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Department of Biotechnology

Molecular and Biological Characterization of the Capripoxviruses and the Development of Molecular Diagnostics Assays.

A dissertation presented to the Department of Biotechnology, the University of National Resources and Life Sciences, Vienna (BOKU), in the partial fulfillment of the requirements for the degree of Doctor of Philosophy

BY

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Abbreviations

CaPV	Capripoxvirus
CARD	Caspase activating recruitment domain
CPE	Cytopathic effect
CTL	Cytotoxic T lymphocyte
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
DIVA	Differentiating Infected from Vaccinated Animals
EEV	Extracellular enveloped virus
ELISA	Enzyme-Linked Immunosorbent Assay
EFSA	European Food Safety Authority
ESH-L cells	Embryonic skin cell line from sheep
EU	European Union
EV	Enveloped virion
G	Guanine
GPCR	G-protein-coupled receptor
GTP	Goatpox
GTPV	Goatpox virus
HRM	High Resolution Melt
ICTV	International Committee for the Taxonomy of Viruses
IEV	Intercellular enveloped virion
IFAT	Indirect fluorescent antibody test
IFNAR	interferon-alpha receptor
IFN	Interferon
IKK	IkB kinase
IKK α	IκB kinase alpha
IMP	Inflammation modulatory protein
IMV	Internal mature virus
IRF3	interferon regulatory factor 3
IL-1β	Interleukin-1 Beta

ISG	Interferon simulated gene
ITRs	Inverted terminal repetitions
Kbp	kilo base pairs
LAMP	Loop-mediated isothermal Amplification
LAV	Live attenuated vaccine
LPS	Lipopolysaccharides
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
MV	Mature virion
MHC-I	Major histocompatibility complex class I
MAVS	Mitochondrial antiviral signalling protein
MDA5	Melanoma differentiation factor-5
mRNA	Messenger Ribonucleic acid
MyD88	Myeloid differentiation primary response 88
NF-κB	nuclear factor kappa B
NK cells	Natural killer cells
nt	Nucleotides
nm	Nano meter
OA3.Ts	Ovine testis cell line
OIE	World Organization for Animal Health
ORF	Open reading frames
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymer chain reaction
PRRs	Pattern recognition receptors
RIG-I	Retinoic acid inducible gene I
RLR	RIG-Like receptor
RNA	Ribonucleic acid
RPO30	RNA polymerase subunit 30 kD gene
SPP	Sheeppox
SPPV	Sheeppox virus
ss	Single stranded
T	Thymine
Th	T helper cells

TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
VACV	Vaccinia virus
vEGF	viral epidermal growth factor
vVEGF	viral Vascular endothelial growth factor.
WAHID	World Animal Health Information Database
WV	wrapped virion
WT	Wild type

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Abstract

Sheeppox (SPP), goatpox (GTP) and lumpy skin disease (LSD) are three important pox diseases of sheep, goat, and cattle. The responsible viruses, sheeppox virus (SPPV) and goatpox virus (GTPV) infecting both sheep and goats, and LSD virus (LSDV) of cattle, belong to the genus *Capripoxvirus* (CaPV) of the family Poxviridae.

Live attenuated vaccines are available for the control of these viruses. However, the efficacy and safety are not always satisfactory; therefore, studies are necessary to understand the immunology of SPPV infections, and we also need tools to monitor the animals to distinguish between vaccinated and infected ones. Besides, molecular characterization can also help to detect new variants of the virus and trace the source of infection during disease outbreaks.

To differentiate LAV SPPV from WT SPPV, first, we identified an 84-nucleotide deletion between the DNA ligase gene and the variola virus B22R homolog gene of LAV SPPV and developed a gel-based PCR method. As this method did not differentiate WT SPPV from GTPV and LSDV, we designed a High-resolution melting (HRM) method exploiting specific markers in the CaPV B22R gene, which was successful in differentiating LAV SPPV from WT SPPV, GTPV, and LSDV. These two assays are a valuable tool for epidemiological investigations during vaccination programs. Second, we investigated LSDVs from East Africa collected between 2007 and 2012. We discovered that LSDV Embu/B338/2011, a field isolate from Kenya presented mixed characteristics of the LSDV vaccine and WT isolates, highlighting the need for constant monitoring of LSDV genetic variability.

Third, we studied the GPCR gene of GTPVs using an alignment-based and an alignment-free method. This work enabled us to follow the segregation of GTPVs based on their geographical origin using the alignment-free method. Thus, this approach can help determine the source of GTPV responsible of an outbreak in disease-free countries and detect the introduction of new strains in disease-endemic countries. Finally, we investigated the expression of thirteen genes involved in the innate immune response in sheep peripheral blood mononuclear cells (PBMC) and simulated in vitro by quantitative real-time PCR. The results showed that both WT and LAV infections in sheep upregulated the expression levels of RIG-1, TNFA, IL-15, and IL-10 ($P < 0.05$). The expression of RIG-1 was significantly higher in WT than LAV. These findings advance our knowledge on SPPV induction of a protective immune response and will further help to develop safer and more potent vaccines against SPP and GTP.

Keywords: Capripoxvirus, Molecular characterization, Assay development, Gene expression

Kurzfassung

Sheeppox (SPP), Goatpox (GTP) und Lumpy Skin Disease (LSD) sind drei wichtige Pockenviruserkrankungen bei Schafen, Ziegen und Rindern. Während Schafpockenviren (SPPV) und Ziegenpockenviren (GTPV) beide sowohl Schafe als auch Ziegen infizieren können, befällt das Lumpy Skin Desease-Virus (LSDV), welches in die Familie der Capripox-Viren (CaPV) gehört, hauptsächlich Rinder. Eine Reihe von Lebendinpfstoffen steht als Präventivmaßnahme zur Verfügung. Allerdings ist deren Wirksamkeit und Sicherheit derzeit nicht zufrieden stellend, wodurch es immer wieder zu Neuinfektionen durch den Impfstoff und fehlenden Impfschutz kommt. Aus diesem Grund ist es wichtig, molekularbiologische Methoden zu entwickeln, um einerseits die Wechselwirkung mit dem Immunsystem der Tiere besser zu verstehen, und andererseits ein breites Monitoring durchführen zu können, welches zwischen infizierten und geimpften Tiers unterscheiden kann.

Um zwischen dem Lebendimpfstoffstamm gegen SPPV (LAV-SPPV) und dem wild-typ SSPV (WT-SSPV) unterscheiden zu können, wurde zunächst eine 84 Nukleotid lange Deletion zwischen dem Ligasegen und dem Vriolavirus B22r homologen Gen, die in LAV-SPPV identifiziert wurde heramngezogen. Die von uns dafür enwickelte gelbasierte PCR-Methode konnte in weiterer Folge jedoch nicht zwischen WT-SPPV und WT-GTPV, beziehungsweise WT-LSDV. Daher wurde eine hochauflösende „High Resolution Melting"-Methode (HRM) entwickelt, die eine genaue Differenzierung der einzelnen Stämme ermöglichte. Beide Untersuchingsmethoden sind wichtige und wertvolle Werkzeuge für epidemologische Untersuchungen während der Impfkampagnen. Wir untersuchten LSDVs aus Ostafrika, die zwischen 2007 und 2012 isoliert wurden. Wir entdeckten, dass LSDV Embu/B338/2011, ein Isolat aus Kenia, eine Mischung aus dem WT-LSDV und dem dazugehörigen Lebendimpfstoffstamm darstellt. Diese Erkenntnis zeigt, wie wichtig ein ständiges Monitoring der im Umlauf befindlichen Virusstämme ist.

Um mehr Einblick in die Epidemologie zu erlangen wurden molkularbiologische Untersuchungen anhand des GPCR-Gens von GTPV durchgeführt. „Alignment"- und „Alignment free" basierte Methodenerlauben die Segregation von GTPV zu verfolgen, und so die Quellen neuer Ausbrüche zu identifizieren, wie auch voraus zu sehen.

Bezüglich besseren Verständnisses der immunologischen Wechselwirkung zwischen Virus und Wirt wurden 13 Gene, die maßgeblich an der angeborenen Immunantwort beteiligt sind, in „Peripheral blood mononuclear cells" (PBMC) aus Schafen mittels quantitativer PCR analysiert. Es zeigte sich, dass vier der analysierten Gene (RIG-1, TNFA, IL-15, IL-10) sowohl in WT-SPPV als auch in LAV-SPPV deutlich hochreguliert waren und ein Gen (RIG-1) in WT-SPPV im

Vergleich zu LAV-SPPV nochmals deutlich erhöht war. Diese und weitere Erkenntnisse über protektive Immunantwort werden dazu beitragen effektivere und sichere Impfstoffe gegen SPPV und GTPV zu entwickeln.

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Introduction

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Introduction

General information of Poxviruses

Poxviruses contain a large linear double-stranded deoxyribonucleic acid (dsDNA) genome that can have a length of 130 to 375 Kbp. This large genome contains genes encoding all the proteins necessary for their unique cytoplasmic replication. Poxviruses replicate almost entirely independently from the infected cell nucleus (Moss, 2013). The most important of the viral proteins are required for transcription, replication, and virion assembly. Besides, several non-essential proteins are playing an important role in modulating the host's immune response to infection (Walsh, 2017; Farré et al. 2017).

Classification of Poxviruses

The International Committee for the Taxonomy of Viruses (ICTV) has divided the Poxviridae family into two subfamilies based on their host range: Entomopoxvirinae (insect-infecting viruses) and Chordopoxvirinae (vertebrate-infecting viruses) (Oliveira et al., 2017). The Chordopoxvirinae consists of eight genera; *Avipoxvirus*, *Capripoxvirus*, *Leporipoxvirus*, *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, *Suipoxvirus*, and *Yatapoxvirus* whereas Entomopoxvirinae subfamily comprises three genera; *Alphaentomopoxvirus*, *Betaentomopoxvirus*, and *Gammaentomopoxvirus* (Oliveira et al. 2017) (Table 1).

Owing to their medical and veterinary relevance (Oliveira et al., 2017), Chordopoxviruses are among the most studied groups in virology.

Poxvirus morphology and genome

Poxvirus particles measure around 250 nm in diameter and 360 nm in length with a characteristic round brick-shape. During their release by budding, the virions will acquire an extra envelope containing cellular lipids and several viral encoded proteins (Appleyard et al., 1971) (Figure 1).

The poxvirus virions also contain a multi-subunit of viral deoxyribonucleic acid (DNA) dependent ribonucleic acid (RNA) polymerase, a messenger RNA (mRNA) capping enzyme and other factors required during the early stages of transcription (Senkevich et al., 2005).

Poxviruses possess of a single linear dsDNA with termini that form covalently closed hairpin loops. These genomes encode between 130 putative genes (Orf virus) to 328 genes (Canarypox virus). Approximately 100 of the genes are common to all chordopoxviruses, and half of those genes are also present in entomopoxviruses (Schramm & Locker, 2005; Willer et al., 1999).

Table 1.Classification of poxviruses and their natural hosts

Subfamily	Genus	Virus species	Host
Chordopoxvirinae	*Orthopoxvirus*	Variola virus	Humans
		Vaccinia virus	Humans, cattle, buffalo, swine and Rabbits
		Cowpox virus	Rodents, cats, cattle, human elephant, rhinoceros, Okapi
		Camelpox virus	Camels
		Ectromelia virus	Mice, voles
		Monkeypox virus	Monkeys, squirrels, anteaters, great apes, humans
		Horsepox virus	Horse
		Taterapox virus	Gerbils
		Racconpox virus	Raccoons
		Volepox virus	Voles
		Skunkpoxvirus	Skunks
	Capripoxvirus	Sheeppox virus	Sheep, Goats
		Goatpox virus	Goats, Sheep
		Lumpy skin disease	Cattle, water buffalo
	Suipoxvirus	Swinepox virus	Swine
	Leporipoxvirus	Hare fibroma virus	*European hare*
		Myxoma virus, Rabbit fibroma virus	*Rabbits*
		Squirrel fibroma virus	Squirrel
	Molluscipoxvirus	Molluscum contagiosum virus	Humans, primates, birds, kangaroos, dogs and equids
	Yatapoxvirus	Tanapox virus	Humans
		Yaba pox virus	Monkeys
	Avipoxvirus	Fowlpox virus, canarypox virus, crowpox virus, juncopox virus, mynahpox virus, pigeonpox virus, psittacinepox virus, quailpox virus, sparrowpox virus, starlingpox virus, turkeypox virus	Birds, chicken, turkey and other
	Parapoxvirus	Bovine popular stomatitis virus	Cattle, human
		Orf virus	Sheep, goats, humans
		Pseudo cowpox virus	Cattle, human
Entomopoxvirinae	*Alphaentomopox virus*	Melolontha melolontha entomopoxvirus	Beetles

	Betaentomopox virus	Amsacta moorei entomopoxvirus	Butterflies, locusts grasshoppers and moths
	Gammaentomop oxvirus	Chironomusluridus entomopoxvirus	Flies and mosquitoes
Unclassified		Poxvirus of fish carp edema and proliferative gill disease virus	Koi (Cyprnus carpio) Athlantic salmon (Salmo salar)
		Squirrel poxvirus	Read and Gray Squirrel
		Mule deer poxvirus	Deer
		Nile crocodilepox virus	Crocodiles

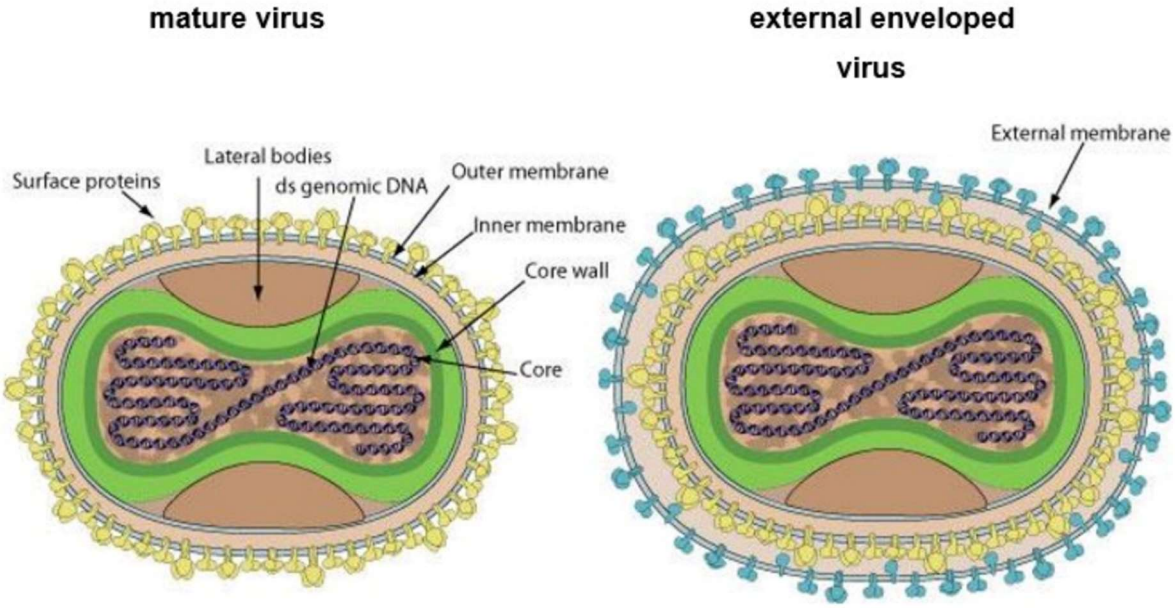

Figure 1. Poxvirus infectious particles. The mature virus (left) has one membrane layer less than the external enveloped virus (right). The virions are brick-shaped or ovoid, with the outer membrane surface composed of a complex combination of viral proteins, which encapsulates a walled, biconcave core and two lateral bodies. Packaged within the core is a collection of viral enzymes involved in virus uncoating and genome replication, and a single molecule of double-stranded DNA (Source: www.poxvirus.org).

The essential genes such as those required for the transcription of viral genes, genome replication, and virion assembly, located in central position of the viral genome, are highly conserved among the various poxviruses (Schmid et al., 2014). Poxvirus genome contains variable lengths of inverted terminal repetitions (ITRs), consisting of identical but oppositely oriented sequences at both ends of the genome (Baroudy et al., 1982; Moss, 2013). Next to the ends of the ITRs, there are short tandem repeated sequences involved in recombination events (Wittek et al., 1980; Figure 2)

The genes are arranged in the genome according to their functions. For instance, those required for virus growth aggregate around the central part of the genome (Gubser & Smith, 2002; Mackett & Archard, 1979). Genes situated outside this region are generally related to immune modulation, providing each poxvirus species with unique and specific characteristics (Gubser and Smith 2002; Seet et al., 2003; Werden et al., 2008).

