and subject to quarantine
- Occasional or few reports
- Evidence of pathogen
- Last reported...

2.11 Genomics of Capripoxviruses.

The Capripox virions have an ovoid shape and their genomes are linear double stranded DNA of ~151kb, containing 156 putative genes with high AT content of 73-75% and shares up to 96% nucleotide identity (Tulman *et al.,* 2002). Like other poxviruses, capripoxviruses contain a central coding region bound by two identical inverted terminal repeats (ITR) regions. Terminal genomic regions data indicate that CaPVs differ in the nature and size of tandemly repeated sequences (Tulman *et al,* 2001). Goatpox and sheeppox viruses share at least 147 putative genes. Lumpy skin disease virus has an additional nine genes that are non-functional in sheeppox and goatpox viruses, some of which are likely responsible for their ability to infect cattle (Tulman *et al*., 2001). Among the 156 ORFs, the essential genes of replication, structure and assembly are located in the central conserved ORFs 024 to 123 and virulence and host range functions are in the terminal variable ORFs 01 to 023 and 124 to 156 (Moss *et al*., 2001). The most important genes in the differentiation of these viruses are RP030 gene (ORF 036) that encodes the RNA polymerase 30 kDa subunit which plays a role in replication; GPCR gene (ORF 011), a host range gene which encodes the G-protein coupled chemokine receptor, a membrane bound protein involved in host immunomodulation; and the P32 gene that encodes the viral coat protein and possesses specific signatures for the CaPVs (Hosamani *et al.,* 2008).

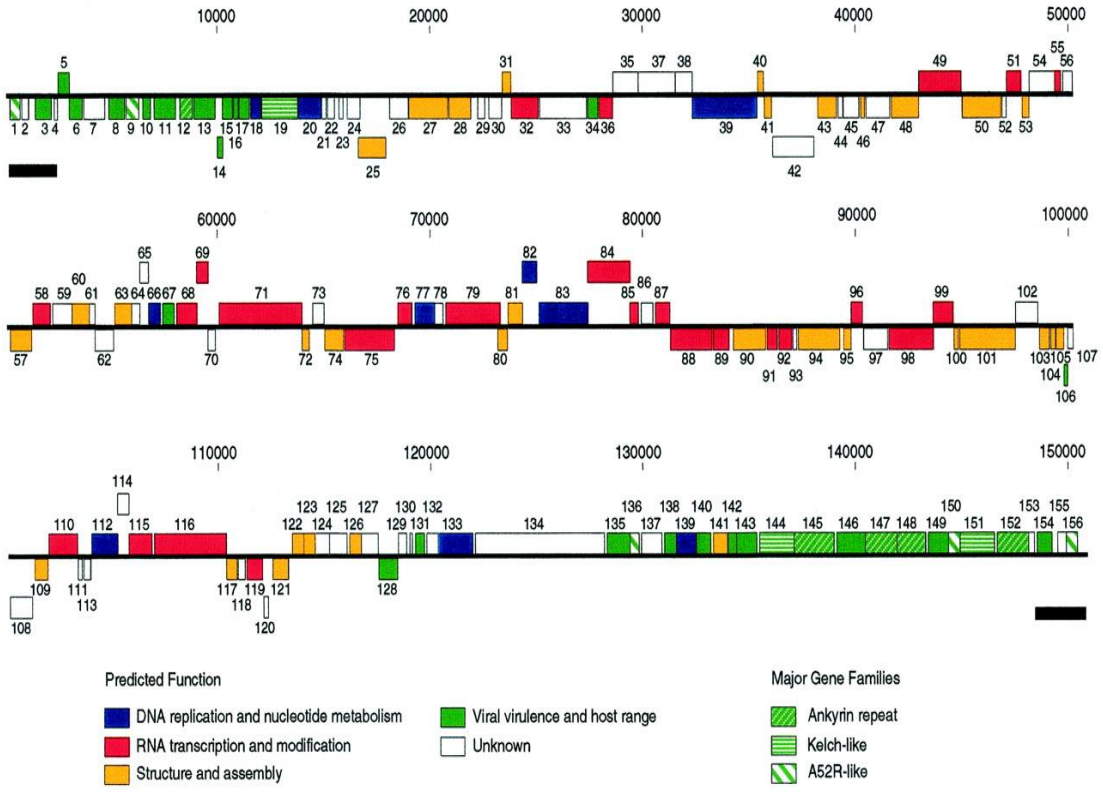

Figure 0.2: Linear map of LSDV genome (Tulman *et al.*, 2001)

ORFs are numbered from left to right based on the position of the methionine initiation codon.
ORFs transcribed to the right are located above the horizontal line; ORFs transcribed to the left are below. Genes with similar functions and members of gene families are coloured according to the figure key. ITRs are represented as black bars below the ORF map

In capripoxviruses, the presence of deletions, substitutions and insertions have resulted in differences in sizes of the ORF of the three viruses with LSDV having 156 ORFs and GPV and SPV 147 ORFs and also the size of different proteins. Nine LSDV genes with likely virulence and host range functions are disrupted in SPPV and GTPV, still some nucleotide differences within the two are associated with host range. Genes affected by insertions, deletions, and substitutions include an LSDV-specific gene (LSDV132) and those similar to IL-1 receptor (Tulman *et al.*, 2002).

2.12 Vaccines

Vaccination is the most effective method of disease control. A variety of attenuated live and inactivated vaccines have been used to provide protection against capripox in disease endemic areas (OIE *Terrestrial Manual* 2010). There are two antigenic forms of capripox virus, the intact virion covered in short tabular elements, and the intact virion additionally covered in host-cell-derived membrane (Tuppurainen *et al.*, 2014). The latter is the form usually produced by the infected animal, whereas the former is that seen when virus is produced by freeze-thawing infected tissue culture (Bhanuprakash *et al.*, 2006). Dead vaccines produced from tissue culture are almost entirely naked virions, and when used as a vaccine do not stimulate immunity to the membrane-bound virion, this partly explains the poor success of inactivated vaccines, the other being that they are less effective in stimulating the cell-mediated immune response, which is the predominant protective response to pox virus infection. Therefore dead capripox vaccine provide at best only temporary protection (OIE *Terrestrial Manual* 2010).

The vaccines currently used against sheeppox, goatpox and LSD in endemic countries are live field isolates (Roth and Spickler, 2003), which have been attenuated by multiple passages in cell culture and in chorioallantoic membranes of embryonated hen's eggs (Davies and Mbugua, 1985). Animals that recover from a virulent capripoxvirus infection generate a lifelong immunity consisting of both antibody and cellular immunity, which can protect the animals from all capripoxvirus isolates (Kitching, 1986). Capripoxviruses infection can also be prevented by administration of anti-capripoxvirus serum (Kitching, 1986), but cell-mediated is likely the most significant component in recovery from infection and in long term protection, evidenced by protection accorded by vaccination where the presence of specific antibodies cannot be detected. Some vaccines might be substantially attenuated in one host but too virulent to be used in another. Although most of the live attenuated vaccines work well, reports of vaccine breakdown, short duration of protection, and low levels of antibody inducement necessitate the need for improved vaccines (Hunter and Wallace, 2001). The availability of whole genome sequenced data

now allows for a more directed approach to vaccine development by targeting genes specifically involved in virulence and host immune system modulation.

Capripoxvirus have also been used as a vector of other viral genes to elicit protective immune responses to a variety of viral pathogens such as rabies (Aspden *et al*., 2002), peste des petits ruminants (Diallo *et al*., 2002) and Rift valley Fever viruses (Hunter and Wallace, 2001).

The fact that capripoxviruses have a single serotype with limited host range can be used to increase the prospect of successfully implementing regional control programs, leading to the elimination of the virus and conceivably global eradication (Babiuk *et al.,* 2008).

2.13 Next Generation Sequencing (NGS)

Next generation sequencing is a novel DNA sequencing techniques that provide high speed and throughput that can produce an enormous volume of sequences (Barzon *et al.*, 2011). NGS is capable of sequencing large numbers of different DNA sequences in a single reaction (i.e., in parallel) The most important advantage provided by these platforms is the determination of the sequence data from single DNA fragments of a library that are segregated in chips, avoiding the need for cloning in vectors prior to sequence acquisition (Barzon *et al.,* 2011). NGS technologies are currently used for whole genome sequencing, investigation of genome, diversity, metagenomics, epigenetics, discovery of non-coding RNAs and protein-binding sites, and gene-expression profiling by RNA sequencing (Behjati & Tarpey, 2013). Typical applications of NGS methods in microbiology and virology, besides high-throughput whole genome sequencing, are discovery of new microorganisms and viruses by using metagenomic approaches, investigation of microbial communities in the environment and in human body niches in healthy and disease conditions and analysis of viral genome variability within the host (i.e., quasispecies) (Grada & Weinbrecht, 2013).

2.13.1 The Basics of Illumina Next sequencing Platform.

All next-generation sequencing (NGS) technologies monitor the sequential addition of nucleotides to immobilized and spatially arrayed DNA templates but differ substantially in how these templates are generated and how they are interrogated to reveal their sequences (Grada & Weinbrecht, 2013)

In principle, the concept behind NGS technology is that the DNA polymerase catalyzes the incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) into a DNA template strand during sequencial cycle of DNA synthesis. During each cycle, at one point of incorporation, the nucleotides are identified by fluorophore excitation. The critical difference is that, instead of sequencing a single DNA fragment, NGS extends this process across millions of fragments in a massively parallel fashion (Barzon *et al.*, 2011). Illumina sequencing by synthesis (SBS) chemistry delivers the highest yield of accuracy, error free reads (Grada & Weinbrecht, 2013).

2.13.2 How Illumina Next generation sequencing Works

The prepared libraries are composed of fragments of double-stranded DNA that can be recognized by the NGS sequencer (Behjati & Tarpey, 2013). The DNA to be sequenced is fragmented down to 200–600 bp, and fragment ends should be enzymatically blunted and flanked by adapter sequences on both sides. A 3′ dA tail is then added using DNA polymerase without 3′–5′ exonuclease activity. dA tailing prevents concatemerization of DNA fragments and allows the use of a dT-tailed adapter, which minimizes adapter dimer formation. A Y-shaped adapter is then ligated using A-T base pairing, and the correctly ligated libraries are amplified. The 5′ end of the Y-shaped adapter contains a phosphate group and the 3′ end contains dT. Phosphorothioate bonds provide resistance to nuclease. During cluster generation which occur on the surface of the flow cell, denatured libraries are annealed to the short oligonucleotides on the surface of the flow cell. The distance between DNA molecules should be long enough to prevent overlapping clusters. The bridging amplification generates clusters and one strand from the double-strand DNA

library is cleaved and washed out for unidirectional sequencing and primers for inserts are annealed for the sequencing of the insert DNA. In each sequencing cycle, protected and fluorescently labeled A, T, G and C bases are applied. After the addition of each nucleotide, the sequencing reaction is stopped, and the image is taken. Because the newly added nucleotides within each cluster are identical, the signal is high enough to be detected by a light sensor. After the image is taken the protection group and the fluorescent molecules are removed (Shin *et al.*, 2014).

When the first-strand sequencing reaction is finished, the synthesized strand is removed and the process is repeated for the opposite strand (figure 2.3).

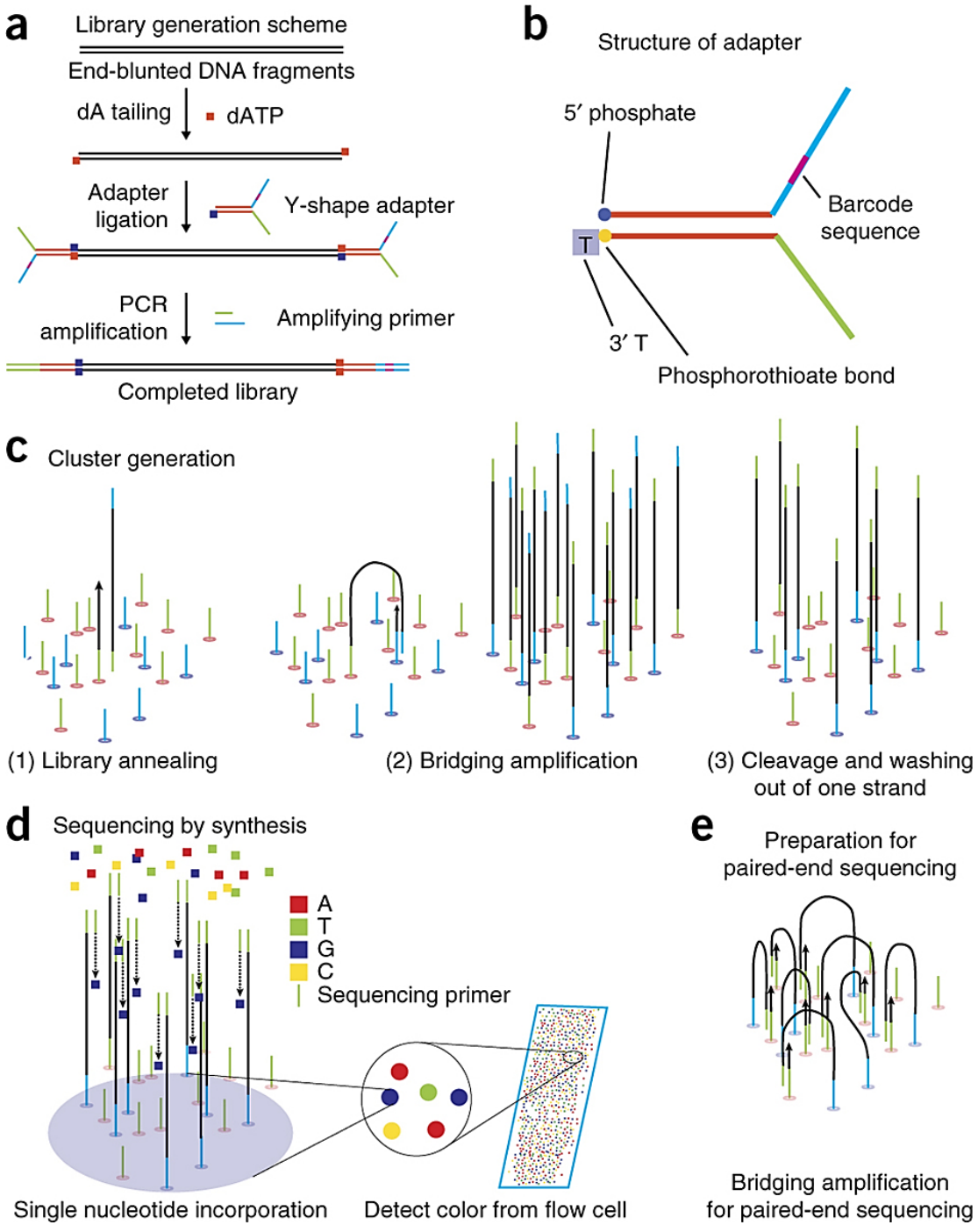

Figure 0.3: **Diagrammatic representation of Illumina Next generation sequencing** (Shin *et al.*, 2014).

(a). DNA to be sequenced is fragmented down to 200–600 bp, the fragments are enzymatically blunted and flanked by adapter sequences. A 3′ dA tail is added using DNA

polymerase without 3′–5′ exonuclease activity. A Y-shaped adapter is then ligated using A-T base pairing, and the correctly ligated libraries are amplified. **(b)** The 5′ end of the Y-shaped adapter contains a phosphate group and the 3′ end contains dT. **(c)** Clusters are generated on the surface of the flow cell. **(d)** Primers for inserts are annealed for the sequencing of the insert DNA. **(e)** The synthesized strand is removed after first-strand sequencing and the process is repeated for the opposite strand.

2.13.3 Paired –End Sequencing

Paired –End Sequencing involves sequencing both ends of DNA fragments in a sequencing library and aligning the forward and reverse reads as read pairs (figure 2.4) (Grada & Weinbrecht, 2013). It generates high-quality, alignable sequence data. In addition to producing twice the number of reads for the same time and effort in library preparation, sequences aligned as read pairs enable more accurate read alignment and the ability to detect indels which is not possible with single–read data. Paired-end sequencing facilitates detection of genomic rearrangements and repetitive sequence elements, as well as gene fusions and novel transcripts (Barzon *et al.*, 2011)

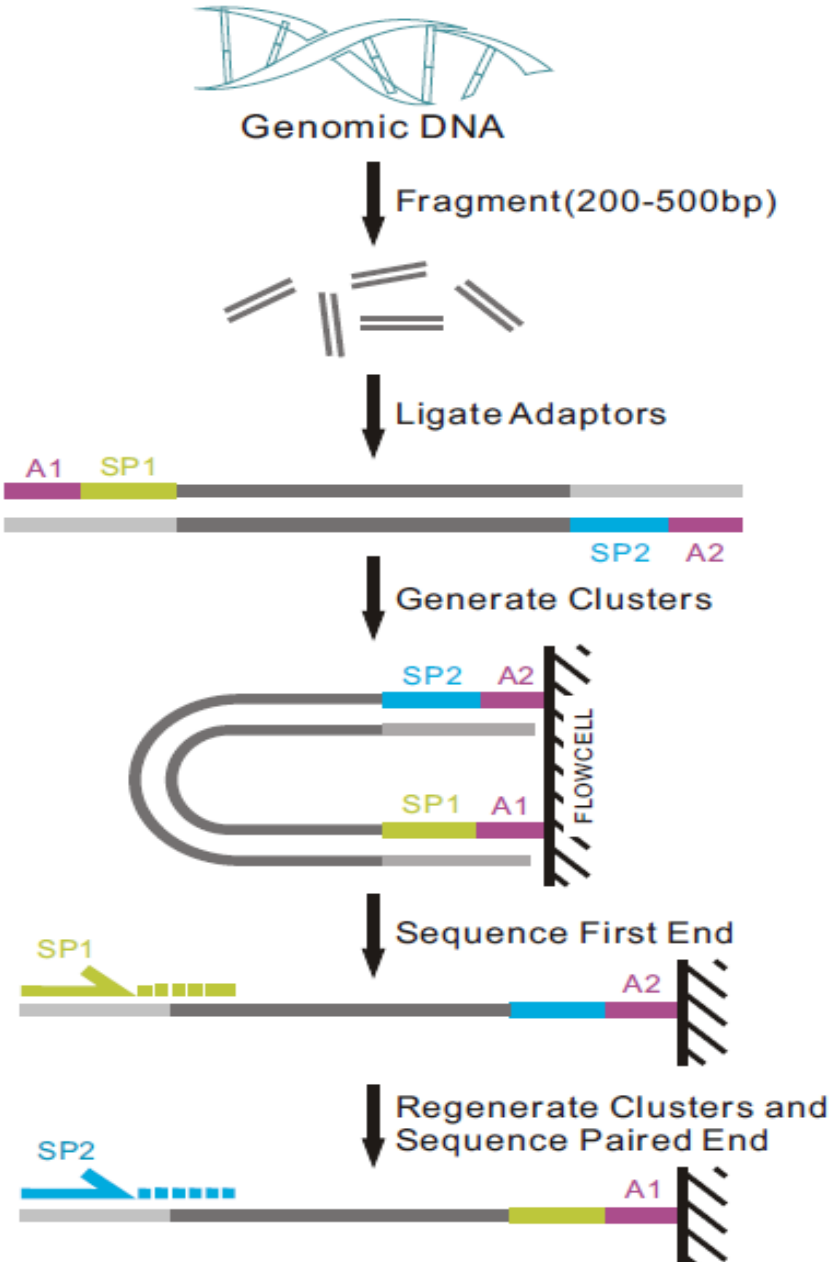

Figure 0.4: Pipeline of Paired-end Sequencing (www.illumina.com).

Genomic DNA are extracted and then fragmented randomly. After electrophoresis, DNA fragments of desired length are gel-purified. Adapter ligation and DNA cluster preparation are performed and subjected to Illumina GA paired-end sequencing. In addition to the sequence information, paired-end strategy allows for the identification of large structural variations and reduces the influence of repeat sequences in assembly.

CHAPTER THREE

MATERIALS AND METHODS.

3.1 Experimental site

This study was done at the Biosciences East and Central Africa (BecA) – ILRI Hub. All the work was done at the BecA laboratories.

3.2 Experimental designs

This was a cross-sectional study covering a period of two years 2013/2014 and 2014/2015. Due to lack of current information on the prevalence of Capripoxvirus, the sample size was determined based on the average samples submitted for Capripox diagnosis on a yearly basis for the last 5 years at the Veterinary Laboratories. The average sample submitted per year from sheep, goats and cattle was determined to be 50 and therefore a total of 100 samples that is, skin scrapings, tissues and blood were targeted in the study. In the course of the study, there were disease outbreaks in Western Kenya, North and West Pokot and 26 samples were collected and these outbreak samples contributed significantly to the study.

3.3 Samples and sampling

A total of 130 samples from different parts of the country (figure 5) were therefore analyzed in this study. These included blood, skin scrapings and skin nodules (tissues) that were already available at the Central Veterinary Laboratories (CVL) – Kabete and some that were collected in the course of the study mostly in reported cases of outbreaks or brought to the Laboratories for diagnosis. The samples also included pure cell cultures from Kenya Agriculture and Livestock Research Institute (KALRO); goatpox virus obtained from goat, sheeppox virus from sheep and lumpy skin disease virus that was obtained from cattle. CVL is the national and referral livestock diagnostic laboratory in Kenya and the available samples included skin scrapings, skin nodules and blood of cattle, goats and sheep from different parts of the country sampled whenever a case is suspected

from symptomatic animals. There has been no Capripoxvirus diagnosis going on at the Laboratories due to challenges of cross reactivity with other viruses and therefore no diagnosis in the entire country. These samples were a representation from different parts of the country, mostly in areas where livestock farming is active (table 3.1).