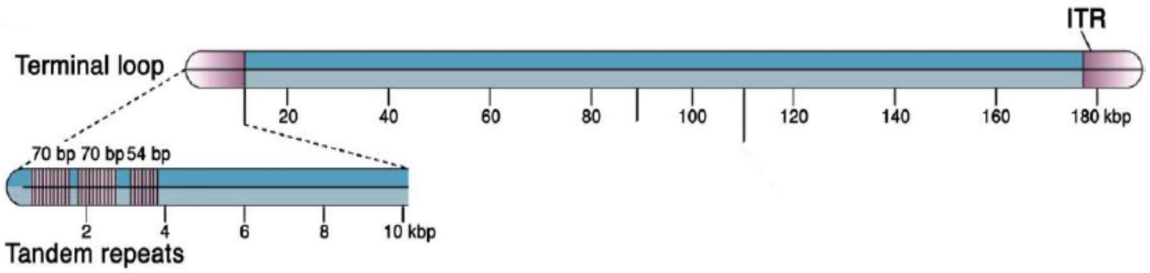

Figure 2. Scheme of the vaccinia (VACA) virus genome: The double stranded DNA with the central core region containing highly conserved genes enclosed by terminal hairpin loops. The enlarged left ITR indicates the typical tandem repeats. (*Source: Principles of Virology*).

The open readings frames (ORFs) of poxviruses are named based on the fragments of the genome resulting from *HindIII* restriction enzyme digestion of the Vaccinia virus strain Copenhagen (VACV-Copenhagen) strain. There are labeled from A to O in decreasing order based on the molecular weight of the fragments. Within each fragment, genes are numbered from left to right (except for fragment C which is reversed). ORFs are given the designation left (L) or right (R), depending on the direction (Werden et al. 2008). For example, A5R is the 5th ORF in the largest *HindIII* fragment and transcribed from left to right, and the protein referred to as A5.

Poxvirus replication cycle

Poxviruses replicate exclusively in the cytoplasm of infected cells in structures called viral factories or virosomes (Moss, 2013; Minnigan & Moyer, 1985). There are different forms of infectious poxvirus particles: the mature virions (MV), the internal mature virus (IMV) and extracellular enveloped virus (EEV). They are structurally similar, but EEV carry an additional outer lipid membrane (Vanderplasschen et al., 1998; Figure 1). A single virion contains the complete transcription system essential for the synthesis of viral mRNA (Li et al., 2009).

Poxvirus infection and replication begins with membrane binding and virion entry into a susceptible cell (McFadden, 2005; Figure 3) through a macropinocytosis process, allowing the release of the core into the cytoplasm. Then, poxvirus RNA polymerase and encapsulated transcription factors initiate the first wave of early viral gene transcription (Broyles, 2003). After the transcription and translation of early genes, the core translocates on microtubules to specific cytoplasmic compartments and forms a viral factory (Tolonen et al., 2001) where the early poxvirus proteins accumulate (Katsafanas & Moss, 2007).

Number of poxvirus gene products are immunomodulatory and host-range factors preparing the intracellular environment for genome replication and transcription of intermediate viral genes (Sarov & Joklik, 1972). Soon after the beginning of DNA replication, the transcription of intermediate genes, encoding for regulatory proteins take place, initiating the transcription of late genes. Then follows the virion assembly with the formation of distinct membrane structures. The late transcription stages require the interaction with host-derived transcription factors (Rosales et al., 1994; Sanz & Moss, 1998; Katsafanas & Moss, 2004).

The newly synthesized viral particles containing structural proteins, enzymes, and early transcription factors are wrapped by membranes derived from virus-modified trans-Golgi or endosomal cisternae, containing at least seven viral proteins, and released from the cell by exocytosis (Smith & Law, 2004; McFadden, 2005).

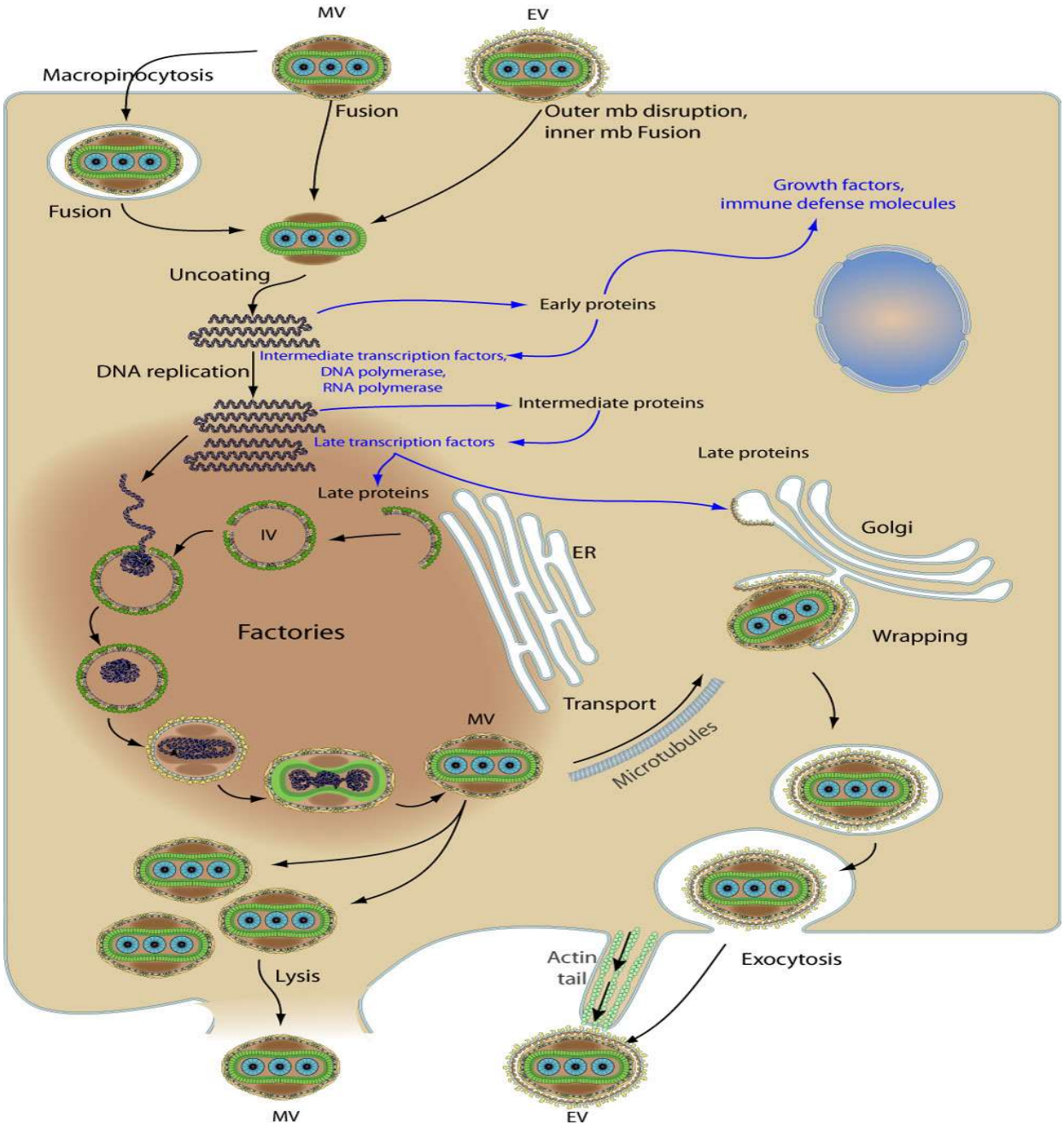

Figure 3. Poxvirus replication cycle. After the entry of EV or MV, the core is released into the host cells cytoplasm. Within the core, the early gene expression starts immediately. In a process, which is also called uncoating the virus DNA is set free and DNA replication starts. As the DNA replication is the pre-requirement for intermediate and late transcription the cascade regulated transcription can proceed. The virus assembly begins with the formation of crescents. After DNA uptake the closed particle matures and are transported to the Golgi apparatus by microtubules. Following Golgi wrapping the WV are directed to the cell surface again mediated by microtubules. The virions are released by budding (enveloped virions) or cell lysis (non-enveloped virions) (Source: McFadden, 2005).

The innate immune system

The interaction between the host immune system and the virus during poxvirus infection is mediated primarily by innate effectors cells such as macrophages, natural killer cells (NK cells) and dendritic cells, whereas adaptive antigen-specific T and B cell responses are essential for the clearance of the virus and the establishment of immunity (Haga & Bowie, 2005). A good understanding of these interaction benefits enormously to design novel vaccines and antiviral drugs.

The rapid detection of invading pathogens and the recruitment and activation of cells of both the innate and the adaptive immune system sketches by the innate immune system is essential for the control of the infection. The innate immune system uses pattern recognition receptors (PRRs) to recognize pathogen-associated molecular patterns (PAMPs). PRRs related to poxvirus are Toll-like receptors (TLR)s, RIG-I-like receptors (RLRs), and DNA sensor molecules. Each TLR detects distinct PAMPs derived from pathogens, such as lipoproteins and lipopolysaccharides (TLR2 and TLR4) (Mukherjee et al., 2016). RIG-I like receptors recognize dsRNA from DNA virus produced during transcription (Kawai & Akira, 2006; Kawai & Akira, 2007). Upon recognition of respective PAMPs, TLRs recruit specific adaptor molecules, such as myeloid differentiation primary response gene 88 (Myd88) and TIR-domain-containing adapter-inducing interferon-β (TRIF). The engagement of RIG-1 like receptors recruits TNF receptor-associated factor-3 (TRAF3), with their specific ligands leading to the activation of the inhibitors of the kB (IkB) kinase (IKK) complex. The IKK complex subsequently phosphorylates IkBα polyubiquitination and proteasomal degradation. This liberates the bound p50: p65, which then translocates to the nucleus (Figure 4).

A common signalling event of the PRRs leads to the activation of transcription factors such as nuclear factor k-light-chain-enhancer of activated B cells (NF-κB) and Interferon regulatory factor 3 (IRF3). The activation of these factors further activates downstream signalling events leading to the secretion of induction of cytokines, chemokines, and type I interferons (IFN). Cytokines including interleukins (ILs), tumor necrosis factor (TNF) and interferons (IFNs) play diverse role, such as initiating cell death and inducing an antiviral state within cells (Akira et al., 2006; Medzhitov & Janeway, 2000; Yoneyama & Fujita, 2010; Kawai and Akira 2011).

After induction, all type I IFNs bind to one common type I IFN alpha receptor (IFNAR) which leads to downstream signalling resulting in the expression of more than 300 interferon-stimulated gene (ISG) (Honda et al., 2006; Samuel, 2001). The ISGs are involved in eliminating viral components from infected cells, inducing apoptosis of infected cells, and confer resistance to viral infection on uninfected cells (de Veer et al., 2001). Nevertheless, the effects of IFNs are not limited to induction of ISGs; they also have a great impact on systemic immunity (Wong and Chen, 2016).

Indeed, it has been reported that IFNs are involved in the maturation of DCs, including the cross-presentation of viral antigens for CD8+ T cell and the activation of NK cells (Burshtyn, 2013; Stetson & Medzhitov, 2006).

Chemokines are also chemoattractant molecules and play a critical role in modulating the innate and adaptive host responses to viral infections. They are a key factor in the initiation of inflammation and recruitment of monocytes, memory T cells, and dendritic cells to the site of infection (Esche et al., 2005).

Poxvirus immune evasion

Poxviruses encode many proteins that are dedicated to host immune evasion. Some of these neutralize complement factors, interferons, cytokines, and chemokines (Seet et al., 2003; Haga & Bowie, 2005) while other inhibit apoptosis or signalling pathways that lead to the production of interferons and pro-inflammatory cytokines and chemokines. The signalling scheme presented in Figure 4 shows cytokine induction via RIG-I and NF-κB pathways. The sites of intervention by several antagonists encoded by poxviruses are indicated (Figure 4). Multiple poxviral immunomodulatory proteins have been identified and broadly divided by function into three strategic classes, which referred to as; virostealth, virotransducers, and viromimetics (Johnston & McFadden, 2004; Figure 5). Virostealth mask the visible signals associated with infection, mostly by downregulating antigen recognition or blocking the presentation of viral antigen to immune cells (Moss & Shisler, 2001; Johnston & McFadden, 2003).

Virotransducers are intracellular viral proteins that inhibit innate antiviral pathways, such as apoptosis, pro-inflammatory cascades, or the induction of the antiviral state (Seet et al. 2003). Virotransducers also target host signal transduction pathways that influence host range. Vaccinia virus also encodes several immunomodulatory proteins that interfere with different pattern recognition receptor (PRR) activation pathways, including toll-like receptor (TLRs) and cytoplasmatic retinoic acid-inducible gene (RIG-I)-like receptors (RLRs) (Haga & Bowie, 2005) Viromimicry includes virokines and viroceptors which mimic host cytokines, chemokines, and their receptors. These proteins establish a protected microenvironment for the virus by blocking extracellular communication signals such as the modulation of IFNs and pro-inflammatory cytokines including tumor necrosis factor alfa (TNF-α) and interleukin 1 beta (IL-1β) (Johnston & McFadden, 2003; Wojdasiewicz et al., 2014).

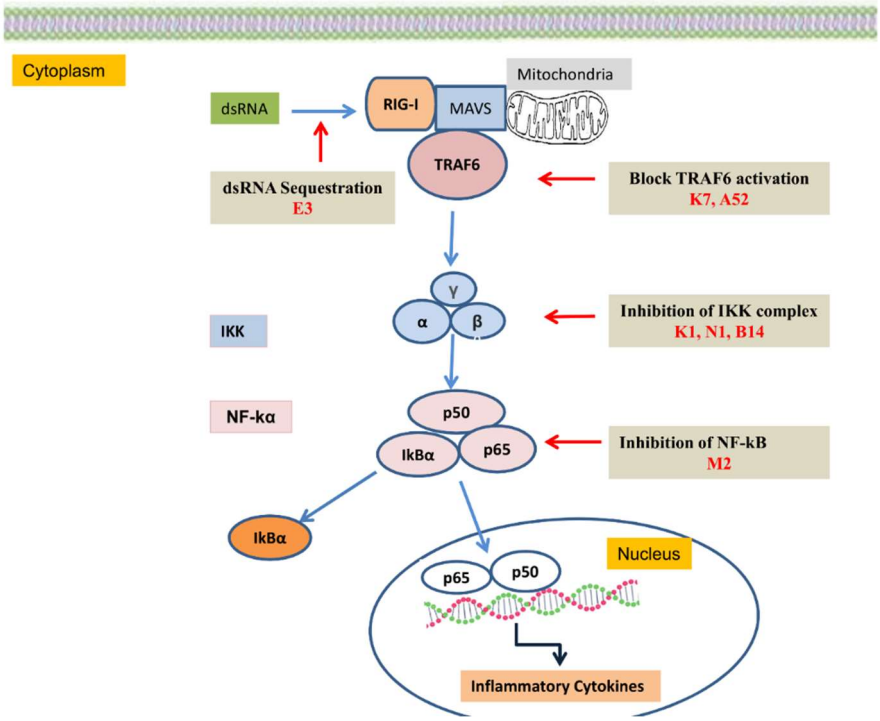

Figure 4. Overview of the RIG-I-like receptors pathway. In the cytosol, viral dsRNAs are recognized by the helicases RIG-I. The N-termini of RIG-I and MDA-5 contain 2 CARD domain that interact with the CARD domain of the mitochondrial adaptor MAVS. MAVS, after recruitment of transactivators, then induces phosphorylation of NF-κB activation leading to the production of pro-inflammatory cytokines.

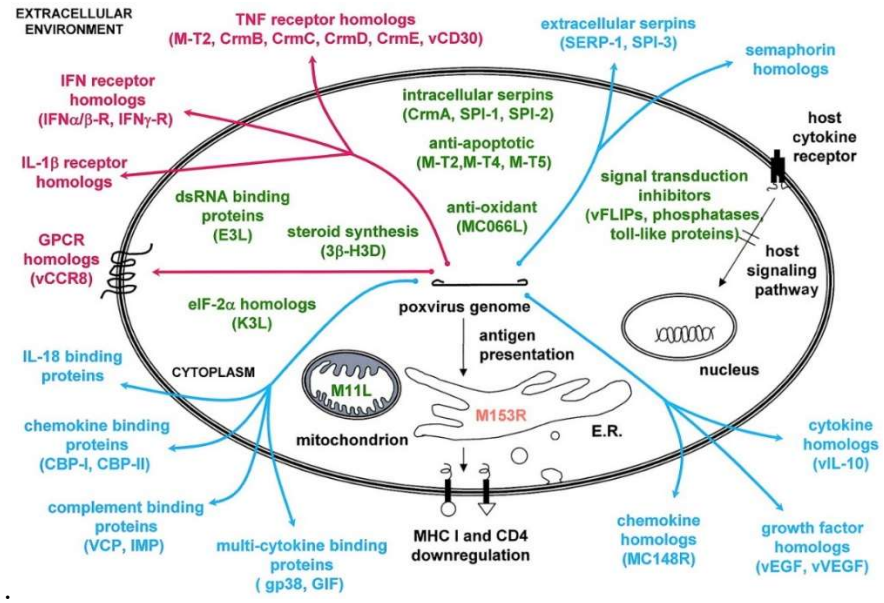

Figure 5. Diagrammatical representation of selected poxvirus encoded immunomodulatory proteins. Poxvirus proteins that participate in virostealth (orange), virotransduction (green), and viromimicry (viroceptors) (red) and virokines (blue) are indicated (Johnston and McFadden, 2003).

Capripoxviruses

The genus *Capripoxvirus* (CaPV) comprises three closely related viruses: Goatpox virus (GTPV), Sheeppox virus (SPPV) and Lumpy skin disease virus (LSDV) (Buller et al., 2005).
CaPVs possess double-stranded DNA genomes of approximately 150 Kbp in size with 147 genes in SPPV and GTPV and 156 genes for LSDV.
The three viruses display close genetic relationship, and a cross-protection can be induced between them (Tulman et al. 2002).

Host specificity of capripoxviruses

CaPVs are considered to be host-specific, causing clinical disease in either sheep, goats, or cattle (Boumart et al., 2016; Tuppurainen et al., 2014). SPPV and GTPV can infect both sheep and goats (Babiuk et al., 2008); however, there is a recent report on GTPV infection in wildlife (Dutta et al., 2019). LSDV causes natural infections in cattle and Asian water (Gari et al. 2011). Clinical signs of LSD have also been reported in impala (Aepyceros melampus) and giraffe (Giraffa camelopardalis) after experimental inoculation with LSDV. Natural infection by LSDV was reported in an Arabian oryx (Oryx leucoryx) in Saudi Arabia, springbok antelope (*Antidorcas marsupialis*) in Namibia, Oryx gazelle in South Africa, and Asian water buffalo in Egypt (Tuppurainen & Oura, 2012). LSDV nucleic acid was detected in skin samples collected from springbok antelope in South Africa (Le Goff et al. 2009; Lamien et al., 2011b). CaPV antibodies were detected in serum samples collected from African buffaloes, greater kudu, waterbuck, reedbuck, impala, springbok, and giraffe (Tuppurainen et al., 2017). However, the role of wildlife in the epidemiology of LSD is not significant in the spread or maintenance of LSD, and there is currently no strong evidence of a wildlife reservoir for CaPVs (Tulman et al., 2001; Hosamani et al., 2004).

Immune response to capripoxvirus infections

Both cellular and humoral immunity are important for the control of CaPVs infections (Joshi et al., 1992). Circulating antibodies derived through natural infection or vaccination can help limit the spread of virus in the animal; however, cell-mediated immune response is essential to eliminates the infection (Carn, 1993; Kitching, 2016).
An animal recovered from natural infection by one member of the genus is protected from infection by another (Kitching, 2003; Ben-Gera et al., 2015). Moreover, passive immunity persists for about six months in calves born form immunized cows (Tuppurainen & Oura, 2012), and colostrums can

interfere with the response to vaccination before six months of age. Affected animals can clear the infections. No carrier state is known to occur (Kitching, 2016).

Geographical distribution of capripoxvirus

SPP and GTP are endemic in Africa, except Southern Africa, and Asia (Beard et al., 2010; Zro et al., 2014). SPP has also re-emerged in south-eastern Europe (e.g. Turkey, Greece, and Bulgaria). The most recent outbreaks of SPP and GTP occurred in Bulgaria, Kazakhstan, Russia, Mongolia, and Greece from 2016 to 2018 (Figure 5).

LSD is currently endemic in Africa except Libya, Tunisia, Algeria, and Morocco (Tuppurainen and Oura 2012).

Until 1984, LSDV was maintained within sub-Saharan Africa, from where it has spread into Egypt in 1988 (Tuppurainen & Oura, 2012).

In 1989, LSDV geographical coverage extended to Middle East, affecting Israel (Yeruham et al., 1995). Then followed several outbreaks in Kuwait, Bahrain, Yemen, and United Arab Emirates. Subsequently, LSDV affected Oman, the West Bank Lebanon, Jordan, and Turkey between 2012 and 2013. LSDV latter spread from Syrian Arab Republic into Turkey (Tageldin et al., 2014; EFSA, 2017; Zeynalova et al., 2016). LSD affected Iran, Azerbaijan, Iraq, Greece, Cyprus, Turkey, Georgia, Russia and Armenia between 2014 and 2016 (Al-Salihi & Hassan, 2015; Tageldin et al., 2014).

LSD entered the European Union (EU) via eastern Greece in 2015 (Agianniotaki et al., 2017) and further spread over the Balkans to Albania, Bulgaria, Kosovo, Montenegro, Serbia and the former Yugoslavian Republic of Macedonia (Beard 2016 ; EFSA, 2016). LSD also spread on the eastern side of the Black Sea, to Armenia, Azerbaijan, Georgia, Kazakhstan, and the Russian Federation (EFSA, 2017). In 2017, outbreaks were registered mostly in Albania, the former Yugoslav Republic of Macedonia, Greece, Turkey, and Russia (EFSA, 2018). In 2018 LSD outbreaks repeatedly reported in Israel, Saudi Arabia, Greece, Montenegro, Georgia, and Russia (https://www.oie.int/wahis_2/public/wahid.php/Countryinformation/Countryhome).

The spread of CaPVs into new areas was predominantly associated with the increase of animal movement through trade (Babiuk et al., 2008) as well as inadequate or the breakdown of veterinary services (Rweyemamu et al., 2006).

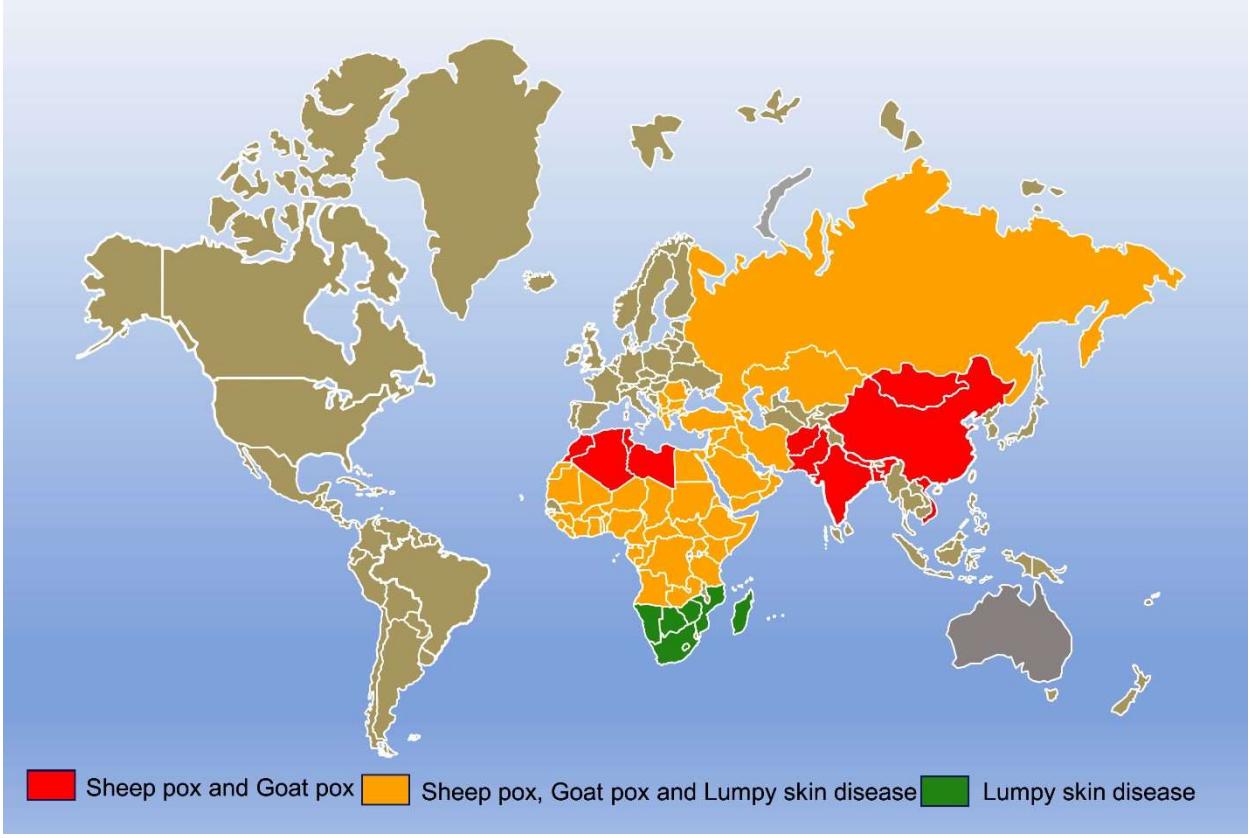

Figure 6. World-wide distribution of Sheep pox, Goat pox and Lumpy skin disease. The red color indicates the presence of SPP and GTP, the orange color shows of SPP, GTP and LSD, whereas green indicate areas affected by LSD only, according to the OIE-WAHID database records by March 2019.

Routes of transmission of Capripoxvirus diseases

GTPV and SPPV transmission occurs by direct contact between an infectious and a susceptible animal. The virus spread through droplets/aerosols via coughing, sneezing, head shaking, vocalizations and breathing, and infects naive animals through inhalation, orally, or via skin abrasions (Bowden et al., 2008). SPPV and GTPV can also be transmitted mechanically, via insect vectors such as stable flies (*Stomoxys calcitrans*) (Kitching & Mellor, 1986; Mellor et al., 1987). As SPPV and GTPV can remain viable for prolonged periods in the environment, fomites can also be a source of transmission.

LSDV spreads mechanically by arthropod vectors (Chihota et al., 2003), via contaminated mouthparts of the vectors. The mechanical transmission of LSDV by the *Aedes aegypti* mosquito (Chihota et al., 2001) and *Rhipicephalus appendiculatus* male ticks has been studied (Tuppurainen

et al. 2015). Blood-feeding arthropods, such as mosquitoes and sand flies, are associated with outbreaks of LSD characterized by generalized lesions.

It was demonstrated that no disease transmission occurs in naive animals housed with LSDV infected animals in the absence of suitable insect vectors (Carn & Kitching, 1995). Direct or indirect contact between infected and susceptible animals is an inefficient method of transmission (Tuppurainen et al., 2015; Carn & Kitching, 1995). LSDV can also be transmitted through semen (Annandale et al., 2014), and the virus can remain in the semen for extended periods (Irons et al., 2005; Osuagwuh et al., 2007).

Clinical disease and pathogenesis

During natural infections, SPP and GTP have an incubation period of 8 to 14 days. Then, the infected animal can exhibit mild to severe clinical signs, depending on the immune status of the host, and virulence of the viral strain (Davies & Mbugwa, 1985). Both SPP and GTP in their acute systemic phase produce fever and generalized lesions in the skin and mucous membranes of the mouth, conjunctiva and nasal cavities as well as lesions in internal organs, especially in the respiratory and digestive tracts (Bowden et al., 2008; Embury-Hyatt et al., 2012). Rhinitis, conjunctivitis, and excessive salivation can also occur throughout the infection. The lesions can cover the entire body, but usually are present mainly in the hairless parts of the skin and mammary glands (Davies & Otema, 1978). During SPPV and GTPV infections, the viremia develops and persists until the host develops adequate response against the virus. The morbidity rate can be as high as 100 % in young animals (Babiuk et al., 2008).

For LSD, the main clinical signs are fever, temperature can exceed 41 °C, and the appearance of nodules on the skin, mucosal membranes, and internal organs. The incubation period of LSD is between 2-4 weeks, under field conditions, and 4-14 days for experimental infections (Carn & Kitching, 1995). Others clinical signs are depression, disinclination to move, inappetence, salivation, lachrymation, and a nasal discharge, which may be mucoid or mucopurulent. Lachrymation can lead to conjunctivitis and, in some cases, corneal opacity and blindness. The nodular skin lesions can cover the entire body or be to a few sites such as the skin of the head, neck, perineum, genitalia, udder, and limbs. Large nodules may become fibrotic and persist for several months; these are referred to as sit fasts (Carn & Kitching, 1995). The scars may remain indefinitely, thus rendering the hide worthless. LSD case-fatality rates and morbidity rates are variable, but in most cases, the disease will lead to a reduction in milk production, sterility in bulls, abortion and significant loss of incomes (Awad et al., 2010; Tuppurainen & Oura, 2012).

Diagnostic tests for capripoxvirus detection

The clinical diagnosis of SPP, GTP, and LSD must be confirmed by laboratory diagnosis.

CaPVs grow well in tissue culture of bovine, ovine, or caprine origin (Binepal et al. 2001; Singh et al., 2001) including primary lamb kidney or lamb testis cells, and cell lines derived from lamb testis (OA3.Ts) (Bowden et al., 2008), embryonic sheep skin (ESH-L cells) (Lamien et al., 2011) and Vero cell (Binepal et al. 2001). In cell culture, CaPVs induce the formation of distinct plaques with a cytopathic effect (CPE) within seven days, although sometimes the procedure may need several blind passages (Diallo and Viljoen, 2007). SPPV and GTPV can also be propagated on the chorioallantoic membrane of embryonated chicken eggs (Binepal et al. 2001). Histopathology, immunohistochemistry and electron microscopy examination of skin nodules are additional options for SPP, GTP and LSD diagnosis (Nagington, 1964; Gulbahar et al., 2006; Bowden et al., 2008).

Serological tests, including virus neutralization test (Babiuk et al., 2008), indirect fluorescent antibody test (IFAT) (Gari et al., 2008) and ELISAs are available for antibody detection (Babiuk et al., 2009).

Several molecular tests, including gel-based PCR and real-time PCR methods, are available for the generic detection of CaPVs (Ireland and Binepal 1998; Heine et al., 1999; Stram et al., 2008, Balinsky et al., 2008; Stubbs et al., 2012; Haegeman et al., 2013), the genotyping of CaPVs (Lamien et al., 2011a, Lamien et al., 2011b, Gelaye et al. 2013) and differentiation of CaPV LAV from WT CaPVs (Abutarbush et al., 2016; Menasherow et al., 2016; Agianniotaki et al., 2017; Chibssa et al., 2018; Chibssa et al., 2019). A high resolution melting curve analysis methods was developed for the detection and differentiation of eight poxviruses of veterinary and medical importance, including LSDV, GTPV, and SPPV (Gelaye et al., 2017). In addition to the above-mentioned methods, the sequencing of the RPO30 and GPCR and P32 genes can also be used for the phylogenetic classification of the CaPVs (Su et al., 2015; Le Goff et al. 2009 ; Lamien et al., 2011; Gelaye et al., 2016; Mafirakureva et al., 2017; Hosamani et al., 2004; Stram et al. 2008).

Prevention and control of capripoxvirus infections

There is no specific antiviral drug available for the treatment of CaPVs infections. In disease-free countries, successful control and eradication of CaPV can rely on early detection of the index case and the rapid implementation of stamping out of all infected and in contact animals, a strict movement control, quarantine and disinfection (Tuppurainen et al., 2017). Any delay in stamping out of infected animals would give time for vectors to become contaminated and transmit the disease (Tuppurainen et al. 2015). In most CaPV endemic countries, a slaughter policy would be

impracticable, and it is impossible to enforce the control of animal movement (Mirzaie et al., 2015). Hence the control strategies emphasize on the use of effective live attenuated vaccines (LAVs) for preventing the CaPV infections (Tuppurainen et al., 2017). LAVs are cheap and provide good protection if sufficient herd immunity, over 80% coverage, is maintained by carrying out annual vaccinations (Kitching 2016). Lack of compulsory and consistent vaccination strategies together with ineffective animal movement control are the most common causes for the uncontrollable spread of CaPVs (Ben-Gera et al., 2015).