A total of 32 tissues (skin scrapings and nodules), 94 blood sample, 3 pure cultures and 1 attenuated vaccine (LUMPIVAX) from KEVEVAPI were analyzed in this study. Details of all the 130 samples are shown in appendix 1. The blood samples were either collected in EDTA tubes or using Whatman FTA (Flinders Technology Associates) cards in the course of the study. Samples were mainly from West and North Pokot, Nyanza, Western, Rift valley, Central, Eastern and North Eastern regions. There were no samples from the coastal areas either previously submitted or collected in the course of the study. Figure 3.1 and table 3.1 shows the different areas where the samples were obtained and the number of samples.

Table0.1: Number of samples analyzed per region (Place of origin)

Region/Counties/Institution	Number of samples Analyzed
Nairobi	37
Homabay	3
Kisumu	3
Siaya	1
Kakamega	2
Bungoma	1
Malaba	2
Nakuru	1
Narok	1
Baringo	8
Kajiado	3
West Pokot	39
Kiambu	1
Nyeri	3
Kitui	4
Meru	2
Isiolo	12
Marsabit	1
Garisa	1
Wajir	1
KALRO	3
KEVEVAPI	1
Total	**130**

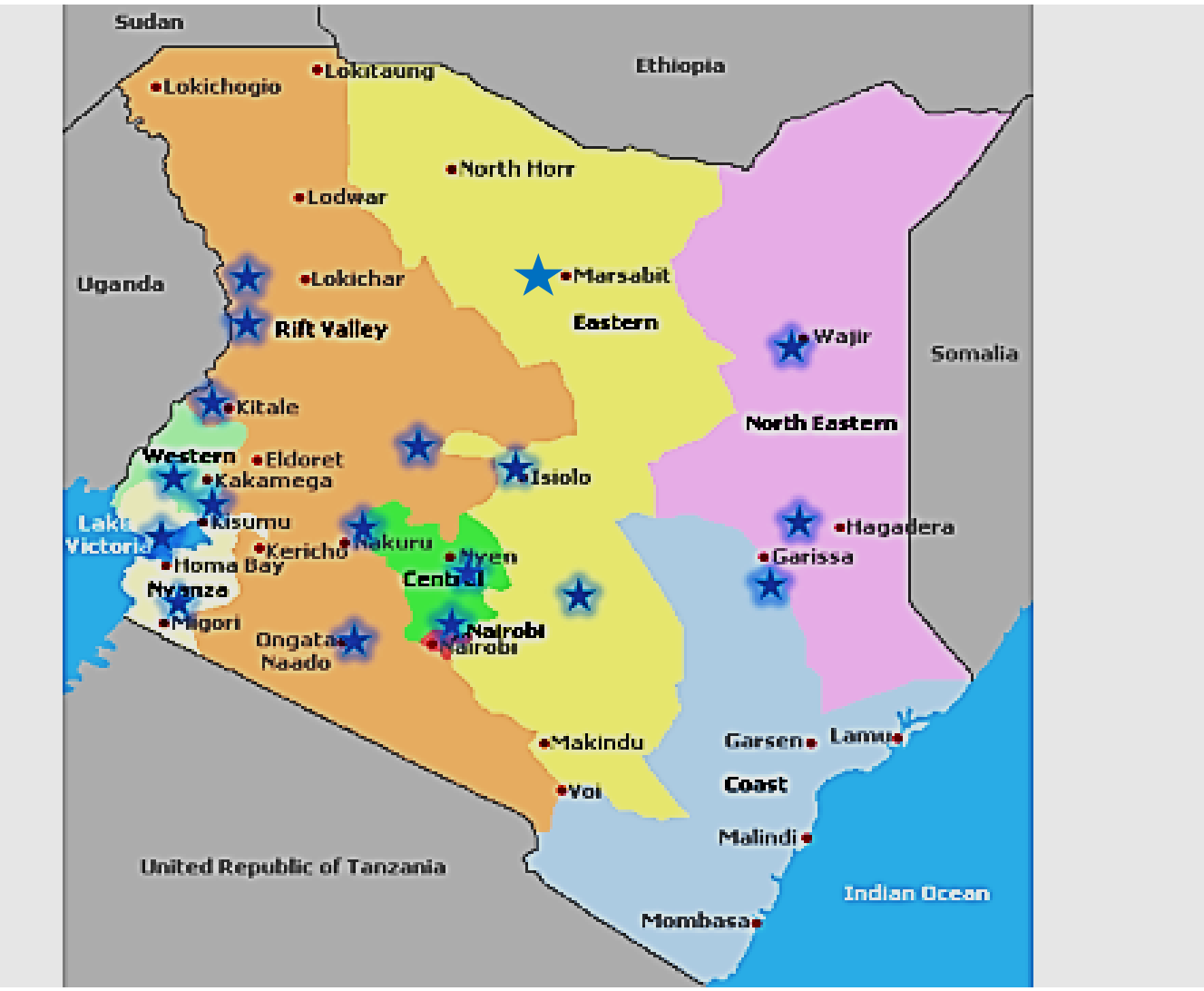

Figure 0.1: Regions where samples were obtained.

3.4 Research Clearance

Clearance to conduct this study was obtained from ILRI Health and Safety Committee and also the DVS - Central Veterinary Laboratories (CVL). The samples used were from symptomatic animals, and Capripox virus being non zoonotic, there were no major challenges and all the biosafety and biosecurity issues were addressed accordingly.

3.5 Methods

3.5.1 Cell Culture

Tissue samples for viral isolation were homogenized in Glasgow Minimum Essential Medium (GMEM) with antibiotics making up to a 50% solution. The homogenized sample were then passed through a sonication procedure to lyse the cell and tissues to release the virus. The samples were centrifuged at 2000 xg for 15 minutes and the supernatant collected and filtered through 0.45µm membrane filter and preserved at -80ºC until use.

Capripoxvirus were grown in lamb testis (LT) cells. 1 ml of buffy coat cell suspension or 1 ml of clarified biopsy preparation supernatant was inoculated on to a 25 cm^2 tissue culture flask to 90% confluent LT, and allowed to adsorb for 1 hour at 37°C. The culture was then washed with warm PBS and covered with 10 ml of a suitable medium that is GMEM, containing antibiotics and 2% fetal calf serum.

The flasks were examined daily for up to 14 days for evidence of cytopathic effect (CPE), and the medium replaced if it appeared to be cloudy. If there was no apparent CPE by day 7, the culture would be freeze–thawed three times (-20°C - 21°C), and clarified supernatant inoculated on to fresh LT culture. Eosinophilic intracytoplasmic inclusion bodies, which are variable in size but are up to half the size of the nucleus and surrounded by a clear halo, are indicative of poxviruses.

At 24 hour post incubation the flask was passed through two regular freeze and thaw processes, and 2ml inoculum harvested, filtered through a syringe filter and again inoculated into next flasks. The medium would be changed after every 48 hours.

3.5.2 DNA Extraction

DNA extraction was done using PureLink® Genomic DNA (Invitrogen) Kit according to the Manufacturer's instructions. This kit was used for all the tissues, blood and cell culture samples.

The Lumpivax vaccine was extracted the same way as cell cultures because it is a live attenuated vaccine

3.5.2.1 DNA Extraction from Tissues

The tissue samples were cut into small pieces with a sterile scalpel or frozen in liquid nitrogen and grind to a fine powder with a mortar and pestle. Lysis Buffer of about 200µl was added to the homogenized sample from step 1 in a 1.5 ml- or 2 ml Eppendorf tubes and then the buffer and sample mixed thoroughly by inverting the reaction vessel several times. The mixture was made as homogeneous as possible.

Approximately, 20 µl of proteinase K (20 mg/ml) was added to the mixture, mixed thoroughly by inverting the tube several times and incubated for at least 4 hours at 55°C with occasional vortexing (if the tissue did not dissolve readily, this incubation was extended overnight at 56°C). During the incubation, the sample was mixed several times thoroughly by inverting to get the tissue material dissolved properly. After the proteinase K digests, the mixture appeared homogenous. RNase (DNase-free, 20 mg/ml), 20µl was then added to the cleared lysate and incubated for a further 5 minutes at 37ºC, 200 µl of lysis/binding Buffer was added and then mixed well by vortexing to obtain a homogenous solution before incubating at 55ºC for 10 minutes to promote protein digestion. After, incubation 200µl of 96-100% ethanol was then added to the lysate and mixed well by vortexing for 5 seconds to yield a homogenous solution. The lysate was then added to the

spin column and the column centrifuge at 1000xg for 1 minute at room temperature, and the collection tube discarded after spinning and the spin column placed into a clean collection tube. The DNA was then washed by adding 500µl of wash buffer prepared with ethanol to the column and the column centrifuged at room temperature at 10,000 xg for 1 minute, the collection tube discarded and then the spin column placed in a clean collection tube. For the second wash, 500µl of wash buffer 2 prepared with ethanol was added to the column and this was centrifuged at a maximum speed for 3 minutes at room temperature, then the collection tube discarded. The column was then placed in a clean collection tube and centrifuged again for 2 minutes to remove any remaining alcohol which could interfere with the downstream applications of the DNA.

The DNA was eluted with warm 20µl genomic elution buffer by placing the spin column in a sterile 1.5 ml microcentrifuge tube and then adding the buffer. This was incubated at room temperature for at least 30 minutes before centrifuging the column at maximum speed for 1 minute at room temperature. The eluted DNA was stored at -20ºC awaiting downstream applications.

3.5.2.2 DNA extraction from blood samples

DNA was extracted from the blood samples using the PureLink® Genomic DNA (Invitrogen) kit. For whole blood, 200µl of blood was put in a sterile microcentrifuge tube and 20µl of proteinase K and RNase added to the sample, mixed well by vortexing and incubated at room temperature for 2 minutes. Subsequently 200µl of the genomic lysis/binding buffer was added and mixed well by vortexing to obtain a homogenous solution. This was then incubated at 55ºC to promote protein digestion. After incubation, 200µl of 96-100% ethanol was added to the lysate, mixed well by vortexing for 5 seconds to yield a homogenous solution. The lysate was added to the PureLink® Spin column in a collection tube and centrifuged at 10,000 xg for 1 minute at room temperature to bind the DNA. The collection tube was discarded and the spin column placed in a clean collection tube before washing the DNA with 500µl of wash buffer 1 and 2 prepared with ethanol. The DNA was then eluted with 30µl Genomic elution buffer and stored at -20ºC.

The dried blood spots on the FTA cards were punched (5-10 punches) into a sterile microcentrifuge tube and 180µl of Genomic digestion buffer and 20µl of Proteinase K. This was mixed by vortexing to ensure that all the pieces were completely immersed in the buffer. This was then incubated at 55ºC with occasional vortexing for 30 minutes to enhance protein digestion. The sample was then centrifuged at maximum speed for 2-3 minutes at room temperature to pellet paper fibers before transferring to a clean microcentrifuge tube. After this, extraction proceeded as for EDTA blood.

3.5.2.3 DNA extraction from Cell Cultures

The Lamb Testis cell cultures were harvested by transferring the cells from the tissue culture flask into tubes and then centrifuging the cells at 250 xg for 5 minutes to pellet the cells and then the medium removed. The cells were then resuspended in 200µl PBS and 20µl of Proteinase K and RNase added to the sample, mixed well by vortexing and incubated at room temperature for 2 minutes. Then 200µl of genomic lysis/binding buffer was added, mixed well by vortexing to obtain a homogenous solution. Then DNA was bonded as in the above steps and eluted in 30µl warm elution buffer as per the PureLink® Genomic DNA kits (Invitrogen) instructions.

3.5.3 Determination of Quality and Quantity of extracted DNA

The quality and quantity of DNA was determined using a NanoDrop® ND-1000. The pedestal was cleaned using soft Kimwipe and the instrument blanked with water to initialize it. 1µl of the extracted DNA was placed on the pedestal and absorbance readings performed at 260nm (A_{260}) where DNA absorbs light most strongly. Cleaning was done between samples. The value generated is the DNA concentration per µl of the sample. Total DNA yield was obtained by multiplying the DNA concentration by the final total purified sample volume.

DNA yield (µg) = DNA concentration × total sample volume (ml)

To evaluate DNA purity, the ratio of the absorbance at 260nm was divided by the reading at 280nm. Good-quality DNA has an A260/A280 ratio of 1.7–2.0. A reading of 1.6 does not render the DNA unsuitable for any application, but lower ratios indicate that more contaminants are present. The ratio was therefore calculated after correcting for turbidity (absorbance at 320nm).

DNA purity (A260/A280) = (A260 reading – A320 reading) ÷ (A280 reading – A320 reading)

3.5.4 Agarose Gel Electrophoresis of Extracted DNA

The concentration and yield of DNA can also be estimated using an agarose gel electrophoresis. The extracted DNA from cell cultures, tissues and blood were also analyzed by agarose gel electrophoresis to determine the concentration and quality. This was done by weighing 0.8g agarose powder and then adding to 100ml of 0.5X TBE electrophoresis buffer, then heated in a microwave oven until completely melted. A volume of 2.5µl gel red was added to the gel (final concentration 0.5µg/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 55ºC, it was then poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel had solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and just covered with buffer. Samples containing DNA mixed with loading dye were then pipetted into the sample wells, the lid and power leads were placed on the apparatus, and a current of 100 volts applied. This was left to run for 45 minutes with continuous monitoring. The flow of the current was confirmed by observing bubbles coming off the electrodes. DNA migrates towards the positive electrode. The distance DNA moved was judged by visually monitoring migration of the tracking dyes. After 45 minutes, the gel was visualized using an ultraviolet transilluminator and gel photos taken.

The DNA concentration and yield was determined by comparing the sample DNA intensity to that of a DNA quantified standard.

3.5.5 Detection of Capripoxvirus using Molecular based methods

3.5.5.1 Conventional Polymerase Chain reactions

Conventional PCR was performed for all the 130 DNA samples extracted. The primers F3 and B3 designed to target the conserved poly (A) polymerase small-subunit gene (ORF068) of the CaPVs genome were used (Das *et al.,* 2012). The primer sequence are as shown below.

Forward primer F3: TGGTAAGTATATTAAAACCAGCAG,

Backward primer B3: GAATCATCCTTTGTGATGCA,

The PCR was carried out with 20µl Bioneer tubes with a dye using ABI 9700 Thermocycler (Applied Biosystems). The reaction master mix consisted of 10µM (each) forward primer (F3) and reverse primer (B3), 25mM Magnesium chloride, 2µl, approximately 10ng/µl template DNA plus the required amount of nuclease-free water to adjust the volume to 20µl. Amplification was carried out under these cycling conditions; initial denaturation at 95ºC for 3 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 65ºC for 30 seconds, elongation at 72ºc for 30 seconds and the final extension at 72ºC for 7 minutes. All the conventional PCR assays were performed in duplicates. The PCR products were subjected to 2% agarose gel electrophoresis.

3.5.5.2 Real time Polymerase Chain Reaction (qPCR)

A highly sensitive quantitative Capripox TaqMan Probe Hybridization assay used for the detection of CaPVs was employed as the gold standard method for validating the LAMP assay in this study. The primers and probes were designed to target the CaPVs conserved poly (A) polymerase small-subunit gene (ORF068), but a different region from that of

conventional PCR and LAMP primers. The sequences of the primers and probes used for real time PCR were as follows:

Forward Primer: 5'-GGCGATGTCCATTCCCTG-3'

Reverse primer: 5'-AGCATTTCATTTCCGTGAGGA-3'

Probe: 5'-6FAM –CAATGGGTAAAAGATTTCTA –MGBNFQ-3'

All the 130 extracted DNA were subjected to real time PCR without normalizing the DNA concentration but using the same quantity of DNA as normalization is not very effective in diagnosis. The reaction master mix consisted of 25µM FAM /MGB probe; 90µM (each) forward and reverse primers, 5µl approximately 10ng/µl of the template DNA, Fast Start Universal Probe master (ROX) Ref.04913957001 and 6.5µl of nuclease-free water to adjust the volume to 25µl. Amplification reactions were performed on an Applied Biosystems 7900HT real time PCR system using a 2 step PCR cycling profile of 95ºC for 5 min and 40 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. All qPCR assays were performed in duplicates except for the sensitivity test that was performed in triplicates. The real time PCR was a 40 cycle reaction with the CT value set at 37.

3.5.5.3 Loop Mediated Isothermal Amplification

The extracted DNA of the 130 samples were subjected to the LAMP assay using VP39 primers derived from Poly (A) polymerase small subunit (Das *et al.,* 2012). The test was run at 60ºC for I hour using the Optigene LAMP machine and the results obtained compared with that of the conventional and real time PCR. This is a real time LAMP reaction. The primer sequence used were as follows:

Forward primer F3: TGGTAAGTATATTAAAACCAGCAG,

Backward primer B3: GAATCATCCTTTGTGATGCA,

Forward inner primer FIP:
TTCATTTCCGTGAGGAATATAGAAAATCTAGTTTAAAATGGCGATG. .

Backward inner primer BIP:
TTCAACCATTTGCGCCTAAAGCTTTATAGGATTACCGCTA.

The final working primer mix for each reaction consisted of 0.2µM (each) F3 and B3, 2µM (each) FIP and BIP and 7.5 µl of Optigene master mix prepared according to Manufacturer's instruction. The final reaction volume used for the assay was 12.5µl including 10µl master mix and 2.5µl of the template DNA in LAMP assay Optigene tubes and incubated at 65ºc for 1 hour in the genie II LAMP reader machine.. The assay was performed in duplicates except for the sensitivity test done in triplicate and monitored in real time by the development of amplification curves.

3.6 Determination of Analytical Sensitivity, Specificity and Limit of Detection

Analytical Sensitivity, Specificity, and Limit of Detection (LOD) was done using a purified concentrated CaPVs DNA. The concentrated Capripoxvirus DNA of 50 ng/µl was serially diluted and the different dilutions subjected both to real time PCR and LAMP Assay to determine the analytical sensitivity. The serial dilutions were also tested in triplicates both by qPCR and LAMP assay as earlier described in section 3.5.5.2 for qPCR and 3.5.5.3 for LAMP assay, to determine the limit of detection (LOD). The analytical specificity was also determined by testing the CaPVs DNA alongside other closely related and "clinical look-a-like" viruses like the orf virus, Camel pox virus, and PPR virus DNA. The PPR viral RNA was reverse transcribed into complementary DNA (cDNA) prior to use as the template for LAMP. These viruses are antigenically related to CaPVs and they cross react when tested using serological tests like ELISA.

3.7 Next Generation Sequencing (NGS) of Capripoxvirus

After analyzing all the 130 samples, 18 LAMP positive samples were selected for deep sequencing. These included 14 samples representing different regions of the country, the 3 CaPVs pure cell cultures and the Lumpivax vaccine as shown in table 4.11, page 63. The sequenced samples were from Kakamega, Kajiado, Meru, Nairobi, Kitui, Bungoma, Kisumu, Homabay, Garisa, Siaya, Baringo, West Pokot and Isiolo. The LUMPIVAX vaccine is a Lumpy skin disease (LSD) vaccine produced by the Kenya Veterinary Vaccine Production Institute (KEVEVAPI). It is an attenuated vaccine. The 3 pure cultures obtained from KALRO were SPV, GPV and LSDV obtained from goats, sheep and cattle respectively. Nextera XT libraries were prepared as per the Illumina Nextera XT Library preparation protocol using extracted DNA. The Nextera XT DNA Library Preparation allows for the preparation of sequencing-ready libraries for small genomes (bacteria, archaea, viruses). The Nextera DNA Library Prep Kit uses an engineered transposome to tagment genomic DNA, which is a process that fragments DNA and then tags the DNA with adapter sequences in a single step. A limited-cycle PCR step uses the adapters to amplify the insert DNA. The PCR step adds index adapter sequences on both ends of the DNA, which enables dual-indexed sequencing of pooled libraries on Illumina sequencing platforms as illustrated in (figure 3.2).

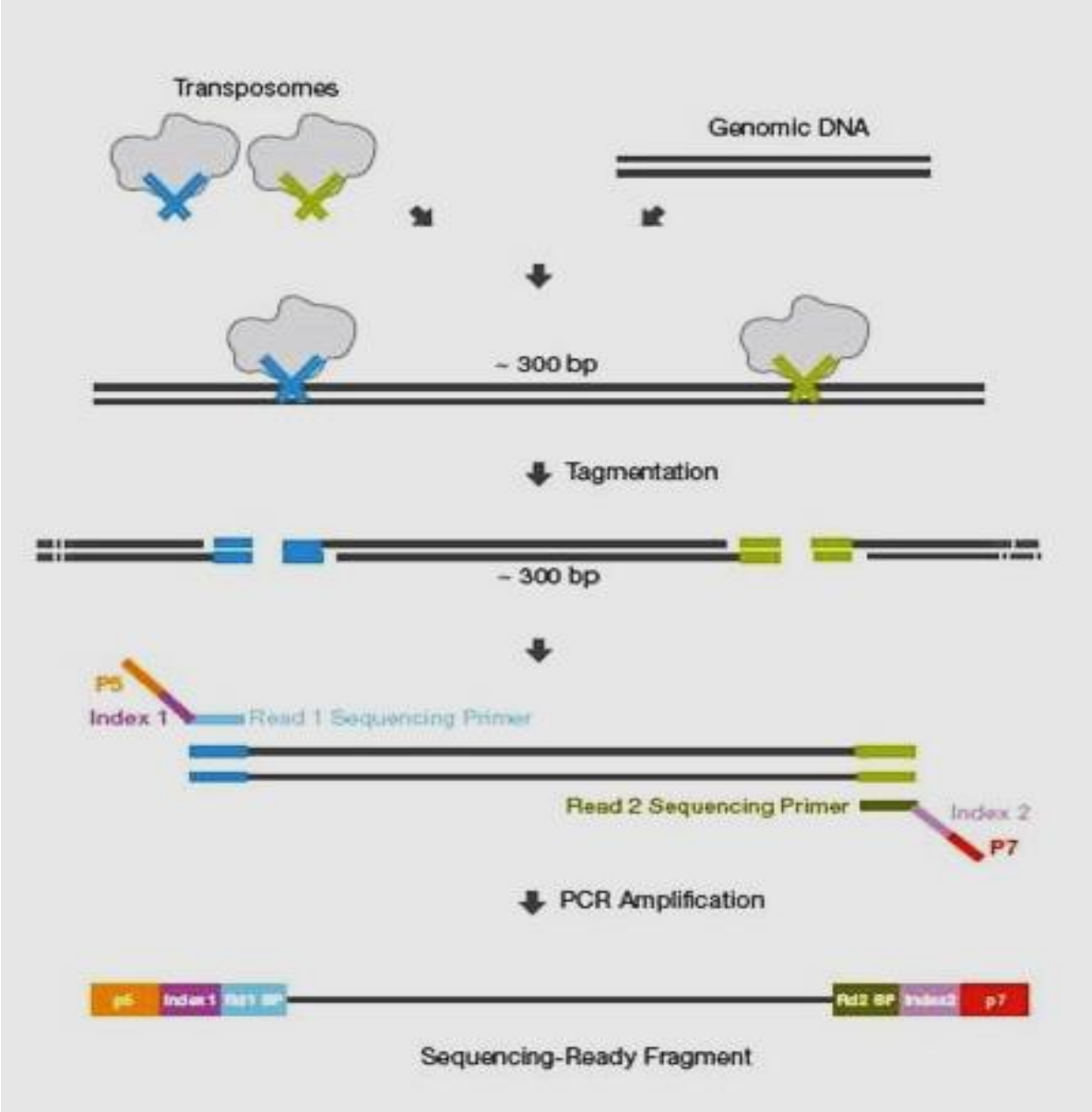

Figure 0.2: Diagrammatic representation of Nextera –XT Library processing (Rizzo & Buck, 2012)

Nextera chemistry simultaneously fragments and tags DNA in single step. The transposomes cuts the DNA to around 300bp long fragments and then tags it with adapter sequences. A simple PCR amplification then appends sequence adapters and sample indices to each fragment.

3.7.1 Genomic Libraries (Nextera XT (FC-131-1024) Libraries) Preparation

3.7.1.1 Input DNA Quantification

DNA extraction was done as described in section 3.5.2 for tissues, blood and cell cultures. The starting DNA material was quantified using Qubit which is a fluorometric based method specific for duplex DNA. Qubit solution was prepared by adding 1µl of dsDNA Qubit broad range reagent to 199µl of dsDNA Qubit broad range buffer, then dispensing 200µl of the Qubit solution to the Qubit tubes and then adding 2µl of the DNA sample. The dsDNA BR standards were used to calibrate the Qubit machine. After determining the concentration, DNA was diluted to 0.2ng/µl. and used as the input DNA for the Nextera XT libraries.

3.7.1.2 Tagmentation of Genomic DNA

During this step, the genomic DNA is tagmented (tagged and fragmented) by the Nextera transposome. The transposome simultaneously fragments the genomic DNA and adds adapter sequences to the ends, allowing amplification by PCR in subsequent steps.

The reagents used at this stage were mixed by inverting the tubes 3-5 times followed by a brief spin in a micro centrifuge, 10µl of tagment DNA (TD) buffer, 5µl of the input DNA at 0.2ng/µl (1ng total) and 5µl of Tagment DNA Enzyme (TDE1) was added to each labeled PCR tube and pipetted up and down 5 times to mix. This was then centrifuged at 280xg at 20ºC for 1 minute before running PCR at 55ºC for 5 minutes and then held at 10ºC.

3.7.1.3 Clean-Up of Tagmented DNA

This step was to purify the tagmented DNA from the Nextera transposome. Clean-up is very important because Nextera transposome can bind tightly to DNA ends, therefore, interfering with downstream processes if not removed.

The obtained tagmented amplicons were neutralized by adding 5µl of neutralize tagment (NT) buffer to each tube containing Nextera XT tagment Amplicon and mixed by pipetting up and down 5 times before centrifuging at 280xg at 20ºC for 1 minute. The tubes were then incubated at room temperature for 5 minutes.

3.7.1.4 PCR amplification of the tagmented DNA

The purified tagmented DNA was amplified via a limited-cycle PCR program. This steps adds indexes [index 1 (i7)] and [index 2 (i5)] as well as common adapters (P5 and P7) required for cluster generation and sequencing.

In this stage Nextera PCR Master Mix (NPM) and index primers: (Index 1- N7XX) & Index 2 – (S5XX) were used. The NPM and Index primers were thawed at room temperature for around 20 minutes and then inverted 3-5 times to mix then briefly centrifuged. Unique indexes were used for each sample. 15µl of NPM, 5µl of Index 1 and 2 respectively were added to each tube, then mixed by pipetting up and down 3-5 times to mix, centrifuged at 280xg at 20ºC for 1 minute before performing PCR. Amplifications were carried out using ABI 9700 Thermocycler with 2 Pre PCR stages at 72ºc for 3 minutes, 95ºc for 30 seconds and then 12 cycles of amplification with each cycle of heat denaturation step (95ºc for 10 seconds), an annealing step (55ºc for 10 seconds), and an elongation step (72ºc for 10 seconds). The final amplification was done at 72ºC for 5 minutes.

3.7.1.5 PCR Clean-up of the generated Library DNA

PCR clean-up was done to purify library DNA using AMPure XP beads. This was to provide a size selection step that removes very short library fragments from the population. It was done using Resuspension buffer (RSB), Ampure XP beads and freshly prepared 80% ethanol. After the PCR, the tubes were centrifuged at 280xg for 1 minute at 20ºC to collect condensation and then the product transferred to the newly labeled tubes. The Ampure beads were vortexed for 30 seconds to mix and then 30µl added to each tube, mixed by pipetting up and down 10 times and then incubated at room temperature without

shaking for 5 minutes. After incubation, the tubes were placed on a magnetic stand for 2 minutes, and then the supernatant removed and discarded with the tubes still on the magnetic stand. The beads were then washed with the freshly prepared 80% ethanol twice by adding 200µl and ensuring that the beads are not resuspended, incubated for 30 seconds after which the supernatant was carefully removed and discarded and the tubes left to air dry. After drying, the tubes were removed from the magnetic stand and 52.5µl of RSB added to each tube, gently pipette mixed up and down 10 times, incubated at room temperature for 2 minutes and then the tubes placed on the magnetic stand for 2 minutes to clear the supernatant after which 50µl (cleaned PCR product) was transferred to clean labeled tubes and stored at -20ºC.

3.7.1.6 Library normalization:

All the reagents used at this stage were thawed to room temperature. 20µl of the cleaned PCR product was transferred to a clean 1.5 ml labeled eppendorf tube and then 45µl of a mixture of library normalization additives 1 (LNA1) and Library normalization beads 1 (LNB1), in the ratio 5.5:1, was added. The tubes were shaken at 1800 xg for 30 minutes and then placed on a magnetic stand for 2 minutes to clear the supernatant. After clearing, the supernatant was carefully removed and discarded, the tubes removed from the magnetic stand and the beads washed with Library Normalization wash 1 (LNW1), by adding 45µl to each tube and then shaken at 1800 xg for 5 minutes. The tubes were then placed on a magnetic stand for 2 minutes and the cleared supernatant removed and discarded. This wash was repeated, and then the tubes removed from the magnetic stand and 30µl of 0.1N Sodium hydroxide added to each well to elute the samples. The tubes were then shaken at 1800 xg to ensure complete elution and then the tubes placed on the magnetic stand for 2 minutes to clear the supernatant. 30µl of the cleared supernatant was transferred to a clean labeled tube containing 30µl of Library normalization storage buffer 1 (LNS1), totaling up to 60µl and then centrifuged at 100 xg for 1 minute.

3.7.1.7 Quality control of the generated Nextera XT libraries:

The quality of the generated libraries was checked using real time PCR. Illumina Nextera PCR cocktail primer containing both the reverse and forward primer (1µl) and Free start universal SYBR green master mix (12.5µl) was used with 1µl of the prepared library. Amplification was done in 2 steps, step 1 (95ºC for 600 seconds) and 20 cycles of step 2 at 95ºC for 10minutes and 60ºC for 45 seconds using the LightCycler® Thermocycler.

The size distribution of some of the prepared libraries were checked by running 1µl of 1:3 diluted library on an Agilent Technologies 2100 Bioanalyzer using a high sensitivity DNA chip. Typical libraries shows a broad size distribution from ~250bp to 1000bp.

3.7.2 Genomic Library sequencing

Library pooling and Miseq Sample Loading: The tubes containing the library were centrifuged at 1000xg for 1 minute at 20ºC to collect condensation, and then each library to be sequenced depending on the outcome of the quality control was mixed up and down 3-5 times. 5µl of each library was transferred to a clean 1.5ml eppendorf tube labeled PAL- Pooled Amplicon Library then mixed well. 30µl of PAL was added to 570µl of Hybridization buffer (HT1) in a tube labeled Diluted Amplicon Library (DAL). This was mixed by pipetting up and down 3-5 times using the same tip to ensure complete transfer. The tube labeled DAL was then mixed by vortexing at top speed, incubated at 96ºC for 2 minutes, and then inverted 2 times to mix before placing in an ice water bath for 5 minutes. After incubation, the tube containing approximately 600µl of diluted Amplicon library was loaded into a thawed Miseq reagent cartridge into the load sample reservoir and then sequenced, following the Miseq System User Guide.

3.8 Data Analysis

3.8.1 Test Validation (Sensitivity and Specificity Analysis)

The diagnostic sensitivity and specificity analysis of LAMP and PCR was done using two by two (2x2) contingency tables. The information obtained by comparing the new diagnostic test with the gold standard (qPCR) was conventionally summarized in a two-by-two table, that is, the number of positives and negative samples per each test as shown in table 2 below.

Table 0.2: A 2x2 contingency table

	Gold Standard Disease Present	**Gold Standard Disease Absent**	
Test Positive	True positives (TP) a	False positives (FP) b	Total Test positives a+b
Test Negative	False negatives (FN) c	True negatives (TN) d	True test negatives c+d
	Total diseased a+c	Total normal b+d	Total population a+b+c+d

Cell ′a′ contains those in whom the test in question correctly diagnosed the disease (as determined by the gold standard). In other words, the test is positive, as is the gold standard. These are the true positives (TP).

Cell ′b′ contains those who have positive results for the test in question but do not have disease according to the ′gold standard test′. The newer test has wrongly diagnosed the disease: These are false positives (FP).

Cell ′c′ contains those who have disease on the ′gold standard test′ but have negative results with the test in question. The test has wrongly labeled a diseased person as ′normal′. These are false negatives (FN).

Cell ′d′ contains those who have no disease as determined by the ′gold standard test′ and are also negative with the newer test. These are true negatives (TN).

Sensitivity which is the ability of a test to correctly classify an individual as ′diseased was calculated as follows:

Sensitivity = a / a+c

= a (true positive) / a+c (true positive + false negative)

= Probability of being test positive when disease present.

Specificity = d / b+d

= d (true negative) / d+b (true negative + false positive)

= Probability of being test negative when disease absent.

Positive predictive value (PPV): a/ (a + b) × 100

= a (true positive) / a+b (true positive +false positive)

=The probability that the subject really has the disease

Negative Predictive Value (NPV): d/ (d + c) × 100

= d (true negative) / d+b (true negative + false negative)

=The probability that the subject really does not have the disease

The LAMP assay analytical specificity and sensitivity was determined from a purified extracted viral DNA as the template.

The analytical sensitivity or the Limit of Detection (LOD) of CaPVs by LAMP was determined using serial dilutions of the DNA extracted from pure cell culture-grown viruses. For performance evaluation the analytical sensitivity was also determined by qPCR. The serially diluted purified DNA was used as the template DNA for both qPCR and LAMP assay.

The comparison of the test methods were done statistically using Fisher's Exact Test at 95% confidence level.

3.8.2 Next Generation Sequencing data analysis.

After sequencing, the data was imported from the Illumina Miseq NGS machine to the using CLC main workbench platform at BecA-ILRI HPC. The obtained data was analyzed by first performing the quality control using FastQc and then the sequences trimmed by SolexaQA, Scythe and Fastx tool kit trimmer to remove poor quality sequences on both ends. Trimming was based on the quality score of 30. The two paired reads of each sequenced sample that is the forward (R1) and reverse (R2) were assembled after trimming using St. Petersburg genome Assembler (SPAdes) program with High Performance Computing (HPC). Many single contigs of different lengths were obtained per sample. A contig is an overlapping sequence data (reads) or clones that form a physical map of the genome used to guide sequencing assembly. The contigs were used for the downstream analysis.

The nucleotide sequence of the reference genomes of GPV: NC_004003, SPV: NC_004002 and LSDV: NC_003027 and other Capripoxvirus strains isolated from different countries were obtained from the NCBI Gene Bank database.

Reference mapping of the obtained sequences was done by mapping each sequence to the reference genome. The genomes were first indexed using ‘bwa’ program before mapping.

Multiple sequence alignment (MSA) of these sequences was performed by MEGA 6 (version 6.0) with ClustalW method and also CLC main workbench 7.1. This was based on RP030, P32 and GPCR genes. The nucleotide sequences of these genes were extracted from the reference genomes and used as references in these analysis as were those of the sequenced samples. The nucleotide sequences were then converted to amino acid sequences and aligned together to determine the presence of specific signatures present in these genes that are used to differentiate the CaPVs.

Phylogenetic analysis was done using MEGA 6 with Neighbor-Joining (NJ), Maximum Parsimony (MP) and Minimum Evolution (ME) and the different trees obtained compared. Reliability of the phylogenetic trees was tested by bootstrap analysis with 1000 replicates. This analysis was based on the RP030, P32 and GPCR genes.

Blast analysis was done using CLC genomic workbench. The obtained contigs were blasted against the NCBI virus database.

CHAPTER FOUR

RESULTS

4.1 Quality of the DNA Extracted

The quality of DNA extracted from the 130 samples were of varied quality based both on electrophoresis and spectrophotometric analysis. This is based on the fact that a good-quality DNA has an A260/A280 ratio of 1.7–2.0 and 80% of the 130 samples analyzed had that ratio, 20% of the samples had a ratio of less than 1.6 (Table 4.1). In electrophoresis, some samples had high concentration of DNA restricting its movement in the gel, yet others were low, and those with possible contamination had some smearing (Figure 4.1) below. Most of the samples showed intact band and all were used for upstream application as primers used for PCR are very specific and there would be no interference

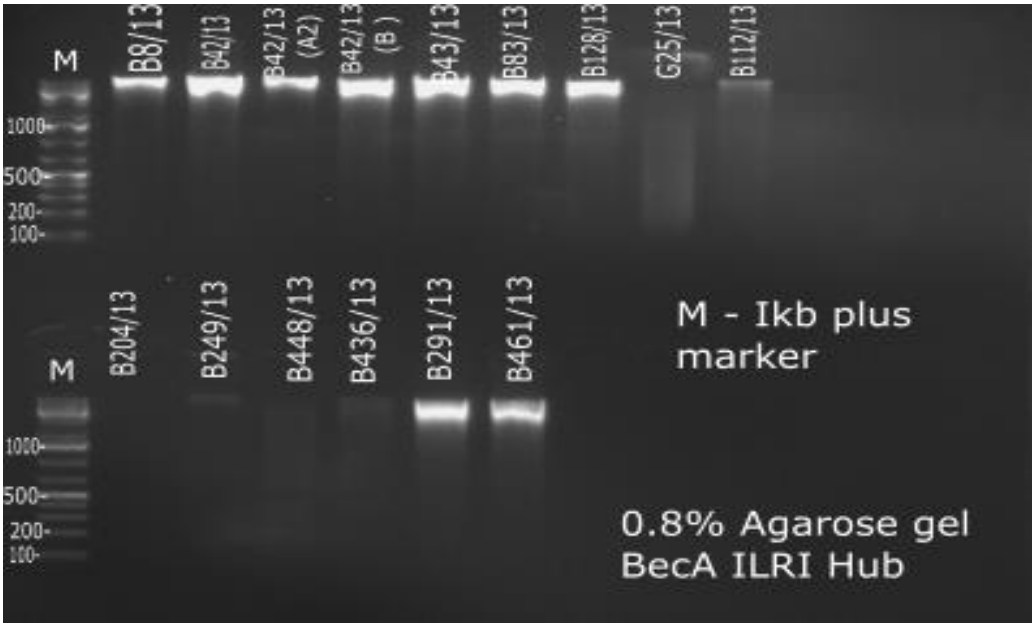

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Figure 4.1: 0.8% Agarose gel of some of the extracted DNA

Samples in lane 1 -7, 11, 14 and 15 had intact unsheared genomic DNA forming a thick band of more than 1kb. Samples in lane 8 and 9 had smear though still indicate the presence of DNA. The lanes labelled M, are 1 kb plus ladder used for genomic DNA as it is always more than 1kb in size.

Table 4.1: A260/280 ratios

Sample ID	User name	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	S. Type	Factor
B8/13	Administrator	139.9	ng/µl	0.797	0.474	1.68	0.72	DNA	50
B42/13	Administrator	331.2	ng/µl	6.624	3.623	1.83	1.69	DNA	50
B42/13 (A2)	Administrator	374.9	ng/µl	1.498	0.872	1.72	0.84	DNA	50
B42/13(B)	Administrator	239	ng/µl	0.78	0.442	1.76	1.16	DNA	50
B43/13	Administrator	297.4	ng/µl	0.548	0.327	1.68	0.62	DNA	50
B83/13	Administrator	298.6	ng/µl	1.971	1.077	1.83	1.36	DNA	50
B128/13	Administrator	334.6	ng/µl	0.693	0.423	1.64	0.58	DNA	50
G25/13	Administrator	20.1	ng/µl	0.402	0.223	1.81	1.08	DNA	50
B112/13	Administrator	24.1	ng/µl	0.283	0.198	1.43	0.43	DNA	50
B204/13	Administrator	14.8	ng/µl	46.1	25.703	1.79	1.9	DNA	50
B249/13	Administrator	52.6	ng/µl	11.05	6.17	1.79	1.83	DNA	50
B448/13	Administrator	32.4	ng/µl	0.648	0.334	1.94	1.68	DNA	50
B436/13	Administrator	46.6	ng/µl	0.932	0.497	1.88	1.52	DNA	50
B291/13	Administrator	475.8	ng/µl	9.517	5.369	1.77	1.55	DNA	50
B461/13	Administrator	510.2	ng/µl	0.205	0.087	2.35	1.04	DNA	50

Representation of NanoDrop readings (Spectrophotometric DNA quantification) of some samples. The concentration was in ng/µl and the quality determined based on the 260/280 values. The quantification was based on factor 50.

4.2 Diagnostic tests.

4.2.1 Conventional PCR

In conventional PCR, 28% of the 130 samples analyzed were positive (detailed data in appendix 1). Gel electrophoresis analysis of the PCR products on 2% agarose gel showed the expected amplicons of about 199 bps for positive samples, (figure 4.2). 72 % of the samples did not amplify and therefore considered negative. Details of conventional PCR results are in appendix 1.