Although most of the LAVs work well, reports of vaccine breakdown, short duration of protection, and low levels of antibody inducement necessitate the search for improved vaccines (Hunter and Wallace 2001). The availability of a whole genome sequence allows for a more directed approach to vaccine development by targeting genes specifically involved in virulence and host immune system modulation.

Importance of Capripoxviruses diseases

CaPVs cause economically important viral diseases leading to significant losses in the livestock industries in affected region due to loss in milk yield, infertility, abortion, and death. Also, production losses in infected animals, restrictions on international trade of live animals and animal products, costly vaccination campaigns and limitations of animal movements can cause significant indirect financial losses (Tuppurainen et al. 2015). Therefore, LSD, SPP, and GTP are categorized by the OIE as notifiable diseases due to their potential for rapid spread and substantial economic impact (Tuppurainen et al. 2017).

Since 2015, the rapid expansion of LSD across many Eastern European countries as well as Russia and Kazakhstan highlighted the emergent nature of the virus in more temperate regions (Bouchemla et al. 2018)

Rationale

Despite repeated incidences of CaPV infections outside their usual endemic regions in Africa (LSD, GTP and SPP) and Asia (GTP, SPP), there is still a substantial gap of knowledge on the biology and molecular epidemiology of the virus, hampering the implementation of proper control and eradication measures worldwide. The management of LSD, SPP and GTP rely on the culling of infected animals, restrictions of animal movements and vaccination (Tuppurainen et al. 2017) using LAVs, which usually provide better and longer protection (Boumart et al. 2016). Though LAVs are not advisable in disease-free zones, the EU authorized emergency vaccination with LAVs during the 2015-2017 LSD crisis (Anon, 2018; Tuppurainen et al. 2015). During vaccination, the appearance of mild to generalized lesions in some vaccinated animals (Yeruham et al., 1994; Tuppurainen and Oura, 2014; Gelaye et al., 2015; Abutarbush et al., 2016) boosted research towards the development of rapid strategies to differentiate LAV LSDV from WT LSDVs (Katsoulos et al. 2018). Although the appearance of lesions in vaccinated animals is also common to SPP and GTP, only LSD cases have received adequate attention. Thus, similar tools to differentiate LAV SPPV from WT SPPV and other CaPVs are still missing. Ideally, such tools must also be compatible with routine needs for CaPV diagnostics, including the detection and genotyping of CaPVs, as both WT SPPV and GTPV can cause disease in sheep and goats (Madhavan, et al., 2016).

In some newly affected countries, an inappropriate selection of LAVs has led to the appearance of generalized lesions in vaccinated animals. Thus, the routine use of LAVs creates a complex scenario where the occurrence of disease in vaccinated flocks could result either from a poor attenuation of the vaccine, or the emergence of new strains, capable of escaping the vaccinal response. A report suggested that such variants could emerge by recombination in infected animals receiving LAVs or during coinfection by two different CaPVs (Gershon et al. 1989). The detection of new strains requires continuous monitoring of virus isolates from both vaccinated and non-vaccinated herds, especially in disease-endemic areas where LAVs are routinely used to control CaPVs. Unfortunately, in Eastern Africa, an LSD endemic area for decades (Davies, 1976, Ali, 1977, (Mebratu et al. 1984), only a few isolates have been characterized (Le Goff et al. 2009 ; Lamien et al., 2011; Gelaye et al., 2015). A comprehensive analysis of isolates recovered from this region before the LSD emergence in Western Asia, Central Asia, and Europe, can provide insights into LDSV genetic diversity and variants encountered in vaccinated herds.

In addition to possible viral recombination, new strains can arise from the importation of sick animals from affected regions (Kitching, McGrane, and Taylor 1986). Hence, the identification of

geographical signatures in CaPV genomes and the development of molecular approaches that enable the segregation of CaPV isolates based on the geographical origin can significantly improve the monitoring of CaPV isolates in disease-endemic regions and tracing the source of infection.

Finally, vaccination failure, and the appearance of generalized lesions in vaccinated animals are also indicative of poor attenuation or low efficacy of LAVs. Hence, it is essential to further understand the mechanisms of host immune system activation by CaPVs. The successful management of LSD outbreaks in Europe since 2017 (Kononov et al. 2019; Tuppurainen et al. 2017) suggest a good performance of the Neethling vaccine which was used to control the disease. In contrast to LSD, there is increased reporting of SPP vaccination failure, creating the need to better understand the induction of host immune response by both LAV SPPV and WT SPPV.

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Aim of the Thesis

The overall aim of this study is to advance the management of capripox diseases through the development of tools and strategies to (1) improve the application of vaccination as a control measure and (2) better analyze the evolution and trace the origin of infection by Capripoxviruses.

The specific objectives are:

I. To identify markers through the comparison of full genomes of CaPVs and develop assays to differentiate LAV SPPV from WT SPPV and other CaPVs.
   a. Use a marker identified in the intergenic region between the DNA ligase gene and the B22R of CaPV to design and validate a gel-based assay to differentiate LAV SPPV from WT SPPV and other CaPVs by conventional PCR (Chapter I).
   b. Exploit CaPV specific markers and deletions found in the B22R gene of LAV SPPV only, to design and validate an HRM assay to differentiate LAV SPPV from WT SPPV and simultaneously genotype CaPVs (Chapter II).

II. To undertake molecular epidemiological studies of CaPVs.
   c. Sequence and compare Eastern African LSDVs collected prior to LSDV incursion in Western and Central Asia, and Europe, to some publicly available LSDVs to assess the diversity of LSDVs. The GPCR and the RPO30 genes of newly sequenced LSDVs from Eastern Africa will be compared to those of LSDVs from the same and other regions available in public databases. An LSDV collected from a vaccinated herd in Kenya, LSDV Embu/B338/2011 will be further analysed by sequencing and comparing its EEV glycoprotein and the partial B22R genes to publicly available sequences of both WT LSDV and LAV LSDV (Chapter III).
   d. The GPCR genes of GTPVs collected from Ethiopia, Ghana, Kenya and Mongolia will be sequenced and analysed together with publicly available sequences of GTPVs from various geographical origin. Multiple sequence alignments and alignment-free methods will be evaluated to develop an approach for GTPV discrimination based on their geographical origins (Chapter IV).

III. To analyse, *in vitro*, the host-pathogen interaction, specially, the differential activation of the innate immune genes by LAV SPPV and WT SPPV.
   e. This will be achieved through the infection of sheep PBMCs with SPPV Djelfa, a WT SPPV from Algeria and LAV SPPV from Morocco, and the analysis of expression of

thirteen different cytokines, using in-house developed real-time PCR methods. The difference in fold change and the correlations between the expressions of the different cytokines will be analysed (Chapter V).

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Chapter I

A gel-based PCR method to differentiate Sheeppox virus field isolates from vaccine strains

Tesfaye Rufael Chibssa**,** Reingard Grabherr, Angelika Loitsch, Tirumala Bharani K. Settypalli, Eeva Tuppurainen, Nick Nwankpa, Karim Tounkara, Hafsa Madani, Amel Omani, Mariane Diop, Giovanni Cattoli, Adama Diallo and Charles Euloge Lamien

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A gel-based PCR method to differentiate sheeppox virus field isolates from vaccine strains

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Abstract

Background: Sheeppox (SPP) and goatpox (GTP) caused by sheeppox virus (SPPV) and goatpox virus (GTPV), respectively of the genus *Capripoxvirus* in the family *Poxviridae*, are severely afflicting small ruminants' production systems in Africa and Asia. In endemic areas, SPP and GTP are controlled using vaccination with live attenuated vaccines derived from SPPV, GTPV or Lumpy skin disease virus (LSDV).
Sometimes outbreaks occur following vaccination. In order to successfully control the spread of the virus, it is essential to identify whether the animals were infected by the field strain and the vaccine did not provide sufficient protection. Alternatively, in some cases the vaccine strain may cause adverse reactions in vaccinated animals or in rare occasions, re-gain virulence. Thus, diagnostic tools for differentiation of virulent strains from attenuated vaccine strains of the virus are needed.
The aim of this study was to identify an appropriate diagnostic target region in the capripoxvirus genome by comparing the genomic sequences of SPPV field isolates with those of the most widely used SPP vaccine strains.

Results: A unique 84 base pair nucleotide deletion located between the DNA ligase gene and the VARV B22R homologue gene was found only in SPPV vaccines derived from the Romanian and Yugoslavian RM/65 strains and absent in SPPV field isolates originated from various geographical locations of Asia and Africa.
In addition, we developed and evaluated a conventional PCR assay, exploiting the targeted intergenic region to differentiate SPPV vaccine virus from field isolates. The assay produced an amplicon size of 218 bp for the vaccine strains, while the SPPV field isolates resulted in a 302 bp PCR fragment. The assay showed good sensitivity and specificity, and the results were in full agreement with the sequencing data of the PCR amplicons.

Conclusion: The developed assay is an improvement of currently existing diagnostic tools and, when combined with a capripox virus species-specific assay, will enhance SPP and GTP diagnosis and surveillance and facilitate epidemiological investigations in countries using live attenuated SPP vaccines. In addition, for laboratories with limited resources, the assay provides a simple and cost-effective alternative for sequencing.

Keywords: CaPV, Sheeppox vaccine, Sheeppox virus, VARV B22R homologue gene, DNA ligase gene

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Background

Sheeppox (SPP) and goatpox (GTP) are caused by sheeppox virus (SPPV) and goatpox virus (GTPV) of the genus *Capripoxvirus* of the family *Poxviridae* [1]. In endemic areas SPP and GTP have a serious economic impact on small ruminant production systems, causing losses in productivity, mortality, damaging skins and hides, as well as inflicting international trade restrictions [2]. They are listed in the group of economically important animal diseases for which outbreaks have to be notified immediately to the World Organization for Animal Health [3].

The main mode of virus transmission is the direct contact between diseased and non-infected animals, but indirect transmission may also occur [4]. Clinical signs of SPPV and GTPV infections are characterized by ocular and nasal discharge and pock-like lesions in the skin and mucosae of the respiratory and gastrointestinal tracts [2, 4, 5]. Most of the isolates are host specific and cause disease mainly in sheep or in goats, whereas some isolates can cause serious disease in both animal species [6].

SPP and GTP are endemic in many African, Middle Eastern and Asian countries and recurrent epidemics have occurred in Greece and Bulgaria in 2013–2014 [7] and in Greece in 2016 and 2017. SPPV is also circulating in the Russian Federation where it causes sporadic outbreaks of disease.

In endemic regions, control of the disease is through effective immunization using killed or live attenuated vaccines derived from SPPV, GTPV or Lumpy skin disease virus (LSDV). In general, live attenuated vaccines are the better choice as compared to inactivated vaccines, as they confer long-lasting immunity. For instance, the Yugoslavian RM65, the Romanian Fanar and KSGP0240 strains, the most commonly used vaccines strains against SPPV, are reported to provide high levels of protection [7]. The Yugoslavian RM65 is widely used in the Middle East, Asia and in the Horn of Africa, while the Romanian Fanar is used in India and Maghreb countries. The Yugoslavian RM65 was attenuated by 30 serial passages on ovine kidney cells, and the Romanian Fanar by 26 serial passages on lamb testis cells [7].

The KSGP 0240 is widely used in several endemic regions in Africa. Nevertheless, KSGP0240 has been shown, by sequencing, to be a LSDV, thus, it can be differentiated from virulent isolates of SPPV using available capripoxvirus genotyping methods [7].

However, when using live attenuated vaccines, the epidemiological investigation of outbreaks can become quite challenging. When outbreaks occur following vaccination, it is essential to identify whether the animals were infected by the field strain because the vaccine did not provide sufficient protection. Alternatively, in some cases the vaccine strain may cause adverse reactions in vaccinated animals or, in rare occasions, re-gain the virulence as suggested by Lee and co-workers for herpesvirus vaccine [8].

Unfortunately, the current live attenuated capripox (CaP) vaccines do not offer the possibility to differentiate vaccinated animals from infected ones. This creates a need to identify a suitable genomic target and develop molecular tools that would enable the differentiation of SPPV field isolates from vaccine strains to rule out the involvement of SPP vaccines during a CaP outbreak in a vaccinated herd. Such a tool will facilitate the management and control of CaP infections in small ruminants.

The present study describes the use of a suitable diagnostic target of the SPPV genome to develop an assay that can discriminate SPP vaccines derived from the Romanian and the Yugoslavian RM/65 strains from SPPV field isolates, to facilitate the diagnosis and surveillance of capripox virus (CaPV) infections in small ruminants.

Methods

Virus and nucleic acid extraction

The information related to the field isolates and vaccine strains of SPPV as well as other CaPVs used in this study are presented in Table 1. Viral multiplication was performed on embryonic skin cell from sheep (ESH-L cells) grown in Hank's Minimum Essential Medium (MEM) supplemented with 10% foetal calf serum and 1% antibiotics. DNA was extracted from infected cell culture supernatants and clinical samples using the AllPrep DNA/RNA extraction kit (QIAGEN) following the manufacturer's instructions. Extracted DNA was eluted with 80 μl elution buffer and stored at − 20 °C until further use.

Primer design and PCR

The target region was selected based on the alignment of the full genomes of three SPPVs, Sheeppox virus 10,700–99 (AY077832), Sheeppox virus A (AY077833) and Sheeppox virus NISKHI, (AY077834), retrieved from GenBank, and the unpublished genome of the Romanian vaccine strain used in Morocco. PCR primers, flanking a unique nucleotide deletion in the Romanian vaccine strain, were designed using Primer3Plus online tool. The target was an intergenic region located between the DNA ligase gene and the VARV B22R homologue gene of CaPVs corresponding to position 121,500–122,799 of SPPV A (AY077833). The primers (Table 2) were designed to amplify amplicons of 302 bp in SPPV field isolates and 218 bp fragments for SPPV vaccines. The specificity of the primers was checked by using the Basic Local Alignment Search Tool (BLAST). The primers were synthesized and purified by HPLC by Eurofins Genomics (Austria).