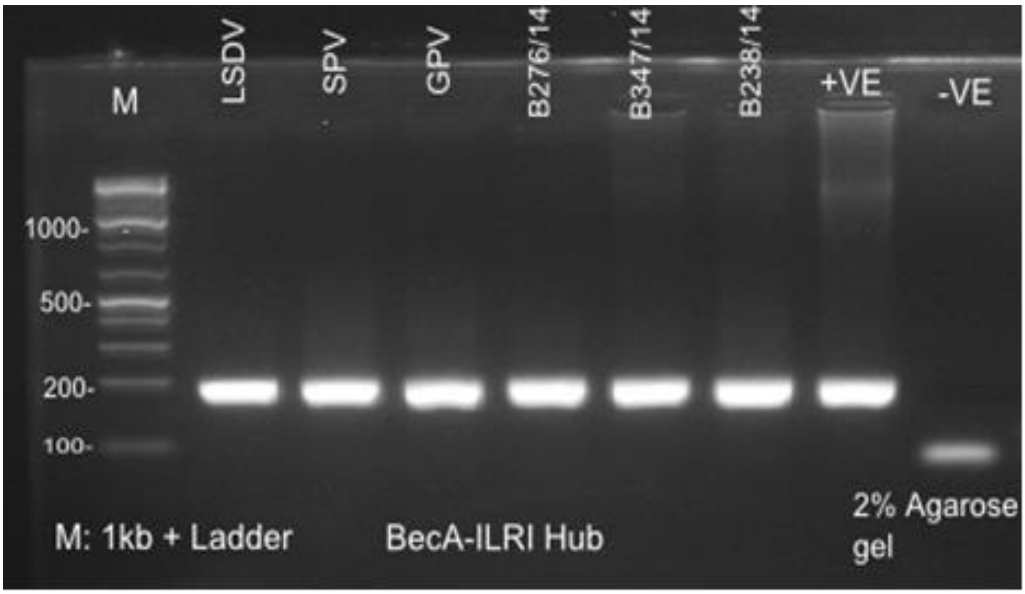

Figure 4.2: 2% Agarose gel of conventional PCR amplicons

Lane 1 contained the 1kb+ DNA ladder used as the marker, lane 2, 3 and 4 had LSDV, SPV and GPV pure cultures and they were all positive. Lane 5 contained sample B276/14, a bovine skin nodule sample from Garisa, Lane 5 contained B347/14, a bovine skin nodule sample from Kajiado, lane 7 contained B238/14, a bovine skin nodule sample from Meru and all were positive and therefore showing a band of 199bp. Lane 8 had the CaPVs positive control. Lane 9 contained the non-template control used as the negative control and therefore a band of approximately 100bp of unused primers due to lack of amplification.

4.2.2 LAMP Assay

The result showed that out of the 130 samples analyzed, 59% were positive by LAMP assay and 41% negative. All the samples that were positive on conventional PCR were also positive on the LAMP assay and details of each sample are shown in appendix 1. There are different methods of visualization in LAMP assay. In this study, the colour change method using hydroxynaphthol blue (Figure 4.3), the real time LAMP using the Optigene 11machine (Figure 4.4) and the gel electrophoresis method (Figure 4.5) were used. Some of the results obtained by hydroxynaphthol blue (HNB) showing colour change are shown below.

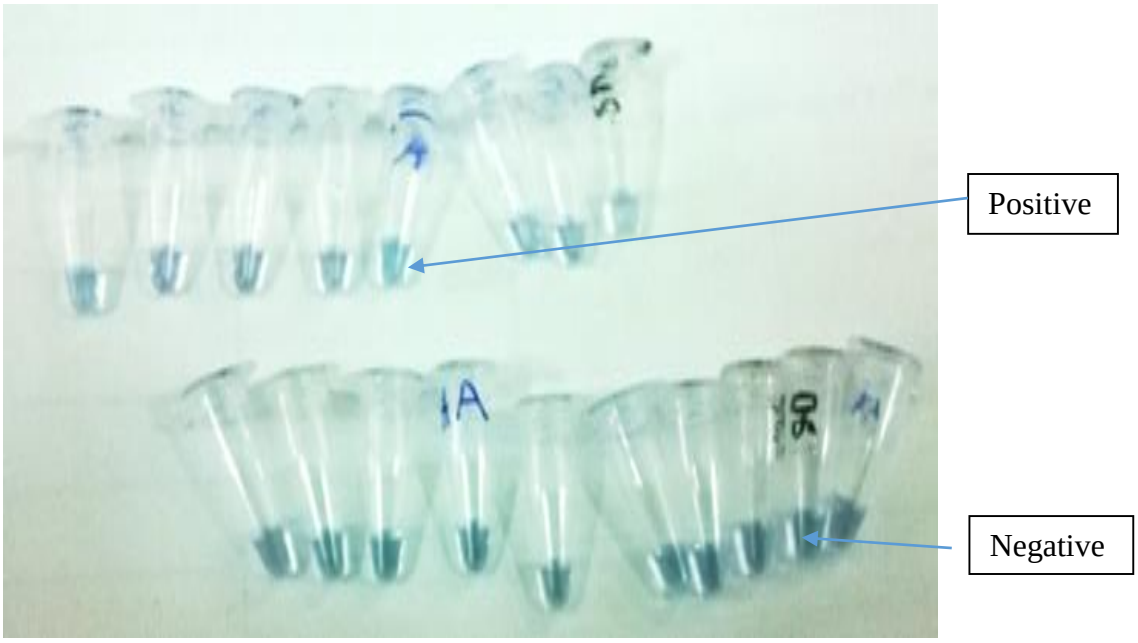

Figure 4.3: LAMP products stained with hydroxynaphthol blue

The upper samples stained sky blue are positive for CaPVs while the samples beneath, that are stained purple are negative.

In the above method, hydroxynaphthol blue, a metal ion indicator was used to detect the product of the LAMP assay. The light sky blue colour is positive while the purple colour is negative. This is a simple method that can be employed in the field where gel electrophoresis is impossible and the LAMP machine is not available

The results from Genie® II LAMP machine were observed as real-time data from isothermal amplification reactions The positive samples were amplified and via the detection channel, curves were produced as shown, (figure 4.4). There were no amplification of the negative samples and negative control and a flat line was observed.

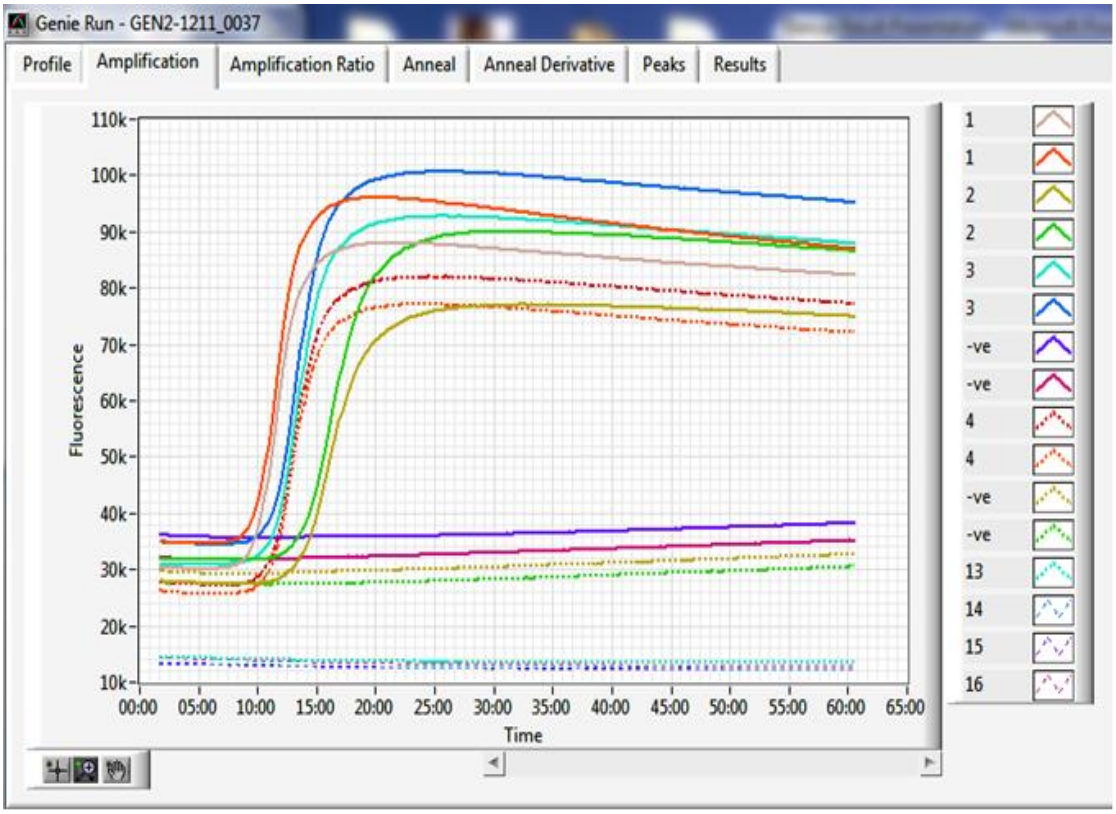

Figure 4.4: Optigene II Machine LAMP Results

LAMP samples were tested in duplicates, 1(B8/13), 2(B42/13), 3(B43/13), 4(B83/13) were positive, showing a sigmoid curve therefore an amplification. Samples 13 &14(B128/13) and 15&16 (B227/13) represented by dotted lines at the bottom were negative showing no amplification but a flat line. The flat lines at the base of the sigmoid curves are negative controls.

The product of LAMP assay gave a series of concatemers of the target region on gel, giving rise to a characteristic "ladder" or banding pattern on the gel, rather than a single band as with PCR a (figure 4.5). This was because this LAMP assay used 4 primers targeting 6 regions within a fairly small segment of the genome leading to a high concentration of the products.

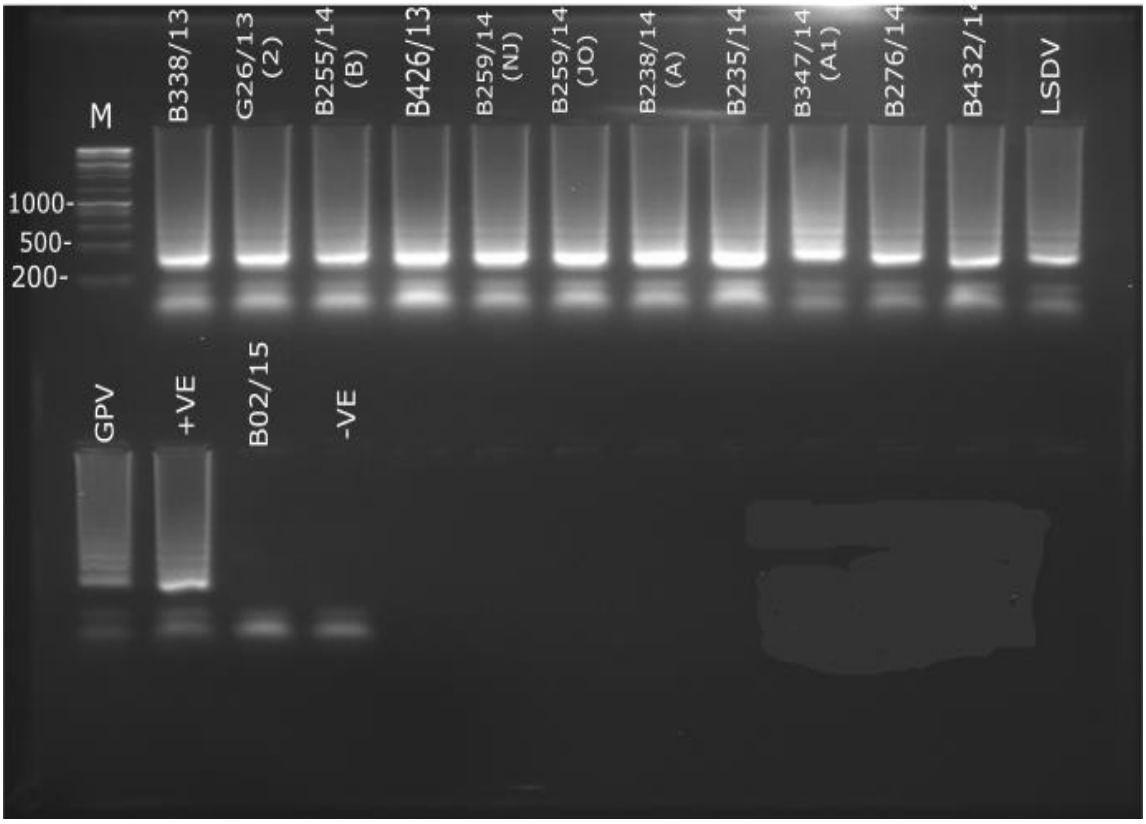

Figure 4.5: 2% Agarose gel of LAMP products

The positive reaction is manifested like a ladder- like pattern on the 2% agarose gel with many bands of different sizes as shown in samples in Lane 1-13 were positive with many bands of between 300 and 600bps. In negative samples there were no bands of more than 180 bps formed, this indicate that there was no amplification reaction in those samples. What is observed in the negative control wells are primer dimers.1 kb + DNA marker was used.

4.2.3 Real time PCR.

A total of 61% of the 130 samples analyzed turned positive by real time PCR which was used as the gold standard method in this study. This included all the samples that were positive on Conventional PCR and LAMP assay. In general, 25 tissues, 50 blood samples and all the 4 cultures were positive. The sigmoid curves represents the positive samples in cases where amplification occurred due to the presence of the targeted sequence (Figure 4.6).

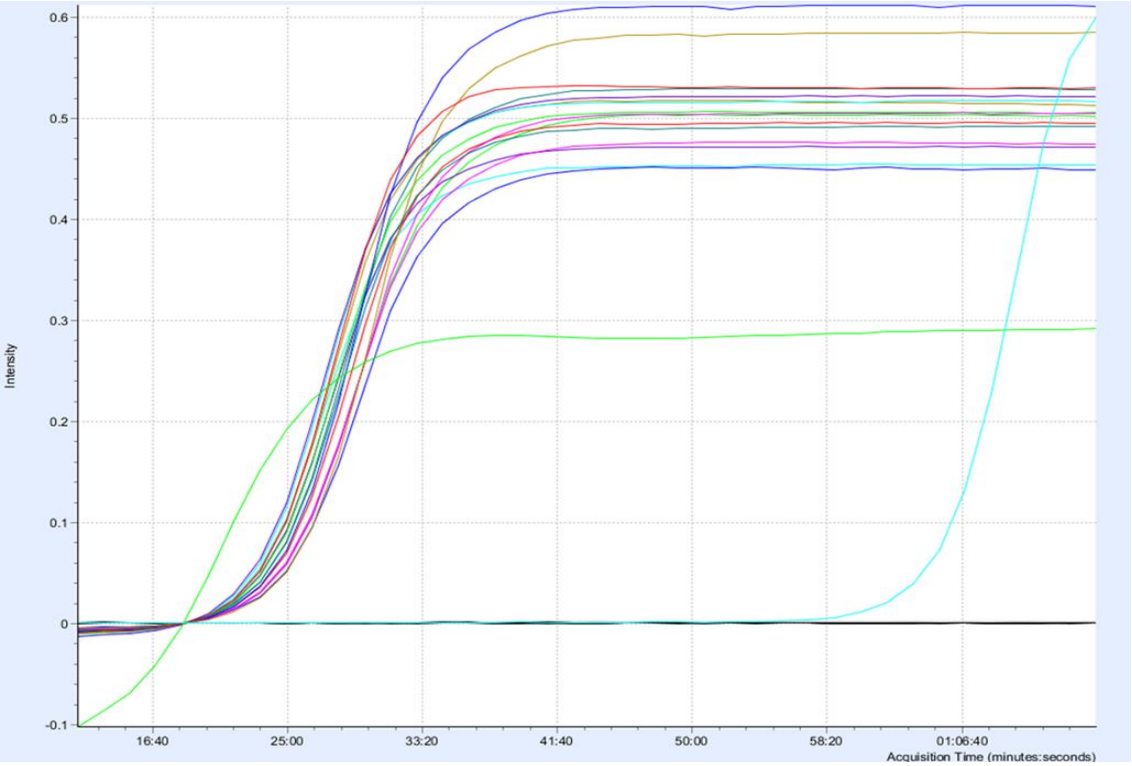

Figure 4.6: Graphical representation of real time PCR.

Amplification of samples in equal amounts was plotted as fluorescence intensity against acquisition time (reaction time), therefore the curves represents accumulation of products over time. The start of the exponential phase of the curves indicates the time taken for target to be detected and therefore reflects the virus titre in the sample. All the samples were positive and samples B347/14 (A3), B276/14, B432/14, LSDV, GPV, SPV, B01/15(1-3) were amplified after around 18 minutes of the reaction. Sample B02/15 (11) was amplified very late after around 55 minutes and this was due to low virus titre. The green amplification curve is the CaPVs plasmid positive control which was detected almost immediately. The baseline fluorescence signals are not shown

4.3 Diagnostic comparison of different Methods.

A total of 130 samples were used for diagnostic analysis and different results were obtained depending on the method. Real time PCR which is the gold standard method showed the highest level of detection at 61%, closely followed by LAMP assay at 59% and conventional PCR at 28%, (Table 4.2 and Figure 4.7).

Table 4.2: Summary of the 130 analyzed samples tested using the 3 tests.

Type of samples analyzed	**No. of samples analyzed**	**Conventional PCR**		**Lamp Assay**		**Real time PCR**	
		Positive	Negative	Positive	Negative	Positive	Negative
Tissues	32	20	12	25	7	25	7
Whole blood	94	12	82	48	46	50	44
Cell cultures	4	4	0	4	0	4	0
TOTAL	130	36	94	77	53	79	51
Percentages	(%)	28	72	59	41	61	39

A total of 32 tissues, 94 blood samples and 4 cell cultures were analyzed. 25 tissues, 50 blood samples and all the cell cultures were positive by the gold standard method; real time PCR. That constitutes 79 samples (61%) of all the samples by this method. A total of 36 samples (28%) were positive by conventional PCR and 77 samples (59%) by LAMP assay.

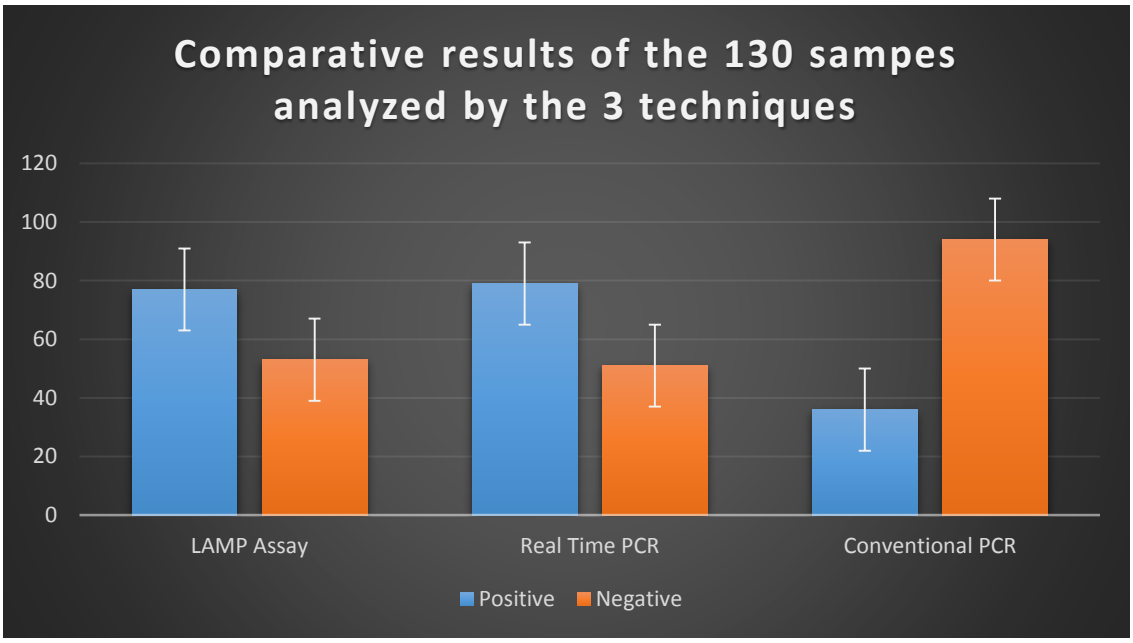

Figure 4.7: Graphical comparison of the 3 tests used in the analysis

The above graph shows the number of positive and negative samples per assay. The blue bar shows the positive samples while the orange bar shows the negative samples. Conventional PCR showed the least number of positive samples and the highest number of negative samples out of the 130 samples analyzed. The real time PCR showed the highest number of positive samples and least negative samples out of the 130 samples, almost similar to the of LAMP assay

Table 4.3: Prevalence/Proportion of detection of CaPVs in different Animals

RESULTS	TYPE OF ANIMAL		
	Cattle	**goats**	**Sheep**
POSITIVE	67	11	1
NEGATIVE	51	-	-
TOTAL	118	11	1
PERCENTAGE PROPORTION OF DETECTION (%)	57%	100%	100%

Out of the 130 samples analyzed, 118 were from cattle, 11 from goats and 1 from sheep. A total of 57% of the cattle samples were positive and all also the samples from goats and sheep. The suspected LSD samples from cattle were 91% of the total samples analyzed

Table 4.4: Diagnostic result per region / institution

Region/Counties/Institution	Total No. of samples Analyzed	No. Negative	No. Positive	Prevalence/ Proportion of detection
Nairobi	37	19	18	49
Homabay	3	-	3	100
Kisumu	3	-	3	100
Siaya	1	-	1	100
Kakamega	2	-	2	100
Bungoma	1	-	1	100
Malaba	2	-	2	100
Nakuru	1	-	1	100
Narok	1	-	1	100
Baringo	8	-	8	100
Kajiado	3	-	3	100
West Pokot	39	23	16	41
Kiambu	1	1	-	0
Nyeri	3	3	-	0
Kitui	4	2	2	50
Meru	2	-	2	100
Isiolo	12	3	9	75
Marsabit	1	-	1	100
Garisa	1	-	1	100
Wajir	1	-	1	100
KALRO	3	-	3	100
KEVEVAPI	1	-	1	100
Total	**130**	**51**	**79**	**61%**

The number of samples obtained from different parents of the country were few except the ones from Nairobi and West Pokot which were many though from the same herd. Active sampling during the study was done in these areas during the study and therefore the high number compared with the rest from other regions where only samples from highly suspected symptomatic animals were brought to the Lab for confirmation and therefore most of them if not all were positive. An exception were samples from Central Kenya (Kiambu and Nyeri) which were all negative.

4.4 Diagnostic Sensitivity and specificity of Conventional PCR, LAMP and qPCR tests

The diagnostic sensitivity of LAMP assay was 97% and specificity, 100% showing a very close agreement and similarity with the qPCR. Table 4.5 shows the 2 by 2 table with positive and negative results based on the real time PCR, the gold standard method. The calculations of specificity and sensitivity are shown below

Table 4.5: Sensitivity and Specificity of the LAMP assay to the gold standard method (real time PCR)

		Truth		
		DISEASE	NON-DISEASE	TOTAL
Test Results	POSITIVE	(True Positive) **77**	(False positive) **0**	(Total Test Positive) **77**
	NEGATIVE	(False Negative) **2**	(True Negative) **51**	(Total Test Negative) **53**
		(Total Disease) **79**	(Total Non-disease) **51**	(Total) **130**

Sensitivity: True Positive/Total disease × 100

77/79 × 100 **= 97%**

Specificity: True Negative/Total Non- disease×100

51/51 × 100 **= 100%**

Positive predictive value: True Positive/Total Test Positive x100

77/77 x 100 = **100%**

Negative predictive value: True Negative/Total Test Negative x100

51/53 x 100 = **96%**

The conventional PCR showed 100% specificity in Capripox detection but with a lower sensitivity of 45.6% compared to that of LAMP assay and real time PCR (table 4.6). The 2 by 2 table was generated from the results that were obtained from conventional PCR analysis against the gold standard method. The sensitivity and specificity were calculated as shown below.

Table 4.6: Sensitivity and specificity of Conventional PCR to real time PCR

		Disease	***Non-Disease***	***Total***
Test Results	***Positive***	**(True Positive)**	**(False positive)**	**(Total Test Positive)**
		36	**0**	**36**
	Negative	**(False Negative)**	**(True Negative)**	**(Total Test Negative)**
		43	**51**	**94**
		(Total Disease)	**(Total Non-disease)**	**(Total)**
		79	**51**	**130**

Sensitivity: True Positive/Total disease ×100

36/79 × 100 **= 45.6%**

Specificity: True Negative/Total Non- disease×100

51/51 × 100 **= 100%**

Positive predictive value: True Positive/Total Test Positive x100

36/36 x 100 = **100%**

Negative predictive value: True Negative/Total Test Negative x100

51/94 x 100 = **54%**

4.5 Analytical sensitivity and specificity of LAMP Assay and real time PCR

4.5.1 Analytical sensitivity

The analytical sensitivity was determined by testing the level of detection of both qPCR and LAMP assay from a serially diluted purified capripoxvirus DNA as described in section 3.6 under materials and methods.

The LAMP assay was able to detect up to a dilution factor of 10^{-16} with a peak/CT value of 29.42, the cutoff point being 30 and an annealing point of 82.56. The results of the different dilutions (Table 4.7).

Table 4.7: LAMP analytical sensitivity

Sample dilution	Peaks	Annealing Point
Neat	15.2	82.17
$10^{-}2$	17.17	82.58
$10^{-}4$	17.32	82.64
$10^{-}6$	19.17	82.54
$10^{-}8$	21.17	82.59
$10^{-}10$	23.05	82.54
$10^{-}12$	25.02	82.32
$10^{-}14$	28.36	82.58
$10^{-}16$	29.42	82.56
$10^{-}18$	_	_
$10^{-}20$	_	_

Key: - Negative (Not detected)

The LAMP reaction was able to detect up to a serial dilution of $10^{-}16$ and this was detected after 29 minutes and 42 seconds of the reaction. The undiluted sample that is the neat was detected only after 15 minutes and 2 seconds due to the high viral titre in it.

On the other hand, qPCR was able to detect up to a dilution factor of 10^{-18} with a peak/CT value of 37.0, the cutoff point set at 37 (any sample with a CT value of 37 and below considered positive) (Table 4.8).

Table 4.8: Real time PCR Analytical Sensitivity Test.

Sample No.	Sample Dilution	CT Value(Mean)
1	Neat	10.07231
2	$10^{-}2$	13.08772
3	$10^{-}4$	17.12098
4	$10^{-}6$	19.72893
5	$10^{-}8$	21.73447
6	$10^{-}10$	24.89356
7	$10^{-}12$	28.07662
8	$10^{-}14$	30.94375
9	$10^{-}16$	34.26325
10	$10^{-}18$	37.00188
11	$10^{-}20$	_
12	$10^{-}22$	_

Key: - Negative (Not detected)

The analytical sensitivity of real time PCR was up to $10^{-}18$ of the serially diluted positive sample with a CT value of 37, being also the cutting point of this reaction. This meant that it was able to detect up to $10^{-}18$. The neat had a CT value of 10 due to the high viral titre/load. The virus was not detected in higher dilution from $10^{-}20$ onwards, they were negative.

4.5.2 Analytical specificity

The analytical specificity was determined using viral DNA extracted from cell culture-grown from the 3 isolates; SPV, LSDV, and GPV. To determine specificity and possibility of cross-reactivity in the LAMP assay and also qPCR with other viruses that may be included in a differential diagnosis of Capripox, the following viruses were tested alongside the pure cultures: Orf virus, Pestes-des-petit-ruminants virus (PPRV), other bacteria like *Mycoplasma capricolum* subsp. *capripneumoniae* (MCCP) that affects goats and also against plant virus; Cassava brown streak virus (CBSV) as an out-group. For performance evaluation the analytical specificity was also determined by qPCR. The results of the analytical specificity of the LAMP assay (table 4.9), it was able to detect the 3 CaPVs but not the others.

Table 4.9: LAMP Analytical Specificity

Wells	**Names**	**Peaks**	**Annealing**
B1	LSDV	9.17	83.01
B2	SPV	16.47	82.31
B3	GPV	16.47	82.27
B4	ORF	_	_
B5	PPR	_	_
B6	MCCP	_	_
B7	CBSV	_	_
B8	BLANK	_	_

Key: - Negative (Not detected)

The LAMP assay was able to specifically detect the 3 CaPVs: LSDV, SPV and GPV and no other closely related viruses and out-groups like PPR and ORF. It was also not able to detect bacteria like MCCP and CBSV from both animals and plants respectively. Therefore very specific to CaPVs.

Table 4.10 shows the analytical specificity of real time PCR. It was able to detect all the 3 CaPVs but not the other viruses as shown.

Table 4.10: Real time PCR Analytical specificity.

Sample No.	Sample Name	CT Value
1	LSDV	11.98234
2	GPV	10.45311
3	SPV	12.32502
4	PPRV	_
5	ORF	_
6	CBSV	_
7	MCCP	_
8	BLANK	_

Key: - Negative (Not detected)

Both the real time PCR and the LAMP assay had the same analytical specificity. They were both able to detect the LSDV, GPV and SPV but no other closely related viruses: Orf and PPR, plant viruses: CBSV and bacteria: MCCP.

4.6 Next Generation Sequencing Data results

The 18 sequenced samples gave a read output of 34,631,717 reads (nucleotides) in total. This was a two paired end sequencing run with a read length of between 35 and 301. It is recommended that at least 70% of the sequences should have a quality score of 30 for the sequenced data to be applied for any analysis. In this study, 81.1 % of the sequences had a quality score of 30, and therefore applicable.

After trimming, a total of 30,355,359 reads were obtained. This was necessary so that the poor quality reads at the ends and the adapters added during library preparation do not interfere with downstream applications.

The highest read was 4,799,728, the least 217,652 and the average of all the sequences was 1,923,984.

Table 4.11 shows the summary of the Illumina Miseq next generation sequencing results:

Table 4.11: Summary of Sanger / Illumina Miseq Sequencing data for Capripoxvirus.

S. No.	Sample ID	Sample type	Place of origin	Sequence Sample ID	Read Length (bp)	Raw data	Trimmed data
1	B347/14	skin nodule	Kajiado	1_S1_L001_R1_001	35-301	217652	217652
				1_S1_L001_R2_001	35-301	217652	204910
2	B238/14	skin nodule	Meru	2_S2_L001_R1_001	35-301	2244735	2244735
				2_S2_L001_R2_001	35-301	2244735	1944666
3	B259/14	skin nodule	Nairobi	3_S3_L001_R1_001	35-301	2282768	2282768
				3_S3_L001_R2_001	35-301	2282768	1924289
4	B255/14	whole blood	Kitui	4_S4_L001_R1_001	35-301	2454479	2454479
				4_S4_L001_R2_001	35-301	2454479	1993153
5	B436/13	skin nodule	Bungoma	5_S5_L001_R1_001	35-301	2188097	2188097
				5_S5_L001_R2_001	35-301	2188097	1851541
6	B43/13	skin nodule	Kakamega	6_S6_L001_R1_001	35-301	2023252	2023252
				6_S6_L001_R2_001	35-301	2023252	1782661
7	B83/13	skin nodule	Kisumu	7_S7_L001_R1_001	35-301	2042713	2042713
				7_S7_L001_R2_001	35-301	2042713	1807306
8	B42/13	skin nodule	Homabay	8_S8_L001_R1_001	35-301	3183260	3183260
				8_S8_L001_R2_001	35-301	3183260	2930910
9	B276/14	skin nodule	Garisa	9_S9_L001_R1_001	35-301	2178008	2178008
				9_S9_L001_9R2_001	35-301	2178008	2010457
10	GPV	cell cultures	KALRO	10_S10_L001_R1_001	35-301	784365	784365
				10_S10_L001_R2_001	35-301	784365	719703

				11_S11_L001_R1_001	35-301	2956905	2956905
11	LSDV	cell cultures	KALRO	11_S11_L001_R2_001	35-301	2956905	2590571
				12_S12_L001_R1_001	35-301	2096291	2096291
12	SPV	cell cultures	KALRO	12_S12_L001_R2_001	35-301	2096291	1668932
				13_S13_L001_R1_001	35-301	2627502	2627502
13	B448/13	Lymph node	Siaya	13_S13_L001_R2_001	35-301	2627502	2299514
				14_S14_L001_R1_001	35-301	468340	468340
14	G25/13	skin nodule	Isiolo	14_S14_L001_R2_001	35-301	468340	424137
				15_S15_L001_R1_001	35-301	772137	772137
15	B235/14	skin nodule	West Pokot	15_S15_L001_R2_001	35-301	772137	723291
				16_S16_L001_R1_001	35-301	811963	811963
16	G26/13	skin nodule	Baringo	16_S16_L001_R2_001	35-301	811963	811963
				17_S17_L001_R1_001	35-301	2595813	2595813
17	B432/14	skin nodule	Kakamega	17_S17_L001_R2_001	35-301	2595813	2595813
				18_S18_L001_R1_001	35-301	4799728	4799728
18	LUMPIVAX	Vaccine	KEVEVAPI	18_S18_L001_R2_001	35-301	4703301	4703301

4.6.1 Denovo assembly and Blasts Analysis

Many single contigs of different lengths were obtained per sample (table 4.12) Contigs of each sequenced sample were blasted against the NCBI viral database to determine its identity. Blasting was specific to a viral database as the entire extracted DNA was sequenced and not only the viral DNA. All the samples hit to either one or all of the CaPVs. Some contigs, when blasted, indicated either an SPV, LSDV or GPV. In sample 1(1_S1_L001_R1_001), for example, a total of 89 of the contigs generated were able to hit to CaPVs, 88 contigs blasted to LSDV and 1 contig to GPV, most likely due to the

close genetically relatedness among the viruses. All the 18 samples showed the presence of CaPVs as shown in table 4.12 and the figures 4.8, 4.9 and 4.10 respectively.

Table 4.12: Summary of contigs generated and Blast analysis results.

Sample No.	**No. of CaPVs Contigs generated per sample**	**No. of Contigs that predicted CaPVs**		
		LSDV	**SPV**	**GPV**
1_S1_L001_R1_001	89	88	-	1
2_S2_L001_R1_001	1	1	-	-
3_S3_L001_R1_001	1	1	-	-
4_S4_L001_R1_001	1	1	-	-
5_S5_L001_R1_001	5	5	-	-
6_S6_L001_R1_001	169	168	1	-
7_S7_L001_R1_001	14	14	-	-
8_S8_L001_R1_001	148	148	-	-
9_S9_L001_R1_001	18	6	5	7
10_S10_L001_R1_001	1	1	-	-
11_S11_L001_R1_001	49	48	-	1
12_S12_L001_R1_001	7	7	-	-
13_S13_L001_R1_001	26	26	-	-
14_S14_L001_R1_001	1	1	-	-
15_S15_L001_R1_001	1	1	-	-
16_S16_L001_R1_001	11	1	10	-
17_S17_L001_R1_001	48	36	1	11
18_S18_L001_R1_001	38	29	1	8

The sequenced Sample 1 (1_S1_L001_R1_001), (B347/14) a skin nodule sample from Kajiado, as generated 89 contigs, 88 of these contigs were specific to LSDV at NCBI and I contig to GPV (Table 4.12). When blasted against the virus database, it showed 95% lumpy skin disease virus and 1% GPV on NCBI. Krona presentation of blast analysis of this sample (figure 4.8).

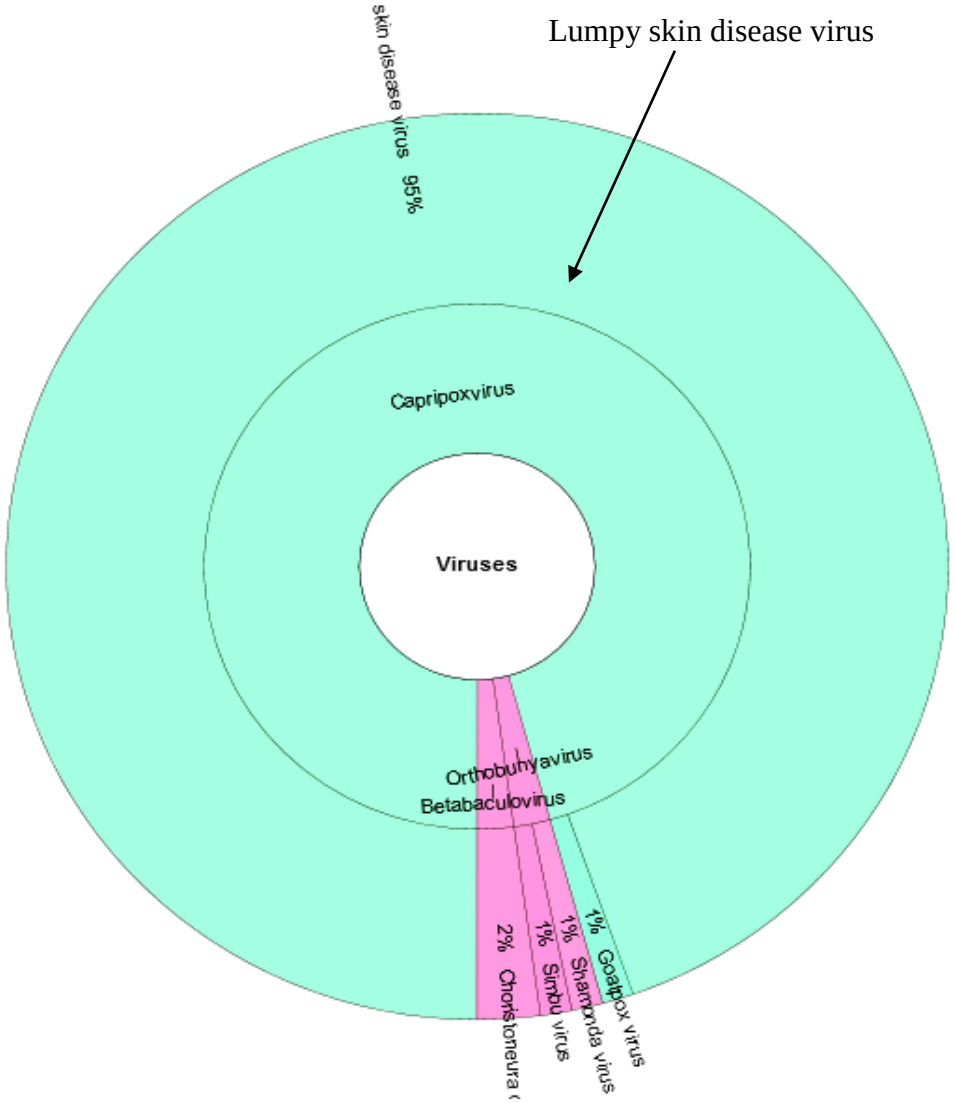

Figure 4.8: Sample B347/14 Krona blast results

Sequenced sample (B347/14), bovine LSDV sample from Kajiado generated 93 contigs and when blasted against the NCBI virus database, 95% were the Lumpy skin disease virus with only 1% similarity to goat pox virus. Other viruses other than the CaPVs like the Shamonda and simbu viruses.

The sequenced sample 9 (9_S9_L001_R1_001), B276/14, a bovine skin nodule sample from Garisa when blasted against the viral database, confirmed the presence of Capripoxvirus mixed infection in that cattle as all the 3 viruses were present, sheeppox virus-13%, goatpox virus-18% and sheeppox virus-15% as shown in figure 15. The results in table 4.12 confirmed this, because out of the 18 contigs generated, 6 predicted LSDV, 5 to SPV and 7 to GPV.

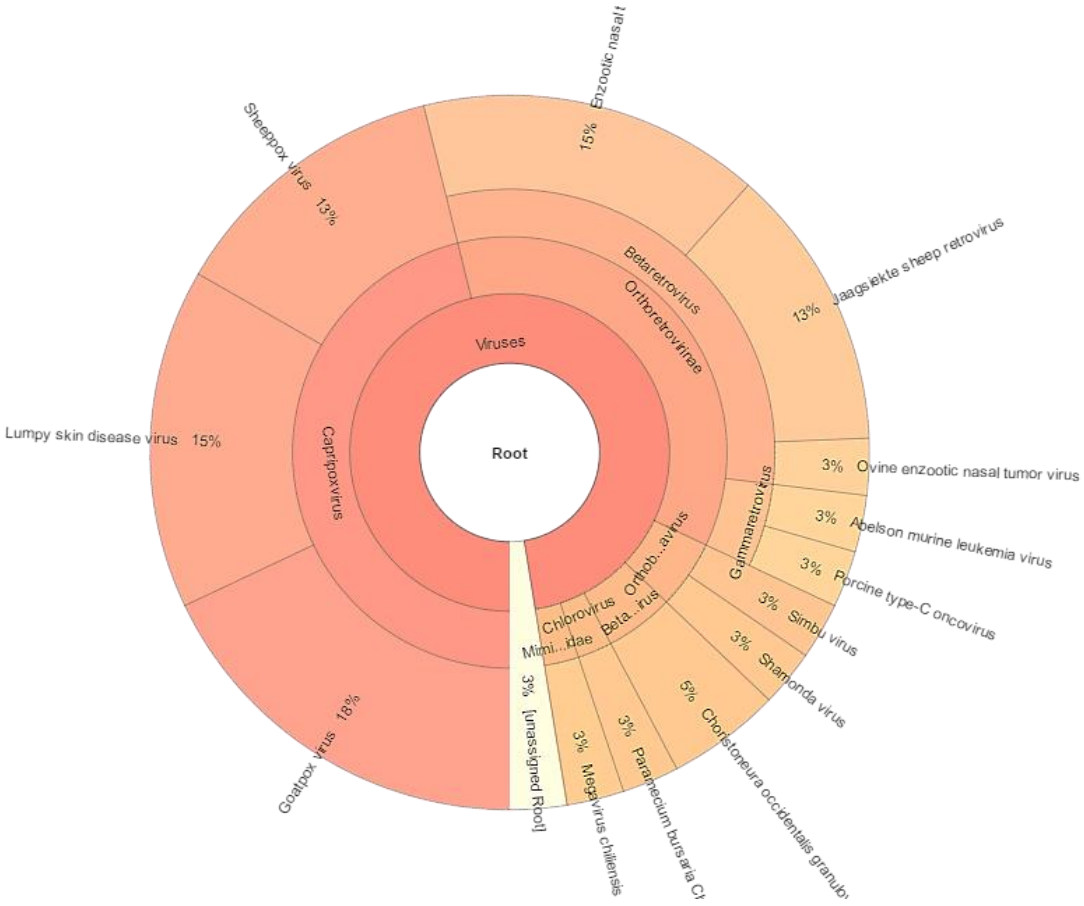

Figure 4.9: Sample B276/14 Krona blast results

Blast analysis of sample B276/14 showed the presence of the three CaPVs: SPV, LSDV and GPV and 13%, 15% and 18% respectively. Viruses other than the CaPVs and therefore not important in this study were present like jaagsiekte sheep retrovirus at 13% and Enzootic nasal tumour virus at 15%. Others present were 5% and less.
The sequenced sample 11 (11_S11_L001_R1_001), LSDV, was a pure culture of

Capripoxvirus obtained from cattle and therefore was referred to as lumpy skin disease virus, but on the blast analysis, it showed 53% sheeppox virus. In the Denovo assembly, 49 contigs were generated and 48 of the contigs predicted SPV as shown in table 4.12. Therefore, an indication of sheeppox virus infecting cattle (Figure 4.10).