PCR was conducted in a reaction volume of 20 μl, containing 500 nM of each forward and reverse primers, 0.2 mM dNTPs, 2.5 U Taq DNA polymerase (QIAGEN), 1× PCR buffer, and 2 μl template DNA. The cycling conditions were as follow: 95 °C for 4 min followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s

Table 1 List of capripoxvirus isolates used in this study

Sample No.	Isolate/Strain name	Source	Sample type	Country	Collection-date	Host
1	SPPV Morocco vaccine	Biopharma/Morocco	Cell culture	Morocco	Unknown	Sheep
2	SPPV Algeria vaccine Lot/7	INMV-LCV/Algeria	Cell culture	Algeria	Unknown	Sheep
3	SPPV Senegal vaccine	LNERV-ISRA/Senegal	Cell culture	Iran	1966	Sheep
4	SPPV PANVAC/6 vaccine	PANVAC/Ethiopia	Cell culture	Kenya	2010	Sheep
5	SPPV Turkey/98 Corum	VCRI-Pendik/Turkey	Cell culture	Turkey	1998	Sheep
6	SPPV Oman/84	IAH-Pirbright/UK	Cell culture	Oman	1984	Sheep
7	SPPV Turkey/98 Denizli	VCRI-Pendik/Turkey	Cell culture	Turkey	1998	Sheep
8	SPPV Algeria/93 Djelfa	INMV-LCV/Algeria	Cell culture	Algeria	1993	Sheep
9	SPPV Algeria/05 Illizi	INMV-LCV/Algeria	Cell culture	Algeria	2005	Sheep
10	SPPV Turkey/98 Sivas	VCRI-Pendik/Turkey	Cell culture	Turkey	1998	Sheep
11	SPPV MOG/SP/T/2/07	IVM/Mongolia	Skin scrapping	Mongolia	2007	Sheep
12	SPPV MOG/SP/T/3/07	IVM/Mongolia	Skin scrapping	Mongolia	2007	Sheep
13	GTPV Iraq/61 Gorgan	IAH-Pirbright/UK	Cell culture	Iraq	1961	Goat
14	GTPV MOG/GP/T/6/08	IVM/Mongolia	Skin scrapping	Mongolia	2008	Goat
15	GTPV MOG/GP/L/5/08	IVM/Mongolia	Skin scrapping	Mongolia	2008	Goat
16	GTPV Awi/O13/2011	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2011	Goat
17	GTPV Bale/O14/2007	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2007	Goat
18	GTPV Giner/O15/2007	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2007	Goat
19	LSDV KS-1	HSL-AGES/Austria	Cell culture	Kenya	1976	Sheep
20	LSDV Egypt/89 Ismalia	HSL-AGES/Austria	Cell culture	Egypt	1989	Cattle
21	LSDV Guder/B5/2008	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2008	Cattle
22	LSDV Humbo/B23/2010	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2010	Cattle
23	SPPV Algeria vaccine Lot/10	INMV-LCV/Algeria	Cell culture	Algeria	Unknown	Sheep
24	SPPV Mauritania/85 Gorgol	LNERV-ISRA/Senegal	Cell culture	Mauritania	1985	Sheep
25	SPPV Turkey/98 Darica	VCRI-Pendik/Turkey	Cell culture	Turkey	1998	Sheep
26	SPPV MOG/SP/T/1/2006	IVM/Mongolia	Skin scrapping	Mongolia	2006	Sheep
27	GTPV Saudi Arabia/93	IAH-Pirbright/UK	Cell culture	Saudi Arabia	1993	Goat
28	SPPV Nigeria/77	IAH-Pirbright/UK	Cell culture	Nigeria	1993	Sheep
29	GTPV Turkey/98 Denizli	VCRI-Pendik/Turkey	Cell culture	Turkey	1998	Goat
30	GTPV Oman/84	IAH-Pirbright/UK	Cell culture	Oman	1984	Goat
31	GTPV MOG/GP/T/4/08	IVM/Mongolia	Skin scrapping	Mongolia	2008	Goat
32	GTPV Yemen/83	IAH-Pirbright/UK	Cell culture	Yemen	1983	Goat
33	GTPV Towele/O17/2013	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2013	Goat
34	GTPV Halasya/G18/2013	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2013	Goat
35	LSDV Galesa/B12/2008	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2008	Cattle
36	LSDV Sodo/B24/2010	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2010	Cattle
37	LSDV Chilimo/B11/2008	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2008	Cattle
38	LSDV Ambo/B8/2008	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2008	Cattle
39	LSDV Toke/B6/2008	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2008	Cattle
40	LSDV Ginchi/B10/2008	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2008	Cattle
41	LSDV Sodo/B22/2010	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2010	Cattle
42	LSDV Adama/B4/2011	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2011	Cattle
43	LSDV Ziway/B3/2011	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2011	Cattle
44	LSDV Asella/B2/2011	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2011	Cattle

Table 1 List of capripoxvirus isolates used in this study *(Continued)*

Sample No.	Isolate/Strain name	Source	Sample type	Country	Collection-date	Host
45	LSDV Arsi/B1/2011	NAHDIC/Ethiopia	Skin scrapping	Ethiopia	2011	Cattle
46	LSDV Sundus/2012	CVRL/Sudan	Skin scrapping	Sudan	2012	Cattle

Abbreviations: *VCRI* Veterinary Control and Research Institute, *LNERV-ISRA* Laboratoire National de l'Elevage et de Recherches Vétérinaires, Institut Sénégalais de Recherches Agricoles, *INMV-LCV* Institut National de la Médecine Vétérinaire, Laboratoire Central Vétérinaire, *IVM* Institute of Veterinary Medicine, *IAH* Institute for Animal Health, *PANVAC* Pan African Veterinary Vaccine Centre, *NAHDIC* National Animal Health Diagnostic and Investigation Center, *HSL-AGES* High Security Laboratory, Austrian Agency for Health and Food Safety, *CVRL* Central Veterinary Research Laboratories

and a final extension at 72 °C for 2 min. PCR products were cheeked by electrophoresis on a 2% agarose gel for 1 h at 100 V.

Preparation of controls

The PCR amplicons of SPPV Denizli and SPPV Morocco vaccine (Romanian vaccine strain) were selected to prepare positive control plasmids, representing the field isolates and vaccine strains respectively. For plasmid preparation, each amplicon was purified and ligated into pGEM-T Easy Vector Systems (Promega). The ligated products were used to transform DH5α competent cells (Invitrogen). Plasmids containing the inserts were purified from the positive bacteria clones using the PureYield Plasmid Midiprep System (Promega) following the manufacturer's instructions. The purified plasmids were sequenced to confirm the presence of the correct target region and quantified using the Quant-iTPicoGreen dsDNA Assay Kit (Invitrogen) on a NanoDrop 3300 fluorospectrometer. The concentration of the plasmids was determined following the steps described by Lamien et al., (2011) and the plasmids were kept at – 20 °C until analysis.

Analytical sensitivity and specificity of the assay

The analytical sensitivity of the method was assessed by amplifying 10-fold serial dilutions, from10^7 to 100 copies/reaction, then follow by 80, 60, 40, 20, 10 and 1 copies/reaction dilutions, of plasmids containing the corresponding target of SPPV Denizli and SPPV Morocco vaccine. The lowest number of viral genome copies that could be detected by the assay was recorded.

The specificity was evaluated by amplifying DNA extracted from all available SPP vaccines and field isolates as well as GTPV and LSDV isolates from various geographical regions (Table 1). The genotype of each sample was confirmed using a capripox species specific PCR (Gelaye et al., 2013). Additionally, the specificity in amplifying only CaPVs was determined by attempting to amplify DNA extracted from Orf virus (ORFV), *Mycoplasma capricolum* ssp. *capripneumonia* (Mccp), Bovine herpes virus (BOHV), Bovine popular stomatitis virus (BPSV) and cDNA derived from peste des petits ruminants (PPR) virus (Additional file 1: Table S1).

Sequence analysis

For all CaPVs used in this study, the targeted region of the genome was sequenced to confirm the accuracy of the assay. Thus, all amplified PCR products of the above described PCR reactions were purified using Wizard SV Gel and PCR Clean Up System (Promega) and sequenced commercially by LGC Genomics (Germany). The sequence data were edited and the fragments assembled using Vector NTI 11.5 software (Invitrogen). Multiple sequence alignments were performed using the CLUSTALW algorithm implemented in BioEdit 7.5 software package to compare SPPV field isolates and vaccine strains. All sequences were deposited in GenBank under accession number MG764242 to MG764286.

Results

Assay design and optimization

Primers were designed to amplify a region between the DNA ligase gene and the VARV B22R homologue gene of SPPV. The region was first selected based on the sequence alignment of three SPPV publicly available full genome sequences and a draft genome sequence of the SPP Morocco vaccine derived from the Romanian vaccine strain. The SPP vaccine had an 84 bp nucleotide deletion as compared to SPPV field isolates (Fig. 1). The initial evaluation of the assay showed that the Morocco vaccine strain could be differentiated from SPPV Denizli field isolate based on the size difference of the produced PCR amplicons: 218 bp for the vaccine strain and 302 bp for the SPPV field isolate. In the subsequent steps, the assay was optimized and further evaluated.

Evaluation of the assay

The optimal assay parameters are presented in Methods. The optimised assay was further evaluated by testing 46

Table 2 Primers used in this study. The length of the predicted amplicons are given

Primer name	Sequences	Length	Amplicon size
SPPV_DIV_Fow	5′-ATCTGCTACAAGTTTTAACGAACTTA- 3′	26	218 bp (SPPV vaccines)
SPPV_DIV_Rev	5′-TGAATGTGATCTCATATCCTTATTG-3′	25	302 (SPPV field isolates and GTPV) and 336 (LSDV)

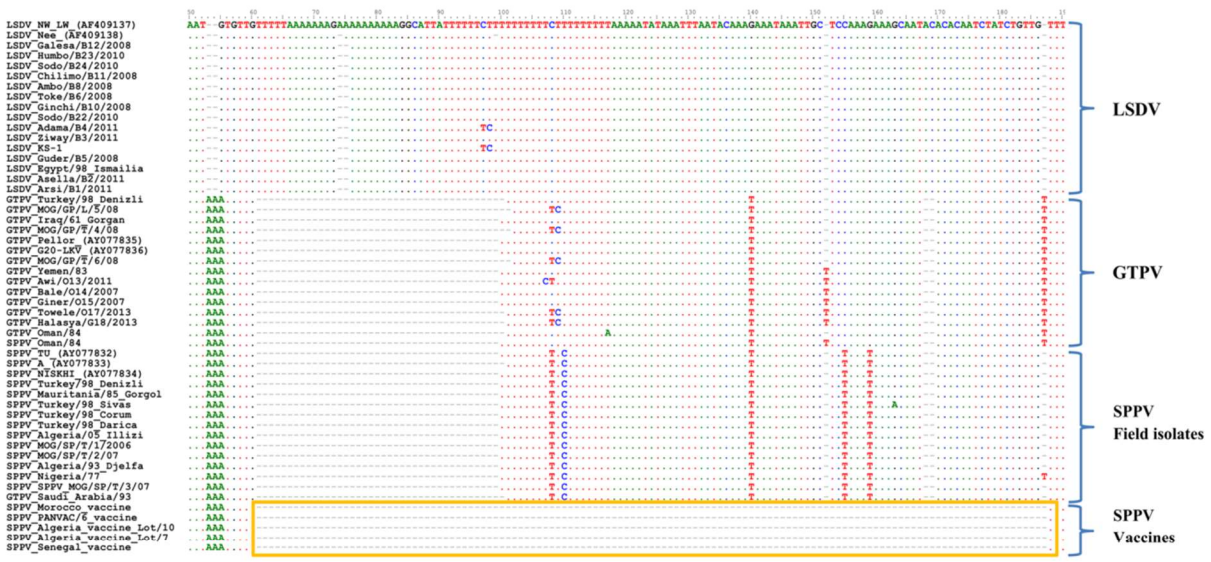

Fig. 1 Multiple sequence alignments showing a 84-nucleotide deletion in SPPV vaccines. The nucleotide sequences of the intergenic region located between the DNA ligase gene and the VARV B22R homologue gene for 45 Capripoxviruses of this study and 7 other retrieved from Genbank were compared

CaPV isolates or clinical samples including 12 field isolates of SPPV, 5 SPP vaccines, 13 field isolates of GTPV and 15 field isolates of LSDV and 1 LSDV vaccine (Table 1). All 5 SPP vaccines produced 218 bp PCR products while the SPPV field isolates produced a 302 bp product (Fig. 2 and Additional file 2: Figure S1). The SPPV field isolates could not be clearly differentiated from GTPVs and LSDVs (Fig. 2 and Additional file 2: Figure S1).

Limit of detection and specificity of the assay

The limit of detection of the assay was evaluated by amplifying 10-fold serial dilutions of plasmids as described in Methods. The results showed that the limit of detection for SPPV field isolates and vaccine strains were 80 and 10 copies/reaction (Additional file 3: Figure S2) respectively. The specificity of the assay was tested by attempting to amplify non-capripoxvirus DNA from Orf virus, *Mycoplasma capricolum* ssp. *capripneumonia* (Mccp), Bovine herpes virus (BOHV), Bovine popular stomatitis virus (BPSV) and cDNA from peste des petits ruminant virus. No amplification was observed (Additional file 4: Figure S3).

Sequencing of the PCR amplicons

All PCR amplicons were sequenced for further validation of this PCR approach. The results of the multiple sequence

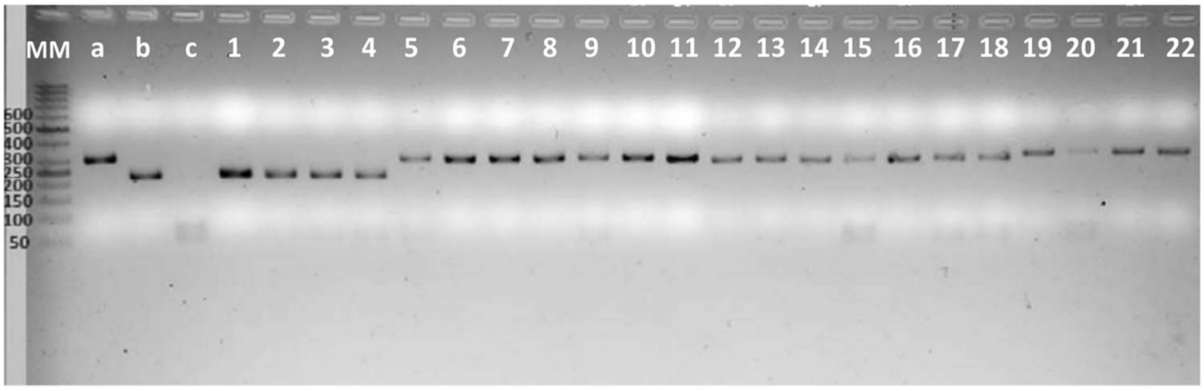

Fig. 2 Gel picture of PCR products for selected capripoxvirus samples. The SPPV vaccines appear to be shorter than SPPV field isolates, GTPV and LSDV due to the 84 bp sequence difference. The PCR products of 218 bp, 302 bp and 338 bp represent SPPV vaccine strains, SPPV field isolates/GTPVs, and LSDVs respectively. MM: 50 bp DNA ladder; a: positive control plasmid of the SPPV field isolates; b: positive control plasmid of the SPPV vaccine strain; c: Negative control; Lanes 4–7: SPPV vaccine strains (sample 1 to 4 of Table 1); Lanes 8–15: SPPV field isolates (sample 5 to 12 of Table 1); Lanes 16–21: GTPVs (sample 13 to 18 of Table 1), Lanes 22–25: LSDVs (sample 19 to 22 of Table 1)

alignment showed the absence of the 84-nucleotide deletion in all SPPV field isolates, GTPV and LSDV. Additionally, the targeted region was found to be well conserved among the SPPV field isolates and the SPPV vaccine strains except for the 84 nucleotides deletion. Ten nucleotide variations were observed between SPPV field isolates and GTPVs. LSDV sequences are longer than SPPV field isolates and GTPVs due to an insertion of 34–36 nucleotides. GTPV Saudi Arabia appeared to be a SPPV and SPPV Oman showed the GTPV specific features. The vaccine KS1 (renamed in our paper as LSDV KS-1) presented a 34-nucleotide insertion as most LSDV of this study.

Discussion

In this study, we identified a suitable target in CaPV genome and developed a PCR method to discriminate SPP vaccine strains from SPPV field isolates as well as from other CaPVs.

By aligning the full genome sequences of SPPV field isolates with the unpublished full genome of SPPV Morocco vaccine, a Romanian strain, we identified a region of 84 bp nucleotide deletion in the vaccine strains. Primers were designed to amplify this intergenic region located between the DNA ligase gene and the VARV B22R homologue gene of CaPV. The sequencing of this region in SPPV vaccine strains and SPPV field isolates available for this study, showed this deletion to be unique to the SPP vaccines derived from the Romanian and the Yugoslavian RM/65 strains produced in Algeria, Egypt, Morocco and Senegal. However, the sequence of the NISKHI vaccine, whose full genome has been previously published [9] did not carry such a deletion. Nevertheless, the use of the NISHKI strain seems to be confined to Russia and countries of the former Soviet Union such as Kazakhstan [10]. By using the targeted intergenic region, the aim of this study was to develop an assay to discriminate the live attenuated Romanian and Yugoslavian RM/65 SPP vaccines strains from virulent SPPV field isolates and other CaPVs.

Interestingly, Romanian and Yugoslavian RM/65 SPPV vaccines produced amplicons of shorter lengths, as compared to field isolates of SPPV and other CaPVs, and thus could be easily discriminated from them. The assay was found to be very specific and sensitive, and the accuracy and reliability was confirmed by sequencing the corresponding amplicons of SPP vaccine strains and SPPV, GTPV and LSDV field isolates available for this study.

The high sensitivity of this assay for SPPV vaccine strain, as compared to the field isolates, is likely due to the shortest amplicon size in the vaccine strains. No amplification was detected for any non-CaPV samples by the assay. However, the assay could not differentiate SPPV field isolates from LSDVs and GTPVs. Although an insertion of 34–36 nucleotides was observed in all LSDVs, making them longer than GTPVs and SPPV field isolates, it was not possible to resolve these differences on the agarose gel. Owing to the availability of species-specific PCR [11–13] for CaPV genotype determination, we suggest that the current assay could be used once the genotype of CaPV is established. Alternatively, the exact capripoxvirus genotype can be determined by sequencing the region targeted in this study. Within the SPPV genotype, the test, undoubtedly discriminates between the virulent field isolates and the vaccines derived from the Romanian and the Yugoslavian RM/65 strains.