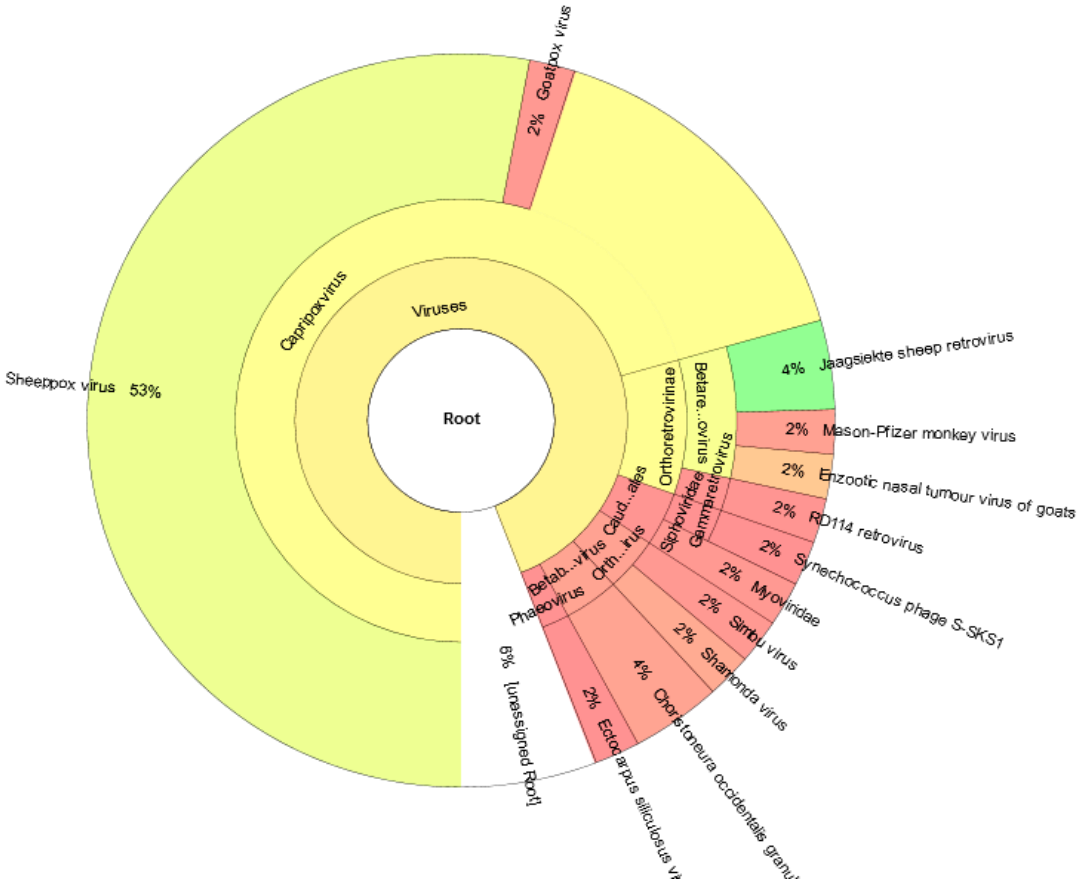

Figure 4.10: Sample 11(LSDV cell culture) Krona Blast results

The LSDV pure culture from KALRO indicated 53% SPV and 2% GPV on blast analysis. Other than CaPVs, viruses not of interest in this study were present though in small percentages of 5% and less.

4.6.2 Reference Mapping

The contigs generated after pairing the two reads [read 1 and 2 (R1&R2)] were mapped to a complete sequenced LSDV, SPV and GPV genomes from the database. The complete genomes of the 3 CaPVs viruses were obtained from the NCBI.TTP site for full original genomes. The CaPVs genomes used were:

GPV: NC_004003 (149,940bp)

LSDV: NC_003027 (150,760bp)

SPV: NC_004002 (149,580bp)

These genomes were indexed before mapping using the bwa program and the results obtained (table 4.13). There was mapping to all the 3 CaPVs due to their genetic relatedness.

Table 4.13: Reference mapping using CLC genomics to the three viruses.

Sample No.	Paired reads LSDV	Paired reads SPV	Paired reads GPV
1	2,021	985	1,011
2	16,500	16,209	16,492
3	21,438	20,448	21,195
4	30,098	29,125	30,077
5	18,232	17,850	18,702
6	13,271	10,983	11,138
7	26,304	13,357	13,826
8	16,275	14,111	14,136
9	148,227	326,061	23,440
10	3,776	4,127	4,105
11	61,732	1,013,816	45,851
12	258,892	36,478	38,567
13	958,808	50,902	58,180
14	2,337	2,383	2,526
15	3,578	3,212	3,415
16	7,623	44,554	7,178
17	73,855	36,868	255,825
18	238,319	112,829	714,024

Due to the 96-98% genome identity between the 3 CaPVs, there was mapping of the reads to all the 3 viruses but at different levels. Some samples had almost the same number of

reads mapping to all the virus like sequenced sample 2, 3, 4 and 5 and therefore quite difficult to differentiate. Yet others had a well-defined differences in numbers like sequenced sample number 17 and 18 where most reads mapped to GPV, 9 and 11 to SPV and 1, 12 and 13 to GPV.

Table 4.14: Reference mapping to LSDV

S. No.	SAMPLE ID	Sample type	Place of origin	Mapped reads	Percentage
1	B347/14	skin nodule	Kajiado	3,858	0.94
2	B238/14	skin nodule	Meru	47,830	1.23
3	B259/14	skin nodule	Nairobi	61,606	1.06
4	B255/14	whole blood	Kitui	87,136	2.19
5	B436/14	skin nodule	Bungoma	53,329	1.44
6	B43/14	skin nodule	Kakamega	33,758	0.95
7	B83/14	skin nodule	Kisumu	51,854	1.43
8	B42/14	skin nodule	Homabay	40,398	0.69
9	B276/14	skin nodule	Garisa	491,854	12.23
10	GPV	cell cultures	KALRO	10,747	0.75
11	LSDV	cell cultures	KALRO	102,259	2.45
12	SPV	cell cultures	KALRO	331,810	9.94
13	B448/13	Lymph node	Siaya	1,065,513	23.17
14	G25/12	skin nodule	Baringo	6,670	0.79
15	B235/14	skin nodule	West Pokot	9,928	0.69
16	G26/12	skin nodule	Isiolo	58,602	3.61
17	B432/14	skin nodule	Kakamega	364,774	7.03
18	LUMPIVAX	Vaccine	KEVEVAPI	1,056,053	11.23

4.6.3 Multiple sequence Alignment.

Multiple sequence alignments of the amino acid sequences of RP030, GPCR and P32 genes with the ones from the database indicated the presence of SNPs including deletions and substitutions (Figures 4.11, 4.12 and 4.13).

The RP030 gene

The RP030 alignment (partially shown in figure 4.11), revealed some missing amino acids in some samples represented as star (*), and also some substitutions. The RP030 gene is conserved and GPV and LSDV have RP030 specific signatures at position 15 and 98, that is LSDV P_{15}, P_{98} while in GPV T_{15}, S_{98} (Shown in the appendix). The gene is 202 amino acid long in LSDV and GPV and 195 amino acid long in SPV.

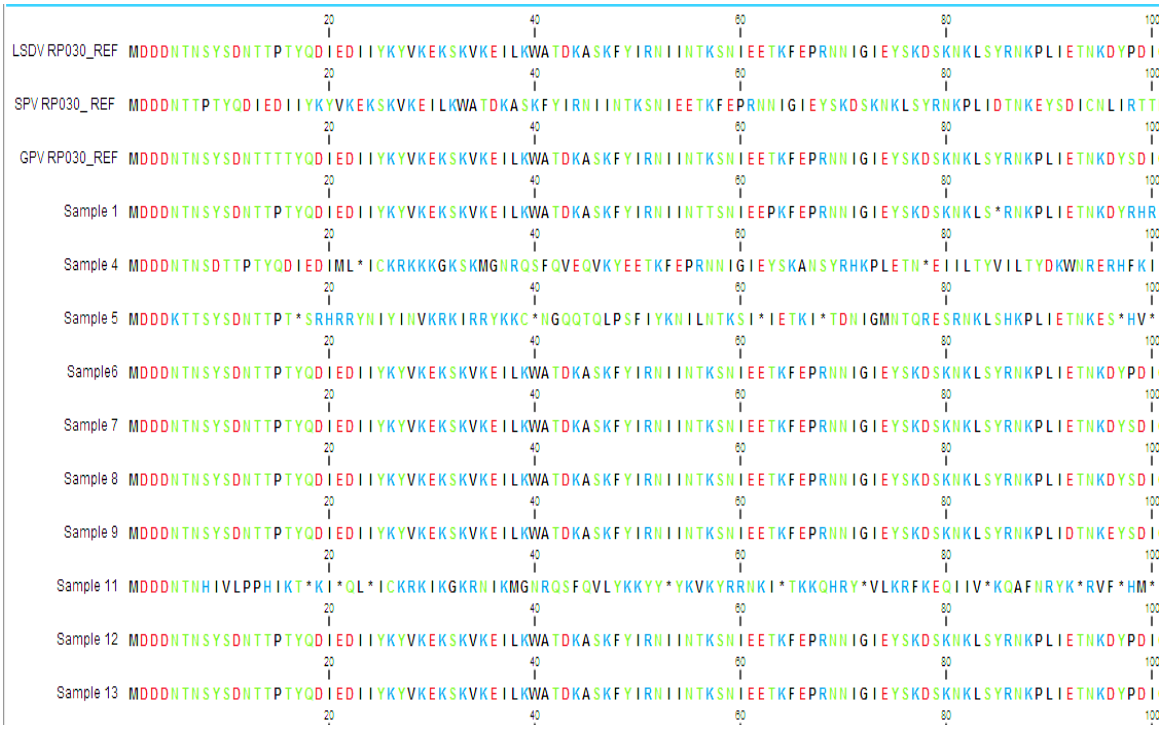

Figure 4.11: Multiple sequence alignment of the amino acid sequence of CaPVs RP030 gene using CLC Main workbench 7.6.1

The amino acid specific signatures of this gene shown by this data were at position 15 and 98 in GPV and LSDV. In LSDV, Proline (P), a conserved polar aliphatic amino acid (aa)

at position 15 is replaced with Threonine (T) a polar hydroxylic amino acid, in GPV. At position 98, Proline (P) in LSDV is replaced by Serine (S) in GPV. Other than the few specific signatures, the gene is quite similar in the two viruses. There are many variations in the SPV aa sequence starting at position 7.

The GPCR gene

The alignment of the GPCR gene amino acid sequences revealed the LSDV characteristic AT signature at positions 11–12 and GPV, YA specific amino acid signatures at the same position (figure 4.12). These signatures are specific for the viruses and bovine sample 17 from Kakamega and Sample 18 (Lumpivax) vaccine having the goatpox virus specific signatures; $N_{6,}$ $G_{10,}$ $Y_{11,}$ $A_{12,}$ $V_{34,\ K49,}$ F99 and $S_{199.}$ Sample 11 showed the SPV specific signature at position 6; (R_6). The gene is 382 amino acid long in SPV and GPV and 375 amino acid long in SPV. The Multiple sequence alignment based on this gene showed the amino acid specific signatures. SPV, GPV and LSDV.

Figure 4.12: Multiple sequence alignment of CaPVs LSDV011- GPCR gene using CLC Main workbench 7.6.1

The LSDV and GPV specific signatures in these gene are at position 11 and 12, that is, Alanine (A) and Threonine (T) in LSDV and Tyrosine (Y) and Alanine (A) in GPV. In SPV, there is a specific signature at position 6, which is Arginine (R) although in LSDV and GPV at this position, there is the amino acid serine (S).This specific signatures therefore differentiate the viruses.

The P32 gene.

The P32 gene, with an ORF LSDV074 and ORF GTPV/SPPV070 is a highly conserved gene. It encodes an envelope protein of 323 amino acid (aa) long sequence in cattle and goats, and 324 aa in sheep. There is an insertion of an aliphatic amino acid leucine (L) at position 63 in sheeppox virus. The identity of these gene in LSDV and GPV 97.83% with signature specific amino acids found at position 26, 135, 289, and 304. The LSDV specific signatures being D_{26}, $F_{49,}$ H_{135} I_{289} and $D_{304,}$ in GPV specific signature being G_{26}, $L_{49,}$ Y_{135}, M_{289}, and $N_{304.}$ (Whole data set not shown). Samples 17 and 18 had the GPV specific signatures and samples 9 and 16 had, SPV specific signatures that is L at position 49, D I at position 55, 56 and Y K at position 60 and 61 (Figure 4.13).

Figure 4.13: Multiple sequence alignment of CaPVs LSDV074 - P32 gene (969bp, 323aa) using CLC Main workbench

Amino acid specific signatures of this gene shown in this data are at position 26 and 49. At position 26, there is the amino acid Aspartic acid (D), in LSDV and SPV but Glycine (G) in GPV. At position 49, Phenylalanine (F) in LSDV is replaced by Leucine (L) in both SPV and GPV. Other specific signatures are found at position 135, 289 and 304 not shown in this data set.

4.6.4 Phylogenetic analysis

Phylogenetic analysis based on GPCR, RP030 and P32 genes was done.

The Phylogenetic analysis of the CaPVs based on the alignment of the nucleotide sequences (64984–65952) of the P32gene that codes for the envelope protein revealed three distinct lineages, that is the LSDV lineage, GPV lineage and SPV lineage. The bootstrap values of 1000 replicates are shown when higher than 70%. The consensus tree was rooted in reference to the different strains of the three poxvirus outgroups and was broken up to show the distant relationship between them and CaPVs. MEGA 6.06 was used for this analysis with Maximum Likelihood method. The red diamond marks shows the analyzed samples while other sequences were obtained from the database (Figure 4.14)

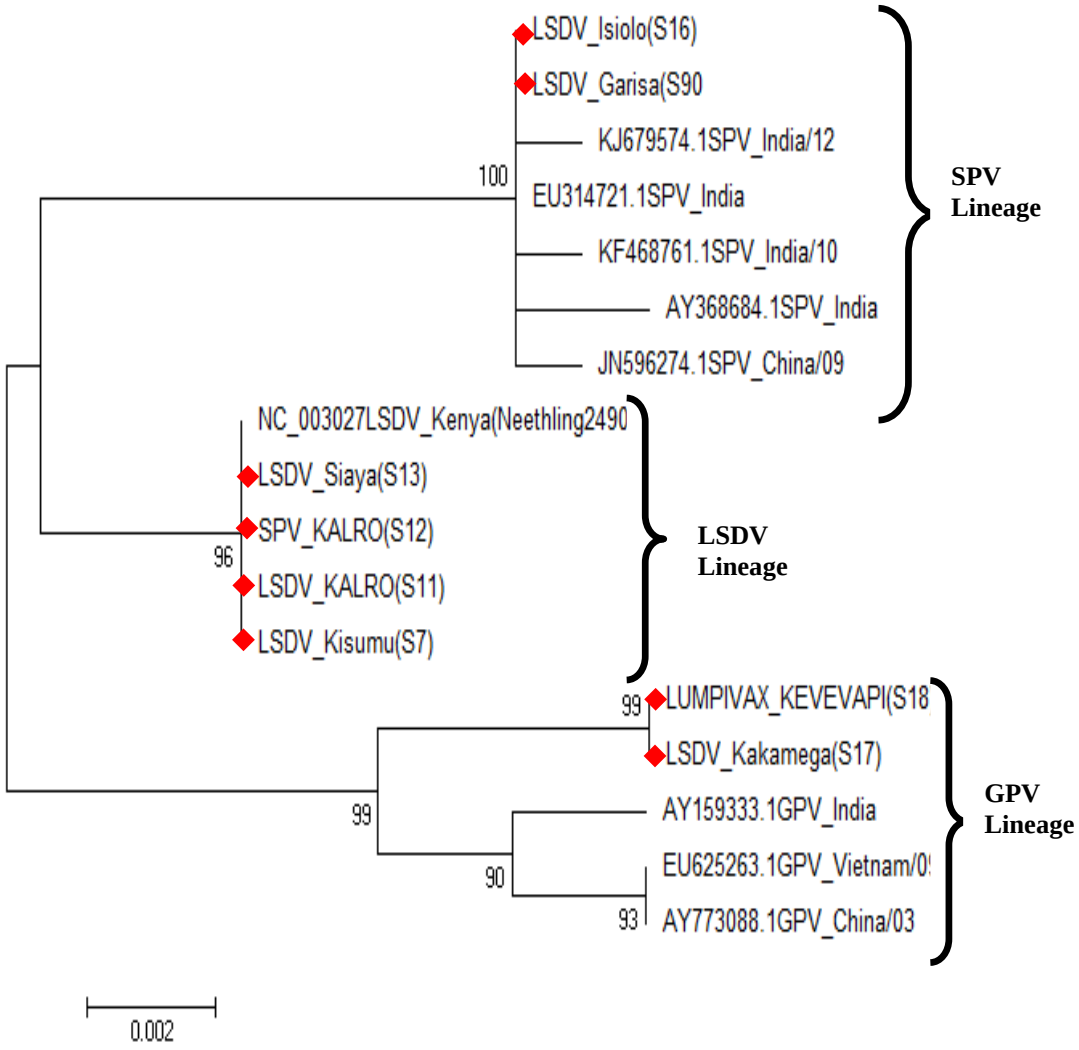

Figure 4.14: Phylogenetic tree of P32 gene of CaPVs samples from different parts of Kenya and other countries.

◆ Sequenced samples

The P32 gene phylogenetic analysis provided good support for the 3 main capripoxviruses lineages: LSDV, SPV and GPV. It showed that the GPV lineages could be divided into 3 sub-clusters, one sub-cluster consisting only of the Kenyan samples: LUMPIVAX vaccine and sample 17 from Kakamega, and the other sub-clusters, GPV from China and Vietnam. The LSDV lineage consisted of the Kenyan samples from the study and the Neethling 2490 strain that have earlier been isolated from Kenya. In the SPV lineage, there were SPV strains from China and India clustering together with LSDV strains from Kenya: Isiolo and Garisa.

The Phylogenetic analysis of the CaPVs based on the alignment of the nucleotide sequences (6973–8118) of the GPCR gene showed the three capripoxvirus lineages with analyzed samples and those from the database clustering differently. The SPV and GPV had two sub clusters each indicating diversity and further differentiation within the groups (Figure 4.15)

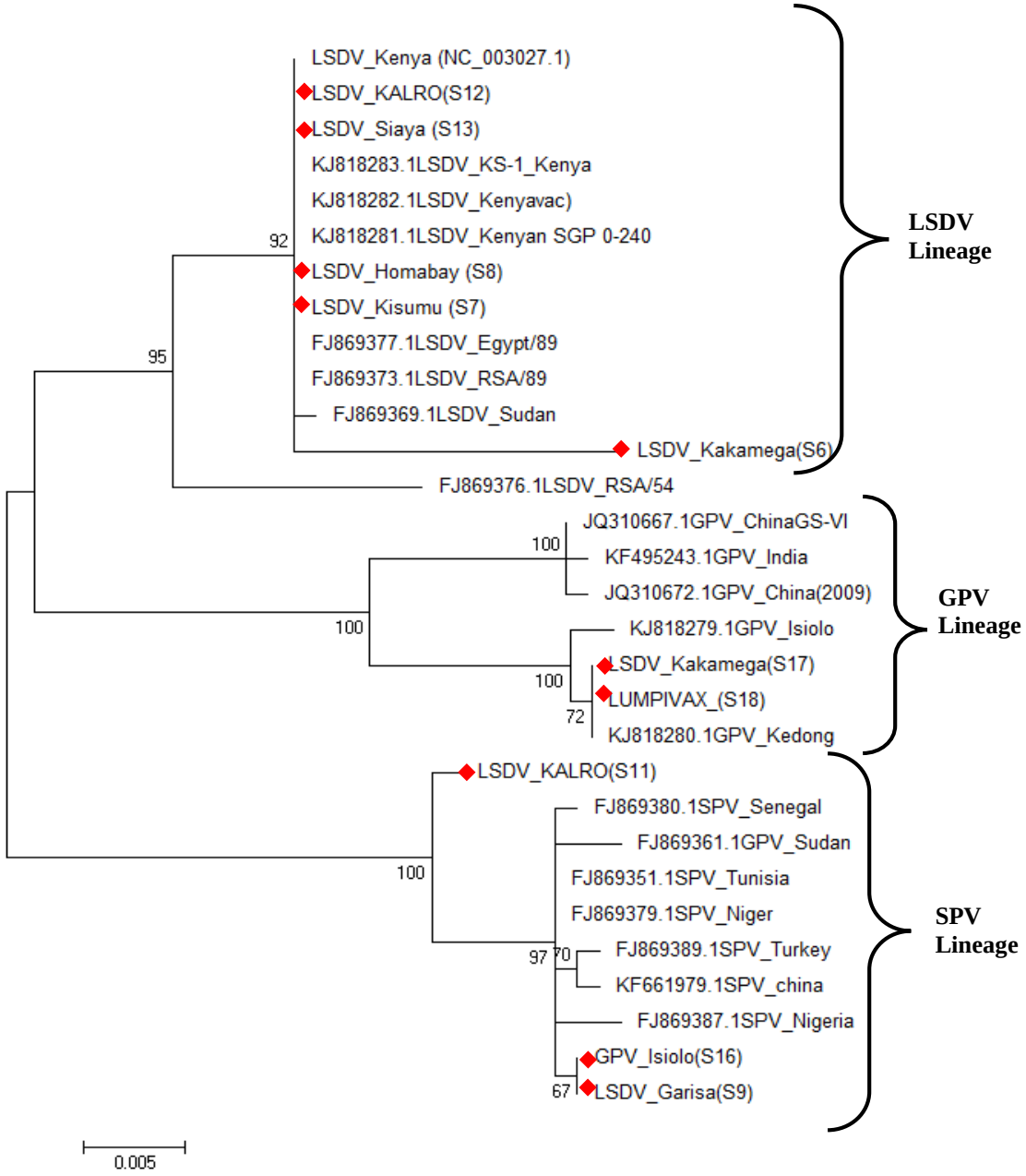

Figure 4.15: Phylogenetic tree of GPCR gene of CaPVs samples from different parts of Kenya and other countries. ◆Represents sequenced samples

The topological structure of the analysis of the GPCR gene could be divide into the 3 capripoxvirus lineages: SPV, LSDV and GPV. The LSDV lineage had 2 sub-clusters with all Kenyan samples clustering in one sub-cluster with strains from Egypt, Sudan and South Africa. The GPV cluster still had the 3 sub-clusters and the SPV lineage; 2 main sub-clusters with one sub-cluster having smaller groupings. One of this groups consisted of GPV sample from Isiolo and LSDV sample from Garisa clustered together.

The phylogenetic analysis based on the RP030 gene that codes for the RNA polymerase subunit 30kD Further supports the clustering of the CaPVs into the three distinct groups. The LSDV lineage had two sub clusters with Kenyan samples in both the groups indicating the presence of diversity within the group. Most of the Kenya strains that have been previously used as vaccines clustered together with strains from Siaya, Kakamega and Kajiado. The Lumpivax vaccine and S17 from Kakamega were clustered as GPV and sample 9 from Garisa and Sample 11 from Isiolo clustering as SPV (figure 4.16).

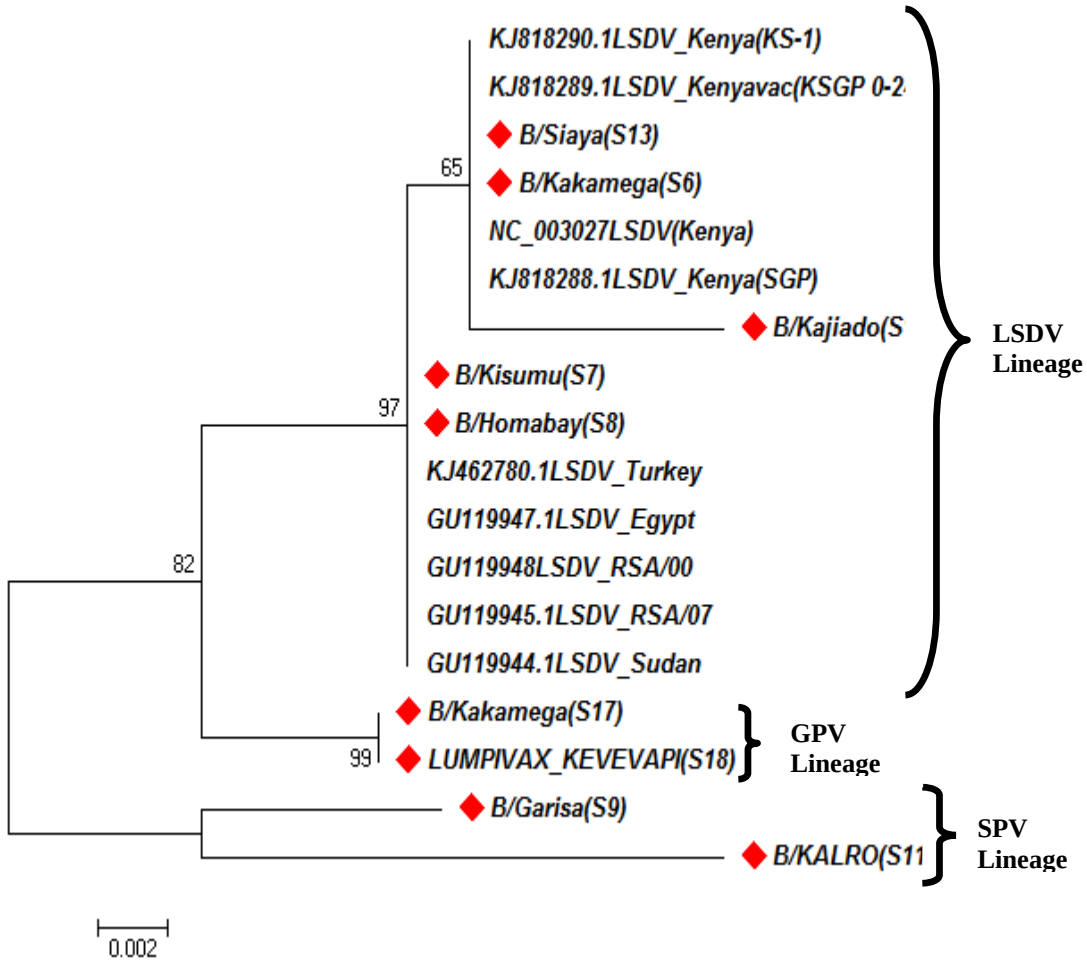

Figure 4.16: Phylogenetic tree of RP030 gene of CaPVs samples from different parts of Kenya and other countries. ♦ Represents sequenced Kenyan samples

The topological structure of the phylogenetic analysis based on the RP030 gene showed the 3 main capripoxviruses lineages: LSDV, SPV and GPV similar to the analysis of the other genes. The LSDV lineage had two main sub-clusters with one sub group consisting of Kenyan LSDV samples some used in this study: S1, S6 and S13 from cattle and others isolated earlier and even used as vaccines in other countries like KJ818289.1 (KSGP 0-24) and KJ818290.1 (KS-1).One of the LSDV lineage sub group consisted of LSDV samples S7 and S8 from Kisumu and Siaya respectively and they clustered together with LSDV strains from Sudan, Egypt, Turkey and South Africa. The GPV lineage clusterd so closely to the LSDV lineage, it had no sub clusters and consisted only of the Kenyan samples: LUMPIVAX vaccine and LSDV sample from Kakamega. The SPV lineage consisted of LSDV samples from Garisa and KALRO.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS.

5.1 DISCUSSION

Capripoxviruses remain a major challenge to farmers in endemic areas like Asia and Africa and presents a real threat of spreading to new geographical regions (Beard *et al*., 2010). Lumpy skin disease is endemic in Africa and has continued to be a big problem to farmers in Kenya as it results in loss of livestock and reduced production of skin, hides, and milk. This has been proved by the fact that out of the 130 samples analyzed, more than half were positive confirming the presence of the disease in the population. That is a disease prevalence rate of 61% was from the population studied. Outbreaks occur in different parts of the country the latest during this study in the year 2014, where outbreaks were reported in western Kenya (Kakamega - Malava) and North and West Pokot. These are areas on the Kenyan border with Uganda, and the transboundary movements of cattle were suspected to have resulted in these infections with approximately 1000 deaths reported before vaccination.

Confirmation of these outbreaks to ensure early control interventions has always been a challenge due to unreliable and nonspecific detection mechanisms and therefore this validated LAMP assay that was used to confirm the recent outbreaks may provide a solution to these problems. The LAMP assay proved to be very sensitive and specific in the detection of Capripoxvirus, with a sensitivity of 97%, that is, it is able to detect 97% of the animals with the disease; and specificity of 100%, that means all the samples with the negative results when using LAMP assay are truly negative. Sensitivity of conventional PCR was 45.6%; it is able to detect 45.6% of the animals with the disease and specificity of 100%, therefore all negative samples are truly negative.

The I00% Positive Predictive value (PPV) of the LAMP assay implies that all those samples that tested positive, the animal of origin had the disease and the 96% Negative predictive Value (NPV), that of all the samples that tested negative, 96% of the animals

do not have the capripox. All the samples that tested positive by Conventional implied that the animals had the disease and therefore a PPV of 100% and 54% of those that tested negative did not have capripox.

The LAMP assay analytical specificity was determined by testing the Capripoxvirus DNA against other closely related viruses like orf and PPR viruses. The Limit of detection (LOD) of the LAMP assay was determined to be 10^{-16} and that of real time PCR 10^{-18} using the same serially diluted sample for both the test. That is, the diagnostic and analytical sensitivity and specificity of real time PCR and LAMP assay were found to be in very close agreement. This is in line with what was proved by the developers of the LAMP assay (Das *et al*., 2012). The statistical analysis of the two methods using the Fisher's Exact Test indicated a p value of 8 (P=.8), at 95 % confidence level, meaning that there is no significant difference between the two diagnostic tests. Considering the simplicity of the LAMP assay compared to the gold standard method qPCR, and therefore its ability to be rolled out to the resource-poor Regional Veterinary Investigation laboratories (RVILs), it offers a better alternative and a reliable technique in CaPVs diagnosis. Real time PCR is complicated and requires delicate equipment that are very expensive to buy and maintain. This, therefore, will ensure quick, reliable and specific detection of the virus which can lead to early interventions in cases of disease presence with appropriate control measures. This assay has also been validated and adopted for CaPVs detection in other East African countries like Tanzania and Uganda.

Next generation sequencing as a technology that has been highly applied and recommended for diagnostic virology, though its main disadvantage has been its high cost (Barzon *et al.*, 2011). This study investigated its reliability in CaPVs diagnosis. It was found to be a 100% sensitive as all the samples sequenced that were LAMP positive, confirmed the presence of CaPVs on all NGS data sequence analysis. Its sensitivity can therefore be compared to that of LAMP assay as it was able to detect up to one contig of CaPVs.

Although Capripoxviruses are considered to be host specific, their host specificity is less stringent and the viruses may cross between the animals in close contact resulting in the genetic adaptation in the new host. A possibility of cross infection has been reported in different areas where the disease is endemic (Babiuk *et al*., 2008),(Le Goff *et al*., 2009). The Kenyan O-240 isolate (KS-1) from a sheep was confirmed to be LSDV (Kitching, 1986). This study also confirmed that some of the samples obtained from cattle turned out to be either SPV or GPV and vice versa on sequence analysis. Sequenced sample 11, a pure cell culture obtained from cattle and so named LSDV, on blast krona analysis turned out to be purely sheeppox virus (SPV). This therefore confirms that CaPVs are not host specific and the naming of the viruses by host of origin could be misleading.

Capripoxviruses are also able to cause mixed infection. The exact pattern of circulation of CaPVs between cattle, sheep and goats is yet to be established but it is a major factor. Blast analysis of sample by Krona of sample 9, a skin nodule bovine sample from Garisa showed a mixed infection of all the three CaPVs at GPV-18%, LSDV-15% and SPV-13%. This could mean that all the 3 viruses contributed to the infection. This shows the importance of one primer that is able to detect all the viruses making it easier to diagnose either one or all of the CaPVs, implement timely and effective control measures and also calls for the development of a combined vaccine.

The phylogenetic analysis showed that the Capripoxviruses studied present are diverse and includes all the three viruses with the three different lineages. The three lineages of CaPVs have been confirmed also by many other scientists (Tuppurainen *et al.,* 2014). The three genes used in the phylogenetic analysis are highly conserved among the CaPVs (Tulman et al., 2002). These genes were P32 gene which codes for the envelope protein, the GPCR which codes for the G-protein coupled chemokine receptor, a membrane bound protein involved in host immunomodulation and the RP030 gene which codes for the poly (A) polymerase small subunit (Tulman *et al.,* 2002). The phylogenetic analysis of the P32 gene showed the 3 main CaPVs lineages (Santhamani *et al*., 2015), with the SPV lineage having a 100% bootstrap value and LSDV and GPV lineages a bootstrap value of 96% and 99% respectively. However, the GPV lineage showed some 3 distinctive subgroups

of between 93-99% bootstrap values. One of the subgroup with a 99% bootstrap value had two Kenyan LSDV samples clustered together. That is, the Lumpivax vaccine (S18) and the sample (S17) that resulted in the most recent outbreak. The sample was obtained from a cattle and the vaccine is a Lumpy skin disease vaccine yet they all clustered together and in the same lineage with other GPVs from India, China, and Vietnam. This could be as a result of a possible mutation in the gene resulting in the differences and hence the formation of the sub-clusters. The LSDV lineage had no subgroups and all the samples clustered originated from cattle except one, (S12) from KALRO that was obtained from a sheep. The SPV lineage showed Kenyan samples (S16) and (S9) both from cattle clustering together with SPV from India China. The P32 gene that codes for the Viral coat protein/envelope protein is more suitable for epidemiological research because it contains more abundant information (Zeng *et al.*, 2014).

The G-protein-coupled chemokine receptor (GPCR) gene has mainly been used in the host-range grouping of capripoxviruses (Le Goff *et al.*, 2009). The phylogenetic analysis using this gene showed three tight genetic clusters consisting of goat pox, sheep pox and lumpy skin disease viruses. However, a few discrepancies exist with the classical virus-host origin nomenclature: a virus isolated from sheep is grouped in the goat poxvirus clade and vice versa. Intra-group diversity was further observed for the goat pox and lumpy skin disease virus isolates. All the samples that were grouped under LSDV lineage were suspected LSDV cases and they all clustered together. The phylogenetic analysis of the GPCR gene revealed the existence of the three distinct lineages (Santhamani *et al.,* 2014) with a bootstrap value of 95% and 100%.The LSDV lineage had all LSDV strains but clustered into two subgroups, with one of the subgroups having only one LSDV strain from South Africa. The GPV lineage had three distinctive subgroups as observed in the P32 gene analysis.

The RP030 gene codes for RNA polymerase subunit 30kD which is a subunit of the RNA polymerase complex that transcribed early, intermediate and late genes (Tulman *et al.,* 2002). CaPVs unlike many dsDNA viruses encodes their own replication machinery and replicate in the cytoplasm (Tulman *et al.,* 2001). The phylogenetic analysis of the gene

that encodes for RNA polymerase subunit also revealed the formation of the three distinct lineages: LSDV, GPV and SPV lineages with the LSDV lineage having two subgroups containing all LSDV stains. In one subgroup, bovine samples (S13) from Siaya, (S6) from Kakamega and (S1) from Kajiado clustered together with previously isolated LSDV strains from Kenyan and have been used as vaccines. The Lumpivax vaccine and the (S17) FROM Kakamega still clustered together under the GPV lineage while the SPV lineage had two samples, (S9), bovine sample from Garisa and (S11), bovine sample from KALRO, a likelihood of it being an SPV isolated from cattle.

Therefore, the phylogenetic study on these 3 genes confirms that the CaPVs can be divided into three distinct clusters, as previously shown using the comparison of the full genome sequences (Tulman *et al.*, 2002). Although GTPV and SPPV are distinct viruses, isolates of both are able to affect cattle in the field with or without differences in the degree of pathogenicity induced. This study shows that GTPV and LSDV are more closely related to each other than to SPPV. This would support the hypothesis that they both emerged from a common ancestor close to the SPPVs, as proposed by others who carried out phylogenetic studies on different genome segments (Hosamani *et al*., 2004; Stram *et al*., 2008) and also this study. This assumption is, however, in contradiction with another study, which concluded that small ruminant poxviruses may have emerged from a common LSDV-like ancestor based on the observation in GTPV and SPPV of disrupted LSDV genes with putative virulence and host range functions (Tulman *et al.*, 2002). The GTPVs, and to a lesser extent the LSDV, lineages show more diversity than the SPPV group in this study.

The multiple sequence alignment of the 3 genes supports the finding of the phylogenetic and blast sequence analysis. These conserved genes have virus specific signatures that can be used to differentiate the viruses and allows the grouping of the CaPVs into the 3 distinct groups (Santhamani *et al*., 2014). These specific signatures are made up of either amino acid deletions, insertions or substitutions which can result into the change of an entire protein. The amino acid sequence analysis confirmed the presence of this specific signatures in different viruses. In the GPCR gene, an amino acid addition or deletion in

positions 10–16 allowed the separation of LSDV isolates from other CaPVs, since they always had the complete sequence (Tulman *et al.,* 2002). Instead, LSDV isolates have the characteristic AT signature at positions 11–12. The gap at positions 10–16 cannot discriminate between SPPVs and GTPVs, since some of the GTPV isolates may display this gap, while others may not (Tuppurainen *et al.,* 2014b). However, GTPV isolates, which do not possess the gap, have a specific YA amino acid signature in positions 11–12 instead. This is why in the phylogenetic analysis of the gene, the GPV lineage had two sub groups both at 100% bootstrap value, with the group containing sample 17 and 18 having the YA amino acid and the other subgroup having gaps in these areas. The molecular characterization of the GPCR allows grouping the CaPVs since (A11, T12, T34, S99 and P199) is the unique signature of LSDVs, (N6, G10, Y11 and A12) or (V34, K49, F99 and S199) are unique signatures of GTPVs and (R6, R34, S99 and T199) is, mostly, a specific signature of SPPVs (Le Goff *et al.,* 2009). The P32 gene has an identity of 97.83% in LSDV and GPV with signature specific amino acids found at position 26, 135, 289, and 304. The entire gene in these two viruses is made up of 323 amino acids. The P32 gene in SPV is very much different from the other two viruses totaling up to 324 amino acid long due to an insertion of an aliphatic amino acid, leucine (L) at position 63. The SPV specific signatures are L at position 49 similar to that of the GPV but others are different from either of the two. There are amino acids D I at position 55, 56 instead of I S in GPV and LSDV, and Y K at position 60 and 61 in place of K F in GPV and LSDV.

The LSDV specific signatures are D_{26}, $F_{49,}$ H_{135} I_{289} and $D_{304,}$ in GPV specific signature being G_{26}, $L_{49,}$ Y_{135}, M_{289}, and N_{304} (Tulman *et al.,* 2002). In LSDV, aspartic acid at position 26, phenylalanine at position 49, histidine at position 135, Isoleucine at position 289 and aspartic acid at position 304 are replaced by glycine, leucine, tyrosine, methionine and asparagine respectively in GPV (Le Goff *et al.*, 2009). Samples 17 and 18 had the GPV specific signatures in multiple sequence analysis supporting their clustering in the GPV lineage in the Phylogenetics. Sequenced sample 9, a bovine skin nodule sample from Garisa and sample 16 a goat skin nodule sample from Isiolo had SPV specific signatures therefore based on the analysis of this gene they are SPV, also confirmed with

phylogenetic analysis. This therefore shows that SPV is able to affect both goats and cattle and therefore not specific to sheep as the name suggests. Multiple amino acid sequence alignment of the RP030 revealed LSDV and GPV specific signatures at position 15 and 98, 102, 135 and 141, that is LSDV $P_{15,}$ $P_{98,}$ $D_{102,}$ N_{135} and H_{141} while in GPV $T_{15,}$ $S_{98,}$ $N_{102,}$ D_{135} and $Y_{141.}$ The results of this analysis supports that of phylogenetic analysis.

This study therefore, supports the findings by IAEA Lab in Austria(Tuppurainen *et al.*, 2014b) and beliefs in some parts of sub-Saharan and central African countries, that the three capripox virus species co-exist causing diseases in cattle, sheep, and goats (Getachew Gari *et al.*, 2015). However, CaPVs nomenclature is still largely based on the host-species name, thus using currently available assays it is impossible to differentiate if the capripox disease in sheep is caused by a GTPV or a goat is affected by SPPV without genetic characterization of the virus isolate. Considering the high cost of gene sequencing, the method is not likely to be applied for routine screening and it is not affordable in most laboratories in the affected regions. It is well established that most of CaPVs strains especially those affecting small ruminants are not strictly host specific and can cross-infect both sheep and goats and cattle (Lamien *et al.*, 2011). The availability of a cost-effective diagnostic tool for routine determination of CaPVs genotype like LAMP assay will, therefore, assist to clarify the epidemiological picture in the affected regions.

The Lumpivax vaccine is an attenuated live vaccine currently used in the control of LSD in Kenya (OIE, 2010). Its phylogenetic analysis in this study showed that it has a goatpox virus lineage and clustered closely together with the Kakamega isolate (S17) which was isolated from the recent outbreak in Kenya. This was depicted through the analysis of all the 3 genes. It, therefore, confirms that the recent outbreak of the disease in cattle that was thought to be Lumpy skin disease virus just because it was in cattle could then have been caused by goatpox virus (GPV). Sequences analysis results have proved that there is a possibility of cross infection and mixed infection, more emphasis could be put on developing a combined vaccine that is able to offer cross protection against all the 3 viruses in all the animals. This can be more economical than using the different vaccines in cases of disease outbreaks or genotyping to determine the virus responsible for disease

outbreak before vaccination can be undertaken. The complains by farmers on the ground of disease upsurge in population after every vaccination could, therefore, be because of the assumption that is made every time there is a disease outbreak in cattle that it is LSDV that is causing the infection, yet it could be any of the 3 CaPVs. This, therefore, calls for the need of a common combined vaccine for all the CaPVs or a broad spectrum vaccine with high efficacy (Abutarbush *et al.*, 2014).