The availability of an easy-to-use molecular method is needed for the identification of SPPV, to rule out the involvement of SPP vaccines following a CaP outbreak in previously vaccinated flock of small ruminants. In Middle Eastern and Asian countries, small ruminants are protected from CaPV infections using various SPPV, GTPV or LSDV derived vaccines, however, SPP vaccines derived from Romanian and the Yugoslavian RM/65 strains are predominant [14]. In Africa, small ruminants are protected against CaPV using either KSGP O-240 and O-180 vaccines or SPP vaccines derived from the Romanian and the Yugoslavian RM/65 strains. Since KSGP O-240 and O-180 strains are of LSDV genotype, the use of a CaPV species-specific assay [11–13] can allow for determination of their involvement when the disease occurs in a previously vaccinated herd. However, if a herd is vaccinated with a SPP vaccine from the Romanian and the Yugoslavian RM/65 strains, the full genome sequencing of the viral isolate, collected during an outbreak, would be required to rule out vaccine involvement when an outbreak occurs. This is time-consuming and cost prohibitive for most laboratories in limited resourced countries. Thus, the identification of suitable target in the viral genome to differentiate SPPV vaccine strains from SPPV field isolates greatly reduces the costs, by allowing the sequencing of small specific genome fragments. Furthermore, this region can be targeted in a simple molecular method such as the PCR approach presented in the work, thereby avoiding the use of sequencing. The current assay is intended to be used by all veterinary laboratories, including those with limited resources. It can also be used as a front-line tool for the direct screening of pathological samples collected during CaPV outbreaks, especially those occurring in previously vaccinated small ruminant populations. A study was recently conducted in Morocco to rule out the involvement of the vaccine strain in 2010 SPP outbreaks using a PCR based approach [15]. When compared to the assay developed by Haegeman and co-workers [15], our method presents the advantage of using only one primer pair to target both viruses, and thus, is much simpler to conduct and interpret. In addition, more vaccine strains and field isolates, from various geographical locations were included in this study, which broadened the scope of the applicability to all countries where SPP vaccines derived from the Romanian or the Yugoslavian RM/65 strains are used.

Conclusions

The molecular assay described herein is a reliable and rapid method that can easily be implemented for the differentiation of SPP vaccine derived from the Romanian or the Yugoslavian RM/65 strains from virulent SPPV field isolates. The method is applicable as a routine tool for outbreak investigations and disease surveillance in both SPP and GTP enzootic and disease-free countries. It is expected that its adoption by veterinary laboratories in CaPV affected countries, will help facilitate the control and management of CaP disease in small ruminants.

Additional files

Additional file 1: Table S1. Non-capripoxvirus samples tested for specificity study. (DOCX 16 kb)

Additional file 2: Figure S1. Gel picture of PCR products for the remaining capripoxvirus samples. These sample were tested in this study, but not presented in Fig. 2 of the manuscript. The PCR products of 218 bp, 302 bp and 338 bp represent SPPV vaccine strains, SPPV field isolates/GTPVs, and LSDVs respectively. First row: MM = 50 bp DNA ladder; a = positive control plasmid of the SPPV field isolates; b = positive control plasmid of the SPPV vaccine strain; c = Negative control; Lane 5 to 15 (sample 23 to 33 in Table 1 of the manuscript). Second row: MM = 50 bp; Lane 2 to 14 (sample 34 to 46 in Table 1 of the manuscript). (PDF 104 kb)

Additional file 3: Figure S2. Determination of the limits of detection of the PCR assay. Defined amount for the plasmid genotype standard (10^4, 10^3, 100, 80, 60, 40, 20, 10, 1 and 0) for SPPV vaccine (A) and SPPV field isolates (B) were tested in parallel reactions and run on agarose gel. (PDF 77 kb)

Additional file 4: Figure S3. Gel picture of PCR for the non-capripoxvirus samples tested in this study. MM = 50 bp DNA ladder; a = positive control plasmid of the SPPV field isolates; b = positive control plasmid of the SPPV vaccine strain; c = Negative control; 1–5 (ORF viruses); 6 (BPSV); 7–8 (Mccp); 9 (cDNA, PPRV); 10 (BOHV-1); 11 (BOHV-2). (PDF 45 kb)

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Availability of data and materials

All relevant information is provided in this current manuscript.

Authors' contributions

Conceived and designed the experiments: CEL, TRC. Performed the experiments: TRC, AO. Analyzed the data: TRC, CEL, RG, AL, AO, TBKS. Contributed reagents/materials/analysis tools: AL, NN, KT, HM, MD, ET. Wrote the paper: TRC, CEL, RG, AD, Supervised the study: AD, GC, RG, CEL. Edited the final manuscript: AD, GC, RG, NN, KT, ET, TBKS, HM, MD. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Additional files

Supplement Table 1: Non-capripoxvirus samples tested for specificity study.

Sample No.	Isolate/Strain name	Source	Sample type	Country	Host
1	ORFV MB38/13 C-2	NVI/Ethiopia	Skin scraping	Ethiopia	Sheep
2	ORFV MB38/13 C-3	NVI/Ethiopia	Skin scraping	Ethiopia	Sheep
3	ORFV MB38/13 C-4	NVI/Ethiopia	Skin scraping	Ethiopia	Sheep
4	ORFV MB38/13 C-5	NVI/Ethiopia	Skin scraping	Ethiopia	Goat
5	ORFV MB38/13 C-6	NVI/Ethiopia	Skin scraping	Ethiopia	Goat
6	BPSV Stamm M1	HSL-AGES/Austria	Cell culture	Germany	Cattle
7	Mccp	NVI/Ethiopia	Pathological lesions	Ethiopia	Goat
8	Mccp	NVI/Ethiopia	Pathological lesions	Ethiopia	Goat
9	PPRV (cDNA)	NVI/Ethiopia	Nasal swab	Ethiopia	Goat
10	BOHV-1 404/2018	HSL-AGES/Austria	Cell culture	Austria	Cattle
11	BOHV-2 95-5/2016	HSL-AGES/Austria	Cell culture	Austria	Cattle

BOHV = Bovine herpes virus; BPSV = Bovine papular stomatitis virus; Mccp = *Mycoplasma capricolum* ssp. *Capripneumonia*; NVI = National Veterinary Institute; HSL-AGES = High Security Laboratory, Austrian Agency for Health and Food Safety; CVRL = Central Veterinary Research Laboratories

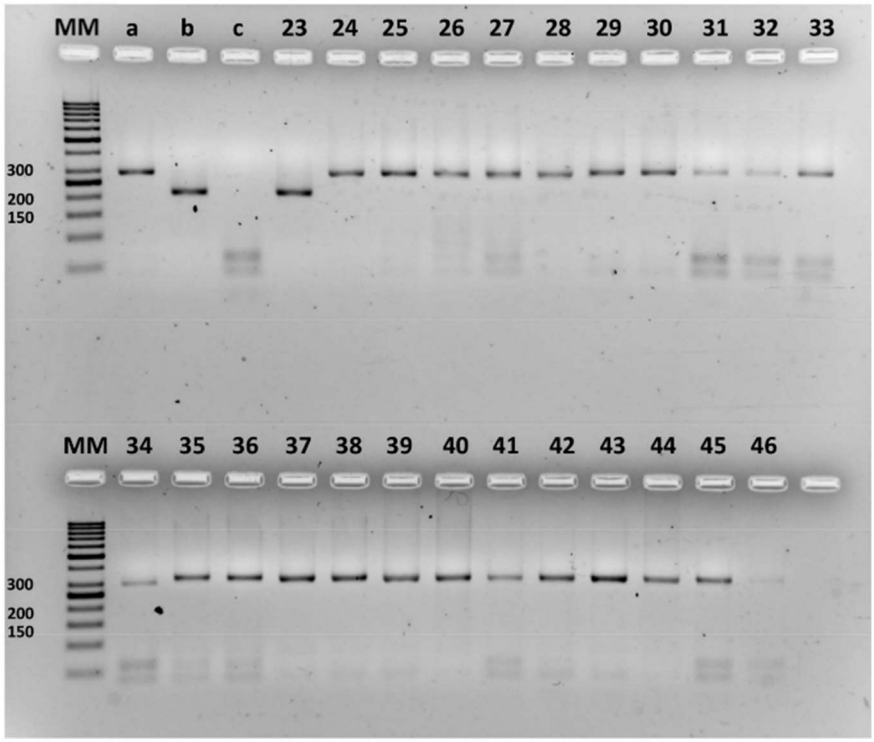

Figure S1. Gel picture of PCR products for the remaining capripoxvirus samples. These sample were tested in this study, but not presented in Figure 2 of the manuscript. The PCR products of 218 bp, 302 bp and 338 bp represent SPPV vaccine strains, SPPV field isolates/GTPVs, and LSDVs respectively. First row: MM = 50 bp DNA ladder; a = positive control plasmid of the SPPV field isolates; b = positive control plasmid of the SPPV vaccine strain; c = Negative control; Lane 5 to 15 (sample 23 to 33 in Table 1 of the manuscript). Second row: MM = 50 bp; Lane 2 to 14 (sample 34 to 46 in Table 1 of the manuscript).

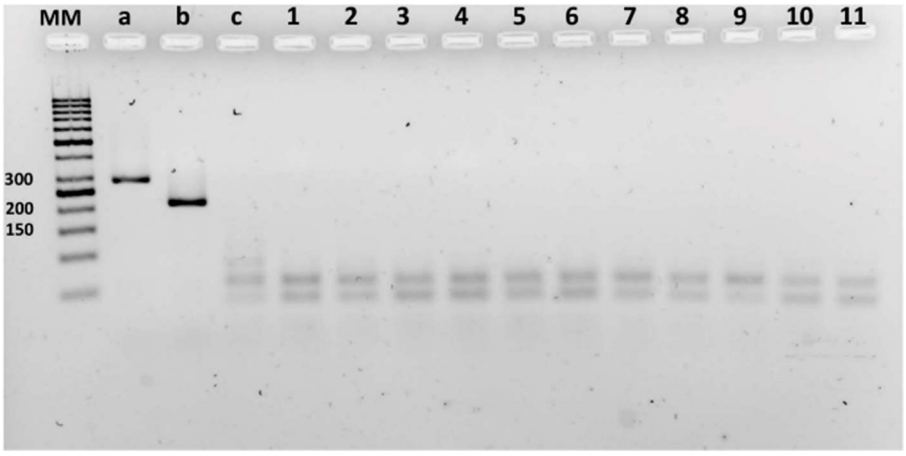

Figure S3. Gel picture of PCR for the non-capripoxvirus samples tested in this study. MM = 50 bp DNA ladder; a = positive control plasmid of the SPPV field isolates; b = positive control plasmid of the SPPV vaccine strain; c = Negative control; 1–5 (ORF viruses); 6 (BPSV); 7–8 (Mccp); 9 (cDNA, PPRV); 10 (BOHV-1); 11 (BOHV-2).

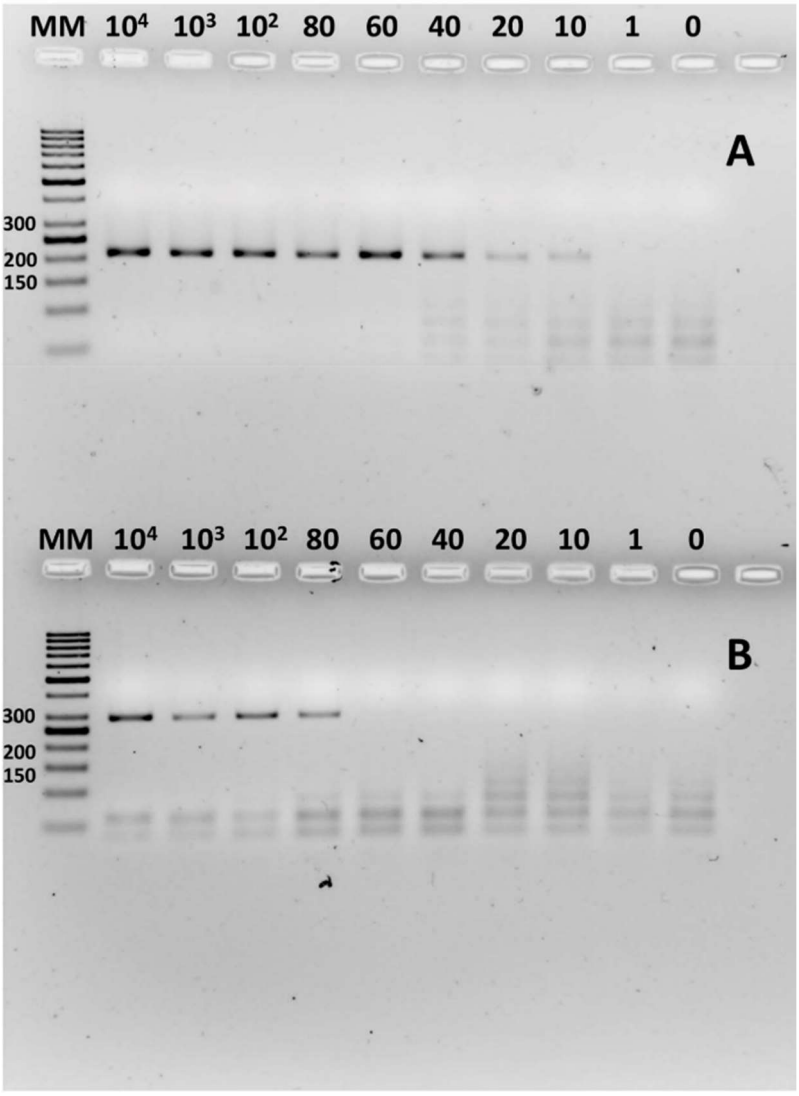

Figure S2. Determination of the limits of detection of the PCR assay. Defined amount for the plasmid genotype standard (10^4, 10^3, 100, 80, 60, 40, 20, 10, 1 and 0) for SPPV vaccine (A) and SPPV field isolates (B) were tested in parallel reactions and run on agarose gel.

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Chapter II

An HRM Assay to Differentiate Sheeppox Virus Vaccine Strains from Sheeppox Virus Field Isolates and other Capripoxvirus Species

Tesfaye Rufael Chibssa, Tirumala Bharani K Settypalli, Francisco J. Berguido, Reingard Grabherr, Angelika Loitsch, Eeva Tuppurainen, Nick Nwankpa, Karim Tounkara, Hafsa Madani, Amel Omani, Mariane Diop, Giovanni Cattoli, Adama Diallo and Charles Euloge Lamien

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An HRM Assay to Differentiate Sheeppox Virus Vaccine Strains from Sheeppox Virus Field Isolates and other Capripoxvirus Species

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Sheep poxvirus (SPPV), goat poxvirus (GTPV) and lumpy skin disease virus (LSDV) affect small ruminants and cattle causing sheeppox (SPP), goatpox (GTP) and lumpy skin disease (LSD) respectively. In endemic areas, vaccination with live attenuated vaccines derived from SPPV, GTPV or LSDV provides protection from SPP and GTP. As live poxviruses may cause adverse reactions in vaccinated animals, it is imperative to develop new diagnostic tools for the differentiation of SPPV field strains from attenuated vaccine strains. Within the capripoxvirus (CaPV) homolog of the variola virus B22R gene, we identified a unique region in SPPV vaccines with two deletions of 21 and 27 nucleotides and developed a High-Resolution Melting (HRM)-based assay. The HRM assay produces four distinct melting peaks, enabling the differentiation between SPPV vaccines, SPPV field isolates, GTPV and LSDV. This HRM assay is sensitive, specific, and provides a cost-effective means for the detection and classification of CaPVs and the differentiation of SPPV vaccines from SPPV field isolates.

Sheeppox (SPP), goatpox (GTP) and lumpy skin disease (LSD) are three important pox diseases of sheep, goat and cattle respectively[1,2]. The responsible viruses, sheep poxvirus (SPPV), goat poxvirus (GTPV) and lumpy skin disease virus (LSDV) are large, complex, double-stranded DNA viruses of the genus Capripoxvirus, subfamily Chordopoxvirinae, family Poxviridae[3]. Due to the economic importance of the cattle, sheep and goat farming industry and the viruses potential for rapid transboundary spread, SPP, GTP and LSD are categorized by the World Organisation for Animal Health (OIE) as notifiable diseases[4,5].