CaPVs strain with a broad host range infection may also serve as a potential vaccine candidate against all capripox diseases after attenuation. Specific, swift and accurate identification of CPVs causative agent can assist in selecting appropriate control and eradication measures, such as most suitable vaccines against the virus during outbreaks (Lamien *et al.*, 2013).

Nevertheless, just one gene is not enough to elucidate the genetic relatedness of capripoxviruses because of their huge size genomes. Multigenetic and genomic level study therefore greatly improves the accuracy and contribute to the general capripoxvirus epidemiological study.

5.2 CONCLUSIONS

The specificity of LAMP was 97% and specificity 100%, and based on Fishers exact test at 95% confidence interval, there is no significance difference between real time PCR and the LAMP assay.

Conventional PCR is not as sensitive as real time PCR and LAMP assay PCR in CaPVs detection.

Next generation sequencing (NGS) is applicable in diagnostic virology with very high sensitivity of 100%. It is important in the discovery, characterization and detection of viruses including novel pathogens. It can successfully be used in investigation of viral diversity, evolution and spread and therefore a very reliable tool in diagnostic virology.

There are 3 CaPVs lineages; LSDV lineage, GPV lineage and SPV lineage based on the phylogenetic analysis.

The CaPVs are no longer host specific. The sequence and phylogenetic analysis of CaPVs based on the three genes; GPCR, RP030 and P32 confirmed the presence of cross infection between cattle, sheep and goats as a result of SPV and GPV and also mixed infection in animals as a result of the presence of more than one of the CaPVs. The analysis of the 3 genetic signatures revealed that they can be used to differentiate and trace the source of capripoxviruses and thus penetrate the epidemic regulation making it possible to prevent and control capripox effectively.

Cattle are the mostly affected by capripoxvirus in Kenya. Lumpy Skin disease is the most common in all the studied population and Lumpy skin disease virus (LSDV) the most common virus. Out of the 130 samples analyzed, 119, (91.5%) were from cattle, 10, (7.7%) from goats and only 10, (0.8%) sample from sheep. Samples were collected based on the clinical symptoms of capripox.

The Lumpivax vaccine currently in use for Lumpy skin disease control has a GPV lineage and not LSDV lineage based on the phylogenetic analysis of the RP030, GPCR and P32 genes.

This study has shown that all the capripoxviruses viruses: SPV, GPV and LSDV are present in Kenya.

5.3 RECOMMENDATIONS

1. Considering the analytical and diagnostic sensitivities of LAMP assay and real time PCR being in close agreement, LAMP assay should be adopted as a diagnostic tool for use in developing countries where resources are limited due to its simplicity and cost effectiveness advantage.
2. Specific, swift and accurate identification of the CaPVs by molecular techniques (LAMP assay and real time PCR) can assist in selecting appropriate control and eradication measures such as most suitable vaccines against the virus during outbreaks and also enable early detection of the virus.
3. Genome sequences of SPV, GPV, and LSDV genomes are very similar to each other, averaging no less than 96% nucleotide identity over their entire length. Therefore, further studies on whole genome sequencing and animal experimentation will decipher the nature of these cross species infecting viruses and build a basis of molecular epidemiological studies.

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APPENDICES

Appendix 1: All the samples Analyzed

S. No.	Sample ID	Sample type	Animal of origin	Place of origin	N.A conc. (ng/ul)	RESULTS		
						LAMP	QPCR	Con PCR
1	B8/13	skin nodules	Cattle	Marsabit	2.9ng/ul	positive	positive	positive
2	B42/13 (A)	blood	Cattle	Homabay	134ng/ul	positive	positive	negative
3	B42/13 (A2)	skin nodules	Cattle	Homabay	145.6ng/ul	positive	positive	positive
4	B42/13 (B)	skin nodules	Cattle	Homabay	171.3ng/ul	positive	positive	positive
5	B43/13	skin nodules	Cattle	Kakamega	557.9ng/ul	positive	positive	positive
6	B83/13	blood	Cattle	Kisumu	311.1ng/ul	positive	positive	negative
7	B83/13 (A)	skin nodules	Cattle	Kisumu	181.3ng/ul	positive	positive	positive
8	B83/13 (B)	skin nodules	Cattle	Kisumu	221.4ng/ul	positive	positive	negative
9	B128/13	skin nodules	Cattle	Nakuru	552.6ng/ul	negative	negative	negative
10	G25/13	blood	Goat	Isiolo	32.4ng/ul	positive	positive	positive

11	G25/13	skin nodules	Goat	Isiolo	46.6ng/ul	positive	positive	positive
12	B227/13	blood	Cattle	Kiambu	39.9ng/ul	negative	negative	negative
13	B112/13	blood	Cattle	Isiolo	331.2ng/ul	negative	negative	negative
14	B115/13	blood	Cattle	Isiolo	74.9ng/ul	negative	negative	negative
15	B200/13	blood	Cattle	Isiolo	39ng/ul	negative	negative	negative
16	B204/13	serum	Cattle	Narok	8ng/ul	positive	positive	positive
17	B249/13(1)	blood	Cattle	Isiolo	27.7ng/ul	positive	positive	positive
18	B249/13(2)	blood	Cattle	Isiolo	82.7ng/ul	positive	positive	positive
19	B249/13(3)	blood	Cattle	Isiolo	55.4ng/ul	positive	positive	positive
20	B249/13(4)	blood	Cattle	Isiolo	63.8ng/ul	positive	positive	positive
21	B249/13(5)	blood	Cattle	Isiolo	69ng/ul	positive	positive	negative
22	B249/13(6)	blood	Cattle	Isiolo	56.5ng/ul	positive	positive	positive
23	B249/13(7)	blood	Cattle	Isiolo	40.5ng/ul	positive	positive	positive
24	B448/13	lymph node	Cattle	Siaya	452.3ng/ul	positive	positive	positive

25	B436/13	skin nodules	Cattle	Bungoma	182.6ng/ul	positive	positive	positive
26	B291/13	skin nodules	Cattle	Nakuru	40.9ng/ul	positive	positive	positive
27	B461/13	skin nodules	Cattle	Malaba	1257.9 ng/ul	positive	positive	positive
28	B461/13	blood	Cattle	Malaba	174.2ng/ul	positive	positive	negative
29	B338/13	blood	Cattle	Kitui	38.2ng/ul	positive	positive	positive
30	G26/13 (1)	serum	Goat	Baringo	10.4ng/ul	positive	positive	negative
31	G26/13 (2)	serum	Goat	Baringo	1O.3ng /ul	positive	positive	negative
32	G26/13 (3)	serum	Goat	Baringo	3.6ng/ul	positive	positive	negative
33	G26/13 (4)	serum	Goat	Baringo	7.1ng/ul	positive	positive	positive
34	G26/13 (5)	serum	Goat	Baringo	8.2ng/ul	positive	positive	positive
35	G26/13 (6)	serum	Goat	Baringo	4.3ng/ul	positive	positive	negative
36	G26/13 (7)	serum	Goat	Baringo	6.2ng/ul	positive	positive	negative
37	G26/13	skin nodule	Goat	Baringo	754.2ng/ul	positive	positive	positive
38	B426/13	skin nodules	Cattle	Wajir	2304.8 ng/ul	positive	positive	positive

39	B255/14 A1	blood	Cattle	Kitui	11.3ng/ul	negative	negative	negative
40	B255/14 A2	blood	Cattle	Kitui	55.7ng/ul	negative	positive	negative
41	B255/14 B	blood	Cattle	Kitui	54.9ng/ul	positive	positive	positive
42	B259/14 (SH)	blood	Cattle	Nairobi (Ruai)	48.6ng/ul	negative	negative	negative
43	B259/14 (KA)	blood	Cattle	Nairobi (Ruai)	11.4ng/ul	negative	negative	negative
44	B259/14 (NJ)	blood	Cattle	Nairobi (Ruai)	5.6ng/ul	positive	positive	negative
45	B259/14 (WAM)	blood	Cattle	Nairobi (Ruai)	9.8ng/ul	negative	negative	negative
46	B259/14 (IN)	blood	Cattle	Nairobi (Ruai)	20.4ng/ul	negative	negative	negative
47	B259/14 (NG)	blood	Cattle	Nairobi (Ruai)	10.1ng/ul	positive	positive	negative
48	B259/14 (SO)	blood	Cattle	Nairobi (Ruai)	31.3ng/ul	negative	negative	negative
49	B259/14 (WA)	blood	Cattle	Nairobi (Ruai)	12.5ng/ul	negative	negative	negative
50	B259/14 (LO)	blood	Cattle	Nairobi (Ruai)	11.3ng/ul	negative	negative	negative
51	B259/14 (SU)	blood	Cattle	Nairobi (Ruai)	16.ng/ul	positive	positive	negative
52	B259/14 (CH)	blood	Cattle	Nairobi (Ruai)	12.1ng/ul	positive	positive	negative

53	B259/14 (AL)	blood	Cattle	Nairobi (Ruai)	22.3ng/ul	negative	negative	negative
54	B259/14 (CE)	blood	Cattle	Nairobi (Ruai)	65.7ng/ul	negative	negative	negative
55	B259/14 (TO)	blood	Cattle	Nairobi (Ruai)	75.9ng/ul	negative	negative	negative
56	B259/14 (ED)	blood	Cattle	Nairobi (Ruai)	21.7ng/ul	negative	negative	negative
57	B259/14 (PA)	blood	Cattle	Nairobi (Ruai)	50.3ng/ul	negative	negative	negative
58	B259/14 (ME)	blood	Cattle	Nairobi (Ruai)	62ng/ul	positive	positive	negative
59	B259/14 (MA)	blood	Cattle	Nairobi (Ruai)	19.5ng/ul	negative	negative	negative
60	B259/14 (JO)	blood	Cattle	Nairobi (Ruai)	17.9ng/ul	positive	positive	negative
61	B259/14 (NA)	blood	Cattle	Nairobi (Ruai)	6.9ng/ul	positive	positive	negative
62	B259/14 (TS)	blood	Cattle	Nairobi (Ruai)	15.6ng/ul	positive	positive	negative
63	B238/14 A	skin nodules	Cattle	Meru	98.7ng/ul	positive	positive	positive
64	B238/14 B	blood	Cattle	Meru	233ng/ul	positive	positive	negative
65	B259/14 (CE)	blood	Cattle	Nairobi (Ruai)	49.8ng/ul	positive	positive	negative
66	B259/14 (SH)	skin nodules	Cattle	Nairobi (Ruai)	56.5ng/ul	positive	positive	positive

67	B259/14 (SO)	skin nodules	Cattle	Nairobi (Ruai)	18.5ng/ul	positive	positive	negative
68	B259/14 (WAM)	skin nodules	Cattle	Nairobi (Ruai)	2.8ng/ul	negative	negative	negative
69	B259/14 (IN)	skin nodules	Cattle	Nairobi (Ruai)	47ng/ul	positive	positive	negative
70	B259/14 (CH)	skin nodules	Cattle	Nairobi (Ruai)	54.4ng/ul	negative	negative	negative
71	B259/14 (KA)	skin nodules	Cattle	Nairobi (Ruai)	143.4ng/ul	negative	negative	negative
72	B259/14 (NA)	skin nodules	Cattle	Nairobi (Ruai)	1.7ng/ul	negative	negative	negative
73	B259/14 (NG)	skin nodules	Cattle	Nairobi (Ruai)	53.8ng/ul	positive	positive	positive
74	B259/14 (ED)	skin nodules	Cattle	Nairobi (Ruai)	36.8ng/ul	negative	negative	negative
75	B259/14 (AL)	skin nodules	Cattle	Nairobi (Ruai)	20.9ng/ul	positive	positive	negative
76	B259/14 (WA)	skin nodules	Cattle	Nairobi (Ruai)	4ng/ul	negative	negative	negative
77	B259/14 (TS)	skin nodules	Cattle	Nairobi (Ruai)	133.9ng/ul	negative	negative	negative
78	B259/14 (LO)	skin nodules	Cattle	Nairobi (Ruai)	37.1ng/ul	positive	positive	positive

79	B259/14 (TO)	skin nodules	Cattle	Nairobi (Ruai)	10.7ng/ul	positive	positive	negative
80	B265/14 (1)	blood	Cattle	Nyeri	9.1ng/ul	negative	negative	negative
81	B265/14 (2)	blood	Cattle	Nyeri	53.6ng/ul	negative	negative	negative
82	B265/14 (3)	blood	Cattle	Nyeri	33.4ng/ul	negative	negative	negative
83	B235/14	skin nodules	Cattle	West Pokot	826.8ng/ul	positive	positive	positive
84	B347/14 A1	skin nodules	Cattle	Kajiado	1372.3 ng/ul	positive	positive	positive
85	B347/14 A2	blood	Cattle	Kajiado	120.2ng/ul	positive	positive	positive
86	B347/14 A3	blood	Cattle	Kajiado	173.4ng/ul	positive	positive	negative
87	B276/14	skin nodules	Cattle	Garisa	135.2ng/ul	positive	positive	positive
88	B432/14	skin nodules	Cattle	Kakamega	896.7ng/ul	positive	positive	positive
89	LSDV	cell cultures	Cattle	KALRO	21.6ng/ul	positive	positive	positive
90	GPV	cell cultures	Goat	KALRO	83.6ng/ul	positive	positive	positive
91	SPV	cell cultures	Sheep	KALRO	24.1ng/ul	positive	positive	positive
92	LUMPIVAX	vaccine	Vaccine	KEVEVAPI	12.3ng/ul	positive	positive	positive

93	B01/15 (1)	blood	Cattle	West Pokot	1500.7 ng/ul	positive	positive	negative
94	B01/15 (2)	blood	Cattle	West Pokot	789ng/ ul	positive	positive	negative
95	B01/15 (3)	blood	Cattle	West Pokot	954.3n g/ul	positive	positive	negative
96	B01/15 (4)	blood	Cattle	West Pokot	456.8n g/ul	negativ e	negative	negative
97	B01/15 (5)	blood	Cattle	West Pokot	143.2n g/ul	negativ e	negative	negative
98	B01/15 (6)	blood	Cattle	West Pokot	56.9ng/ ul	negativ e	negative	negative
99	B01/15 (7)	blood	Cattle	West Pokot	98.5ng/ ul	positive	positive	negative
100	B01/15 (8)	blood	Cattle	West Pokot	13ng/ul	negativ e	negative	negative
101	B01/15 (9)	blood	Cattle	West Pokot	65.41n g/ul	negativ e	negative	negative
102	B01/15 (11)	blood	Cattle	West Pokot	56.7ng/ ul	negativ e	negative	negative
103	B01/15 (12)	blood	Cattle	West Pokot	76.2ng/ ul	negativ e	negative	negative
104	B01/15 (13)	blood	Cattle	West Pokot	89.7ng/ ul	positive	positive	negative
105	B01/15 (14)	blood	Cattle	West Pokot	41.4ng/ ul	positive	positive	negative
106	B01/15 (15)	blood	Cattle	West Pokot	62.1ng/ ul	positive	positive	negative

107	B01/15 (16)	blood	Cattle	West Pokot	58.3ng/ul	negative	negative	negative
108	B02/15 (1)	blood	Cattle	North Pokot	10.3ng/ul	negative	negative	negative
109	B02/15 (2)	blood	Cattle	North Pokot	113.6ng/ul	negative	negative	negative
110	B02/15 (3)	blood	Cattle	North Pokot	65.7ng/ul	positive	positive	negative
111	B02/15 (4)	blood	Cattle	North Pokot	32.3ng/ul	negative	negative	negative
112	B02/15 (5)	blood	Cattle	North Pokot	16.2ng/ul	positive	positive	negative
113	B02/15 (6)	blood	Cattle	North Pokot	31.7ng/ul	negative	negative	negative
114	B02/15 (7)	blood	Cattle	North Pokot	24.1ng/ul	positive	positive	negative
115	B02/15 (8)	blood	Cattle	North Pokot	11.9ng/ul	positive	positive	negative
116	B02/15 (9)	blood	Cattle	North Pokot	14.7ng/ul	negative	negative	negative
117	B02/15 (10)	blood	Cattle	North Pokot	17.9ng/ul	negative	negative	negative
118	B02/15 (11)	blood	Cattle	North Pokot	32.7ng/ul	negative	positive	negative
119	B02/15 (12)	blood	Cattle	North Pokot	75.4ng/ul	negative	negative	negative
120	B02/15 (13)	blood	Cattle	North Pokot	205.7ng/ul	negative	negative	negative

121	B02/15 (14)	blood	Cattle	North Pokot	47.1ng/ ul	negativ e	negative	negative
122	B02/15 (15)	blood	Cattle	North Pokot	19.6ng/ ul	negativ e	negative	negative
123	B02/15 (16)	blood	Cattle	North Pokot	45.7ng/ ul	negativ e	negative	negative
124	B02/15 (17)	blood	Cattle	North Pokot	376.9n g/ul	negativ e	negative	negative
125	B02/15 (18)	blood	Cattle	North Pokot	639.2n g/ul	negativ e	negative	negative
126	B02/15 (19)	blood	Cattle	North Pokot	265.3n g/ul	negativ e	negative	negative
127	B02/15 (20)	blood	Cattle	North Pokot	2004.1 ng/ul	negativ e	negative	negative
128	B02/15 (21)	blood	Cattle	North Pokot	897.0n g/ul	negativ e	negative	negative
129	B02/15 (22)	blood	Cattle	North Pokot	439.3n g/ul	negativ e	negative	negative
130	B02/15 (23)	blood	Cattle	North Pokot	126.4n g/ul	negativ e	negative	negative

Appendix 2: Amino acid Sequences of the GPCR gene of other CaPVs strains from used in analysis

Name	Size	Start of sequence	Latin name
FJ869351SPV	375	MNYTLRTVSSSNITTIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Sheeppox virus Tunisia/01 9P2
FJ869361GPV	375	MNYTLRTVSSSNITTIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Goatpox virus Sudan
FJ869369LSDV	378	MNYTLSTVSSATMYNSSSNITTIATTIISTISTNQNNVTTPSTYENTTTI	Lumpy skin disease virus Sudan/06 Obied
FJ869373LSDV	378	MNYTLSTVSSATMYNSSSNITTIATTIISTISTNQNNVTTPSTYENTTTI	Lumpy skin disease virus RSA/07 Brahman
FJ869376LSDV	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	Lumpy skin disease virus RSA/54 Haden
FJ869377LSDV	378	MNYTLSTVSSATMYNSSSNITTIATTIISTISTNQNNVTTPSTYENTTTI	Lumpy skin disease virus Egypt/89 Ismalia
FJ869379SPV	375	MNYTLRTVSSSNITTIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Sheeppox virus Niger/88
FJ869380SPV	375	MNYTLRTVSSSNITTIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Sheeppox virus Senegal Sangalcam/88
FJ869387SPV	375	MNYTLRTVSSSNIITIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Sheeppox virus Nigeria/77
FJ869389SPV	375	MNYTLRTVSSSNITTIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Sheeppox virus Turkey/98 Van2
JQ310667GPV	375	MNYTLSTVSSSNITTIATTIISTILSVISTNQNNVTTPSTYKNTTTISNY	Goatpox virus
JQ310672GPV	375	MNYTLSTVSSSNITTIATTIISTILSVISTNQNNVTTPSTYKNTTTISNY	Goatpox virus
KF495243GPV	375	MNYTLSTVSSSNITTIATTIISTILSVISTNQNNVTTPSTYKNTTTISNY	Goatpox virus

KF661979SPV	375	MNYTLRTVSSSNITTIATTIISTILSRISTNKNNVTTPSTYENTTAISNY	Sheeppox virus
KJ818279GPV	382	MNYTLNTVSGYAMYNSSSNITTIATTIISTILSVILTNQNNVTTPSTYKN	Goatpox virus
KJ818280GPV	382	MNYTLNTVSGYAMYNSSSNITTIATTIISTILSVILTNQNNVTTPSTYKN	Goatpox virus
KJ818281LSDV	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	Lumpy skin disease virus
KJ818282LSDV	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	Lumpy skin disease virus
KJ818283LSDV	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	Lumpy skin disease virus
REF_LSDV011	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	Lumpy skin disease virus NI-2490
S7	382	MNYTLSTVSSATMYNSSSNITTIATTIITTIISTISTNQNNVTTPSTYEN	
S8	382	MNYTLSTVSSATMYNSSSNITTIATTIISTIISTISTNQNNVTTPSTYEN	
S9	380	MNYTLSTVSSATMYNSSSNITTIATTIISTILSRISTNKNNVTTPSTYEN	
S11	379	MNYTLRTVSQPCIIVAVILPL*LLQLLVQFSVEFQQIKIMLQRLQLMKIR	
S12	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	
S13	382	MNYTLSTVSSATMYNSSSNITTIATTIISTILSTISTNQNNVTTPSTYEN	
S16	376	MNYTLRTL*QLVAVILPL*LLQLLVQFSVEFQQIKIMLQRLQLMKIRQQY	
S17	382	MNYTLNTVSGYAMYNSSSNITTIATTIISTILSVILTNQNNVTTPSTYKN	
S18	382	MNYTLNTVSGYAMYNSSSNITTIATTIISTILSVILTNQNNVTTPSTYKN	