SPP and GTP are endemic in Africa, except southern Africa, across the Middle East and Asia[6,7]. SPP incursions have also been reported in Bulgaria and Greece[7]. In early 2018, outbreaks of SPP in Greece went uncontrolled despite implementation of an extensive stamping out campaign[7]. According to the World Animal Health Information System (WAHIS) web portal[WAHID][8], the disease is also present in Russia with several sporadic outbreaks reported between 2008 and 2019. The endemic geographic range of LSD was limited to the African continent including Madagascar[2,9] until recently when the disease emerged in the Middle East before spreading into Asia and Europe. Outbreaks in Albania, Bulgaria, Greece, the Former Yugoslav Republic of Macedonia,

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Figure 1. Multiple sequence alignments of partial B22R gene sequences of 20 representative capripoxviruses. Four SPPV vaccine strains, 7 SPPV field strains, 5 GTPV and 4 LSDV field strains were compared. Two series of deletions in the SPPV vaccines (21 and 27 bp) are highlighted in the red boxes. Conserved nucleotides to the reference LSDV sequence are shown as dots while the nucleotide mismatches are represented with the corresponding character.

Montenegro, Serbia, and the Russian Federation, increase the disease risk for the neighboring countries in the region[7,10].

Live attenuated SPPV, GTPV and LSDV derived vaccines are widely used in many endemic African, Middle Eastern and Asian countries to control SPP and GTP[11,12]. As live poxviruses may cause adverse reactions in vaccinated animals, it is crucial to use diagnostic tools that can rapidly and specifically differentiate virulent SPPV field isolates from attenuated SPPV vaccine strains. This would help to elucidate the origin of disease in the event of mild or systemic post-vaccination reactions in vaccinated animals[13]. Similar assays have proven to be very useful in cattle to differentiate the LSDV Neethling vaccine from LSDV fields isolates in Israel[14], Greece[15] and Russia[16].

Routine capripox diagnosis would be improved if one assay was designed to provide both differentiation of SPPV field isolates from SPPV vaccine strains and simultaneously classify the sample into one of three known species: SPPV, GTPV and LSDV. Several tests have addressed the differentiation of SPPV vaccine strains from SPPV field isolates[13,17], or the genotyping of CaPVs[18–20], however, a single assay to perform both methods has not been described. This can be accomplished by identifying a suitable genetic marker in the CaPV genome that will allow for the development of a unique, cost-effective test that can be used for both outbreak sample screening and epidemiological investigations in previously vaccinated herds.

With the new generation of dyes and technological improvements in qPCR platforms, high-resolution melting (HRM) curve analysis has become a powerful technique for genotyping microbes, offering the possibility of developing methods other than sequencing for rapid pathogen typing[19,21–24], including the differentiation of vaccine strains from field isolates[14,16].

In this paper, we describe the identification of a novel target in the capripox genome and the development of an HRM curve analysis to differentiate SPPV vaccine strains from SPPV field strains and simultaneously identify the three species of CaPVs to support epidemiological surveillance and disease diagnosis during the vaccination period.

Results

Assay Design. The analysis of multiple-sequence alignments of CaPV genomes, including a SPPV vaccine from Morocco, enabled the identification of a region with a 21-nucleotide and 27-nucleotide deletion, unique to the SPPV vaccine. This region, within the CaPV homolog of the variola virus B22R gene, also harbored specific nucleotide differences between SPPV field isolates, GTPV and LSDV (Fig. 1). To investigate the uniqueness of these deletions to SPPV vaccines, we sequenced and analyzed the corresponding region in SPPV vaccines from Algeria, Senegal and the Pan African Veterinary Vaccine Center of the African Union Commission (AU-PANVAC). The nucleotide sequences generated in this study have been deposited in GenBank under accession numbers MK005931 to MK005967. The comparative analysis of the nucleotide sequences showed the presence of these two deletions in only the SPPV vaccines, as compared to SPPV field isolates, GTPVs and LSDVs (Supplementary Fig. S1). The sequencing results also confirmed the presence of species-specific nucleotide differences between the SPPV field isolates, GTPV and LSDV. We identified 6 single-nucleotide differences when comparing SPPV field isolates to LSDVs: C/G, T/C, C/T, G/A, A/T, G/A, of which only four nucleotide changes (C/G, G/A, A/T, G/A) can influence the melting temperature. Comparing GTPVs with LSDVs, 7 nucleotide differences were found: G/A, T/A, A/G, C/G, G/A, A/G, G/T, including three aggregate changes that could be exploited during the melting curve analysis: T/A, C/G, G/T.

This region was then targeted to design another set of primers for the amplification of a 110 bp fragment in SPPV vaccine strains and a 158 bp fragment in SPPV field strains, GTPV and LSDV.

Subsequently, we tested the expected melting profiles and melting peaks of the predicted amplicons for SPPV vaccine strains, SPPV field isolates, GTPVs and LSDVs using the uMelt software. The results show four different and distinct melting peaks for SPPV vaccine strains, SPPV field isolates, GTPVs and LSDVs (Supplementary Fig. S2), indicating that the anticipated PCR products could be used to differentiate these four genotypes by HRM.

PCR Optimization. Following an initial testing of a panel of primers, the pair of forward and reverse primers indicated in Table 1 was used for optimization and evaluation of the HRM method. During optimization, the most critical parameters were primer concentration, annealing temperature and time, number of cycles and

Method	Primer Name	Primer sequence (5′–3′)	Amplicon size
HRM	Cap_ B22RDIV_For	TATGGATTTAGGAGTAGA	158, 110 (Field & vaccine)
	Cap_ B22RDIV_Rev	GCTTTACTTTAATATCATTG	
Sequencing	B22R_ seqHRMFor	TAACGGCATATTGTCTGAATC	250 bp
	B22R_ seqHRMRev	GCTTTACTTTAATATCATTG	

Table 1. Primers used in this study for the HRM assay and for sequencing.

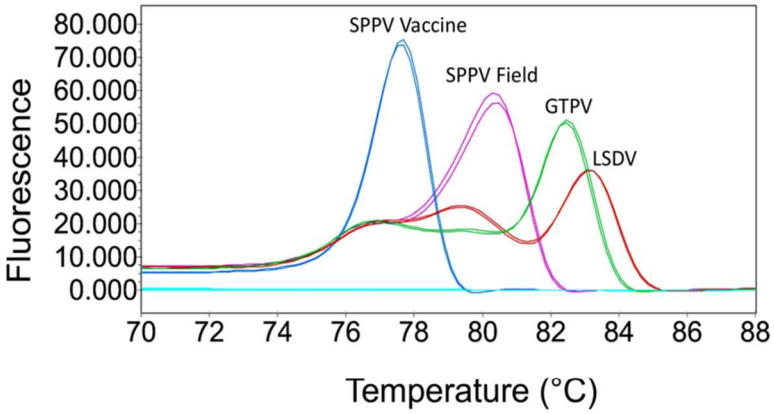

Figure 2. Melting curve variance of SPPV vaccine and three CaPVs Species. The melting curve and melting temperature for SPPV vaccine and each CaPVs species can be well-discerned.

number of acquisitions during melting. Once the assay was optimized, we evaluated the difference between Tms of all four genotypes using plasmids containing the targeted sequences for SPPV Morocco vaccine, SPPV Turkey/98_Denizli, GTPV Awi/O13/2011 and LSDV Guder/B5/2008. The results show that the SPPV vaccines could be distinctly differentiated from SPPV field isolates and at the same time, the assay could classify CaPV isolates into one of three species (Fig. 2). The average Tm values with the control plasmids (at 10^4 to 10^6 copies per reaction) were 76.90 ± 0.13 for SPPV vaccine, 80.27 ± 0.11 for SPPV field isolates, 81.58 ± 0.06 for GTPV and 82.29 ± 0.07 for LSDV.

Improved Data Analysis using HRM Software. To enhance and clarify the data analysis, we used the HRM software to further analyze the melting curves of the PCR amplicons. The HRM analysis of the normalized melting (Fig. 3a) and differential curves (Fig. 3b) were clearly distinct between SPPV vaccine strains, SPPV field isolates, GTPV and LSDV, producing four independent clusters, readily and consistently resolved (Fig. 3a,b). The HRM analysis results were consistent with the conventional melting curve analysis; however, the processed graphs displayed by the HRM software were easier to interpret.

Limit of Detection of the Assay. The limits of detection, with ≥95% confidence limits, were: 38.04 (31.1–54.14), 17.80 (14.62–26.46), 16.27 (12.63–25.53) and 17.01 (13.22–26.64) copies per reaction for SPPV vaccine, SPPV field isolates, GTPV and LSDV respectively.

Discriminating Power of the Assay. To study the discriminating power of the HRM assay, 61 DNA samples extracted from SPPV vaccines, cell culture supernatants and clinical samples of CaPVs field isolates, from various geographical locations were screened (Supplementary Table S1). From the 61 samples, we identified SPPV vaccines (n = 4), SPPV field isolates (n = 14), GTPVs (n = 11) and LSDVs (n = 32). The overall Tm ranges, illustrated in Fig. 4, show a clear separation between all four genotypes. The Tm values for the samples were very similar to those of the control plasmids for each genotype: 76.73 ± 0.35 for SPPV vaccine, 80.02 ± 0.16 for SPPV field isolates, 81.66 ± 0.22 for GTPV and 82.29 ± 0.10 for LSDV. One-way ANOVA determined that the average Tm between SPPV vaccines, SPPV field isolates, GTPVs and LSDVs were significantly different (P = 0.000).

We also tested artificial mixtures of control plasmids at 10^4 copies/µl, at five proportions (1/9, 2/8, 5/5, 8/2 and 9/1) to analyze the potential of the assay to detect coinfection of SPPV vaccine with SPPV field isolates or GTPV or LSDV. At all proportions tested, we observed two readily distinct peaks for the mixture SPPV vaccine/GTPV (at Tm 76.26 for SPPV vaccine and 81.58 for GTPV), and the mixture SPPV vaccine/LSDV (Tm 76.58 for SPPV vaccine and 82.36 for LSDV). In contrast, for the mixture SPPV vaccine/SPPV field produced two readily distinct peaks (Tm 76.38 for SPPV vaccine and 80.25 for SPPV field isolates) at the proportion of 9/1 only. The mixture at the proportion 8/2 presented only a shoulder peak at Tm 76.38 (for the vaccine) and the SPPV field peak at Tm 80.25. The SPPV vaccine peak was absent at other proportions of the mixtures SPPV vaccine/SPPV field. Overall, there was a small shift in the Tm of SPPV vaccine in the mixtures as compared to SPPV vaccine tested alone.

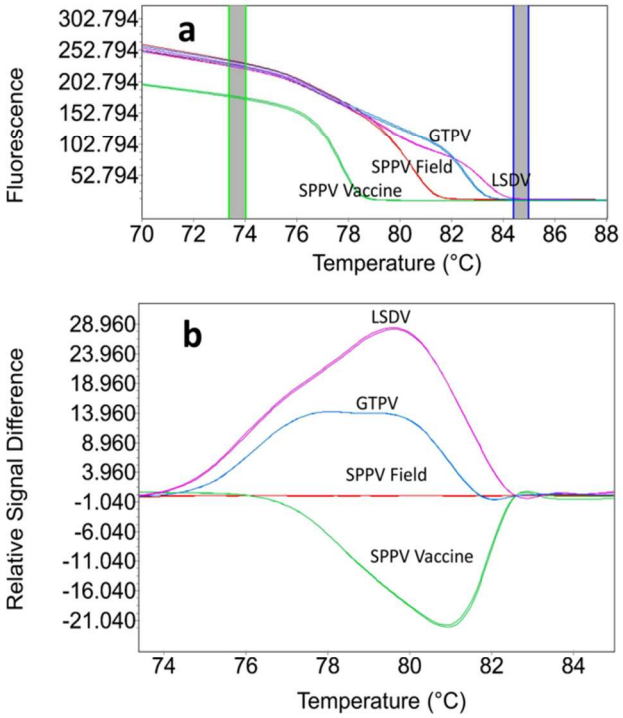

Figure 3. Normalized HRM plots for SPPV vaccine and other CaPVs. (**a**) The graphs visualize the representative profiles of normalized melt curve and (**b**) difference melting curve plots. Each of the genotype species were given a different colored line. The two dark columns in the melting curve plot (**a**) represent pre and post melting normalization regions. In the difference plot (**b**), SPPV field isolates have been selected as the reference for displaying the deviations between SPPV vaccine from GTPV and LSDV field isolates.

Specificity. The specificity of the assay was evaluated by testing 13 DNA extracts from other ruminant pathogens (Supplementary Table S2). No amplification was recorded with these non-capripoxvirus pathogens including parapoxviruses.

Cross-platform Compatibility Test. To assess the cross-platform compatibility of this method, we performed the assay using a selected panel of samples including both plasmid and viral DNA extracted from cell culture supernatants and clinical samples on four different real-time PCR instruments: CFX96 (BioRad), QuantStudio 6 (LifeTechnologies), RotorGene Q (Qiagen) and LightCycler 480 (Roche). This assay was successfully run on all four platforms, utilizing the same mixture and protocol, and succeeded in differentiating SPPV field isolates from SPPV vaccine strains as well as placing the samples in the correct genotypes. (Supplementary Fig. S3). With the various platforms, we noticed a slight shift in the Tm values of the amplicons from one instrument to another (Table 2).

Discussion

We have described the identification of specific genetic profiles of CaPV genomes and their use in an HRM-based assay to differentiate SPPV vaccines from SPPV field isolates and further classify CaPVs into SPPV, GTPV or LSDV.

Analysis of the partial fragment of the B22R gene in 37 representative SPPV vaccines, SPPV field isolates, GTPVs and LSDVs available for this study, confirmed two sets of deletions (21 bp and 27 bp) that are specific to the SPPV Romanian and Yugoslavian RM65 vaccine strains, which are used in Morocco, Egypt, Senegal and Algeria and the Romanian Fenner strain (MG000157), which is used as a SPPV vaccine in India. However, the comparisons showed that these deletions are absent in the B22R gene of SPPV NISKHI, an SPPV vaccine used mainly in Russia and countries of the former Soviet Union such as Kazakhstan[25–27]. A previous study reported size differences among CaPVs in the B22R gene and other genes, in the terminal genomic regions, encoding for proteins involved in viral virulence and host range[25]. Although the B22R gene of SPPV NISKHI displayed similarity to virulent SPPVs, prominent differences are present in two of the five CaPV ankyrin repeat-containing genes; a single in-frame stop and a frameshift mutation in SPPV NISKHI SPPV138 and SPPV141, respectively, which resulted into two smaller ORFs, suggesting a different mechanism of attenuation[25,27] as compared to the SPPV vaccines targeted in this study. Both mutations involved only a single nucleotide and were absent in the draft genome used to design our assay. Similarly, those mutations were absent in the publicly available genome of SPPV Romanian Fenner (MG000157), a vaccine strain comparable to those tested in this study.

The comparative analysis of the partial B22R sequences of SPPV field isolates, GTPVs and LSDVs generated in this study with those retrieved from full genomes in public databases also confirmed the presence of

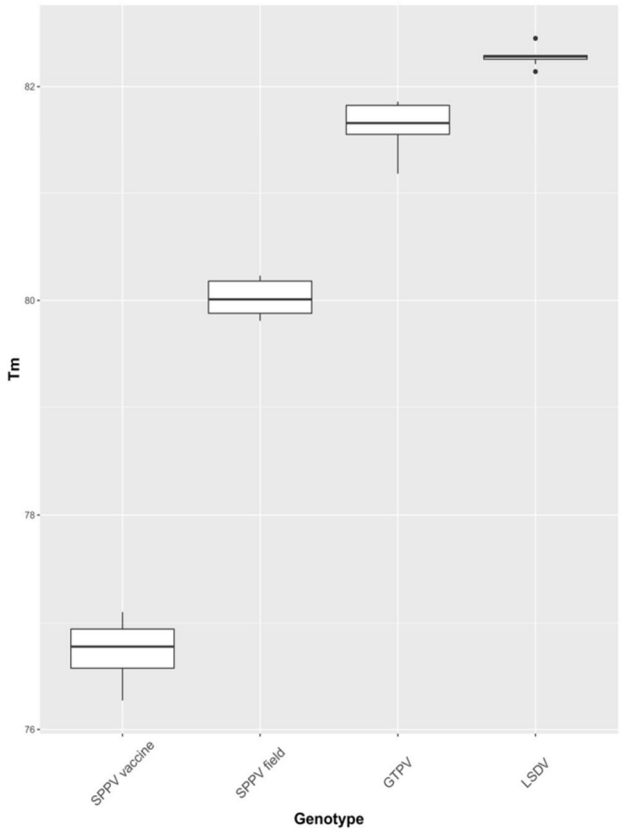

Figure 4. Box plots of the melting temperatures (Tms) of SPPV vaccines and other CaPVs species. The differences in Tm for SPPV vaccine, SPPV field, GTPV and LSDV are shown. The ANOVA test showed a significant difference in average Tm values between the genotypes (P = 0.000).

Virus Genotype	Real time PCR machines with Tm values			
	CFX 96 (Bio-Rad)	LC 480 II (Roche)	QS 6 (Life tech)	RG-Q (Qiagen)
SPPV vaccine	75.8–76.3	76.59–76.88	76.45–76.74	77.2–77.54
SPPV field	78.9–79.4	79.87–80.26	79.74–80.22	80.46–80.9
GTPV	80.4–80.4	81.39–81.52	81.29–81.38	82–82.2
LSDV	80.9–80.9	82.07–82.16	81.96–82.06	82.6–82.66

Table 2. Cross-platform testing of the HRM assay. Four different real-time instruments: the LigthCycler 480 (LC 480 II), QuantStudio 6 (QS 6), RotorGene Q (RG-Q) and CFX 96 were used for assay evaluation. The respective amplicon melting temperature values are indicated for SPPV vaccine and other CaPVs.

species-specific signatures that were well-conserved within each of these 3 species, with minor sequence variability noted for both SPPV field isolates and GTPVs (Supplementary Fig. S1). The HRM typing results were in full agreement with sequence results of the PCR amplicons for all 37 samples that were sequenced and the sequence variability had no impact on the melting temperatures within each genotype. Thus, classification of the CaPV isolates into SPPV, GTPV and LSDV using the current HRM assay was consistent with previous methods[18–20], for all 61 samples in this study. Of interest, is that the assay identified and correctly assigned previously characterized isolates into the correct CaPV species[20]: SPPV KS1, collected from sheep, was identified as a LSDV; SPPV Oman, GTPV Kitengela/O58/2011 and GTPV Kitengela/O59/2011 collected from sheep were identified as a GTPVs; GTPV Saudi Arabia, collected from a goat was identified as a SPPV. Similarly, two recent isolates, GTPV_Awi/O13/2011 and GTPV_Bale/O14/2007, both collected from sheep were identified as GTPVs.

Though we only tested LSDVs from Africa, our sequence analyses showed that LSDVs from Israel (KX894508), Greece (KY829023), Serbia (KY702007) (Supplementary Fig. S1), and Russia (MH893760), were 100% identical to African LSDVs on the targeted fragment of the B22R gene.

Our experiments produced melting curves (Fig. 2) with similar shapes to the uMelt predicted melting curves (Supplementary Fig. S2), with different Tm values, thus illustrating the usefulness of the uMelt simulation software[28] for the design of HRM-based assays.

The HRM assay displayed good sensitivity, comparable to a previously reported dual-hybridization probe assay for CaPVs' classification[20]. It was highly specific, with no misidentification of genotypes and no reactivity with other ruminant pathogens tested in this study.

The current HRM assay is easy to perform, interpret and is compatible with various qPCR platforms. Indeed, with all four qPCR instruments tested in this study, the analysis of the melting curves was sufficient to differentiate SPPV vaccines from SPPV field isolates and discriminate between SPPV, GTPV and LSDV. Additionally, we found that using the HRM analysis software enables a better visual representation for the discrimination of different clusters, especially when several samples are analyzed in a single run.

In SPP and GTP endemic countries, the Yugoslavian RM65, the Romanian and the KSGP 0240 strains are the most widely used live attenuated vaccines to protect small ruminants against SPP and GTP[12,13]. The KSGP 0240 vaccine strain which is used in several SPP and GTP endemic countries[12] was shown to be LSDV[29,30], therefore, it can be differentiated from SPPV field isolates using species-specific PCR methods[18–20]. The Yugoslavian RM65 strain which is mainly used in the Middle East, Asia and the Horn of Africa[12], and the Romanian strain, used in India and the Maghreb region[12], are all SPPV derived vaccines. In the event of capripox outbreaks in small ruminant herds, following the administration of a vaccine based on one of these SPPV strains, it is essential to determine whether the disease is caused by a field isolate or a side effect of the vaccination. Our assay presents the advantage of enabling such a differentiation between SPPV vaccines from SPPV field isolates, and the classification of CaPVs into one of its three species. Two previous reports have described methods to differentiate SPPV vaccines from SPPV field isolates[13,17], however these methods could not directly classify the CaPV species without additional genotyping methods or sequencing. SPPV and GTPV are not strictly host specific. Similarly, LSDV has been detected in antelope, and the KS1 isolate which was initially collected from small ruminants, has been shown to be LSDV based on sequencing[20,29]. Thus, to implement efficient CaPV disease control measures, it is essential to use specific tools for the accurate identification of circulating isolates in sheep, goats, cattle and wildlife. Indeed, despite the cross-protection displayed by CaPVs, the use of homologous vaccines is preferred as it is believed to provide better protection[7,12].

Though several assays are available for CaPV species determination by PCR and real time PCR[18–20,30], none are intended for differentiation of SPPV vaccines from SPPV field isolates. Various research groups developed assays to differentiate the LSDV Neethling vaccine from LSDV field isolates in cattle[14,15], and furthermore genotype CaPVs[16]; however, those assays are unable to differentiate SPPV vaccines from SPPV field isolates. Therefore, it is expected that this HRM assay will be used as a direct means for screening samples from suspected capripox cases collected in unvaccinated flocks, and to undertake epidemiological investigations when a capripox outbreak occurs following vaccination in a small ruminant or cattle herds.

Materials and Methods

Viruses and Nucleic Acid Extraction. A total of 61 CaPV strains were used in this study: four SPPV vaccine strains derived from the Romanian and Yugoslavian RM/65 strains and 57 field isolates of CaPV including cell culture supernatants, and field samples collected from outbreaks at various geographical locations (Supplementary Table S1). In addition, field samples (n = 13) confirmed to be free of CaPV but infected with other ruminant pathogens were used to evaluate the specificity of the new assay (Supplementary Table S2). DNA was extracted from tissue culture supernatant and clinical samples using the AllPrep DNA/RNA extraction kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The DNA samples were used immediately or kept at −20 °C until use. All pathological samples and viral isolates were handled within the biosafety level 3 facility of the Institute for Veterinary Disease Control, Austrian Agency for Health and Food Safety, Austria.

Target Gene and Primers Design. In order to identify regions with unique differences between SPPV vaccines and SPPV field isolates, we scanned the sequence alignments of a draft full genome SPPV vaccine from Morocco and representative field isolates and vaccines of all three CaPV species. The sequences were retrieved from Genbank (accession numbers are indicated in Fig. 1) and aligned using MAFFT (http://mafft.cbrc.jp/alignment/server). To broaden the scope of the assay to enable the classification of CaPVs, we selected the final region based on the following: (1) the selected region offered markers for an unequivocal differentiation of SPPV vaccines, derived from the Romanian and Yugoslavian RM65, from SPPV field isolates; (2) the region presented enough nucleotide differences between the SPPVs, GTPVs and LSDVs that enabled the separation of these three species by HRM; (3) the differences between SPPV, GTPV and LSDV were well conserved within each CaPV species, thus representing strong species-specific signatures for genotyping. A region within the B22R gene of CaPVs which contains two separate nucleotide deletions of 21 and 27 nucleotides in length was selected. The same region also presented species-specific signatures for SPPV, GTPV and LSDV. A primer pair (Table 1) flanking this region was selected (positions 122045 to 122202 in SPPV A genome) to amplify and sequence the targeted regions in all SPPV vaccines, and in some representatives of SPPV field isolates, GTPV and LSDV available in our laboratory. Another set of primers (positions 121952 to 122202 in SPPV A genome) was designed for evaluation in the HRM assay (Table 1). To confirm the suitability of these primers in the HRM assay to differentiate SPPV vaccines from SPPV field isolates with the simultaneous classification of CaPVs, we performed an in-silico simulation, with the predicted PCR amplicons for SPPV vaccines, SPPV field isolate, GTPV and LSDV, using the uMelt software[28]. uMelt is a flexible web-based tool for predicting DNA melting curves and denaturation profiles of PCR products. The above-mentioned primers were selected using Allele ID version 6 software (Premier Biosoft International, Palo Alto, CA, USA), synthesized and purified by reverse phase high-performance liquid chromatography by Eurofins, Germany. The specificity of the primer sequences were checked using the Basic Local Alignment Search Tool (NCBI/Primer-BLAST). From now on, we will refer to each of these four groups, SPPV vaccines, SPPV field isolates, GTPVs and LSDVs, as genotypes.

Positive Controls. Positive control plasmids, containing the target gene fragment of the B22R gene of SPPV Morocco vaccine, SPPV Turkey/98 Denizli, GTPV Awi/O13/2011 and LSDV Guder/B5/2008 isolates were constructed by ligating the respective PCR products into the pGEM-T Easy Vector System (Promega, Madison, WI, USA) as per the manufacturer's instructions. The ligated products were used to transform E. coli DH5α competent cells (Invitrogen, Carlsbad, CA, USA). The positive clones were propagated, and the plasmid DNA extracted using the PureYield plasmid Midiprep System (Promega, Madison, WI, USA). The purified plasmids were quantified with the Nanodrop 3300 Fluorospectrometer using the PicoGreen dsDNA reagent kit (Thermo Fisher Scientific, Waltham, MA, USA) per manufacturer's instructions and sequenced to confirm the presence of the correct insert. The copy number was calculated as described by Lamien *et al.*[20].

HRM Analysis. The 10 µl PCR reaction mixture contained 1X Lightscanner master mix (BioFire Defense, Utah, USA.), 250 nM of each primer (Cap_ B22RDIV_For and Cap_ B22RDIV_Rev) and 2 µl of sample DNA. The PCR reaction was performed in a LightCycler 480 II Real time PCR Detection System (Roche Diagnostics Corporation, IN, USA) with an initial denaturation at 95 °C for 4 min, followed by 42 cycles of 95 °C for 5 sec, 58 °C for 5 sec and 72 °C for 5 sec. The PCR products were then denatured at 95 °C for 30 sec, cooled to 65 °C for 1 min, and melted from 65 °C to 90 °C at a rate of 100 acquisitions per °C. For each set of reactions, positive control plasmids and negative controls consisting of nuclease-free water in place of the template DNA were included. High Resolution Melting (HRM) curve analysis was performed using the LightCycler 480 Gene Scanning Software (Roche) to analyze the data and melting profiles of SPPV vaccines, SPPV field isolates, GTPVs and LSDVs. Normalized melt curves and difference plots were obtained by analyzing the active melt region and designating the pre and post melt regions.

Limit of Detection. Each plasmid was 10-fold serially diluted from 10^7 to 10^1 viral copies/µL using Herring sperm DNA matrix (5 ng/mL) and kept at 4 °C until further analysis. First, the linearity of the assay was analyzed for each viral species to determine the efficiency and dynamic range of the assay. Subsequently, the limit of detection (LOD) of the method was assessed by amplifying eight different concentrations (100, 80, 60, 40, 20, 10, 5 and 0 viral copies/µL) of each plasmid, corresponding to each of SPPV vaccine, SPPV field isolate, GTPV and LSDV. Five replicates of different plasmid concentrations were tested on four separated occasions, and the proportion of positive results were determined for each concentration. The LOD of the current assay was determined by probit regression analysis.

Discriminating Power and Specificity of the Assay. The discriminating power of the developed HRM assay was evaluated using DNA (n = 61) extracted from suspected capripox clinical samples and CaPV infected cell culture suspensions from sheep, goats and cattle from different geographical regions (Supplementary Table S1). The accuracy of the assay results was confirmed by comparing the HRM genotyping results with the sequencing data of the amplicons. The specificity of the method was also evaluated by testing DNA (n = 13) extracted from Mycoplasma mycoides subsp. mycoides Small Colony (MmmSC), Orf virus (ORFV), Bovine herpes virus 1 and 2, and cDNA from Peste des Petits Ruminants virus (PPRV), (Supplementary Table S2).

Statistical Analysis. All statistical analyses were performed in R (version 3.4.1) via Rstudio (version 1.1.383). A One-Way ANOVA test and Tukey multiple comparisons of means were performed in R to determine whether the average Tm differences between the genotypes were significant. In addition, box and whisker plots were constructed to illustrate the differences between the Tm of the four genotypes using the ggplot2 package in R. For LOD determination, the LC_probit function of the ecotox package in R was used to calculate the predicted limits of detection and their confidence limits (CL) using a probit analysis. The dose-response curves were plotted using ggplot2 in R.

Cross Platform Compatibility Test. The cross-platform compatibility of this assay was evaluated using various real time PCR instruments. Thus, using the same PCR mix and protocol as in the LightCycler 480 II (Roche) instrument, PCR and melting curve acquisition analysis was also conducted using the CFX96 Real Time PCR system (Bio-Rad, Hercules, CA, USA), QuantStudio 6 Flex Real Time PCR system (ThermoFisher Scientific Inc., Waltham, MA, USA), and Rotor Gene Q Real Time PCR cycler (Qiagen).

Nucleotide Sequencing and Analysis. A 250 bp region of the B22R gene containing the targeted HRM fragment was amplified by PCR for selected samples, using the primers B22R seqHRM-For and B22R-seqHRM-Rev (Table 1), and sequenced. The 20 µl PCR reaction mixture contained 500 nM of each primer, 0.2 mM dNTPs, 2.5U Taq DNA polymerase (Qiagen), 1X PCR buffer, and 2 µl template DNA. The cycling conditions were as follows: 95 °C for 4 min, followed by 35 cycles at 95 °C for 30 sec. 58 °C for 30 sec, and 72 °C for 30 sec, and a final extension at 72 °C for 2 min. PCR products were cheeked by electrophoresis on a 2% agarose gel for 1 h at 100 V. The PCR products were purified using Wizard SV Gel and PCR Clean Up System (Promega) and sequenced commercially by LGC Genomics (Germany). The sequence data were edited, and the fragments were assembled using Vector NTI 11.5 software (Invitrogen). Additional nucleotide sequences of the corresponding regions for SPPV, GTPV and LSDV isolates were retrieved from GenBank for comparative analysis. Multiple sequence alignments were performed using the CLUSTALW algorithm implemented in BioEdit 7.5 software package.

Data Availability

DNA sequences have been deposited in GenBank and the accession numbers were provided in the manuscript. All the remaining datasets generated during this study are available from the corresponding author on request.

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Author Contributions

C.E.L. and T.R.C. conceived and designed the experiments. T.R.C. and A.O. performed the experiments. T.R.C., C.E.L., R.G., A.L., A.O. and T.B.K.S. analyzed the data. A.L., N.N., K.T., H.M., M.D. and E.T. contributed reagents/materials/analysis tools. T.R.C., C.E.L., R.G., F.J.B. and A.D. wrote the paper. A.D., G.C., R.G. and C.E.L. supervised the study. F.J.B., A.D., G.C., R.G., N.N., K.T., E.T., T.B.K.S., H.M. and M.D. edited the final manuscript.

Additional Information

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Supplementary information

Supplementary table S1. Capripoxviruses tested by the HRM for this study.

	Isolate name	Origin	Year	Source	Type of samples	Host	Tm	Genotype
1	LSDV_Egypt/98_Ismailia	Egypt	1998	HSL-AGES	CC	Cattle	82.27	LSDV
2	LSDV_Ziway/B3/2011	Ethiopia	2011	NAHDIC	SL	Cattle	82.27	LSDV
3	LSDV PANVAC-7	Egypt	NA	PANVAC	CC	Cattle	82.27	LSDV
4	SPPV_KS-1	Kenya	1976	HSL-AGES	CC	Sheep	82.14	LSDV
5	LSDV Marsabit/B291/2007	Kenya	2007	Kenya	SL	Cattle	82.21	LSDV
6	LSDV Arsi/B1/2011	Ethiopia	2011	NAHDIC	SL	Cattle	82.21	LSDV
7	LSDV Sudan North/2011	Sudan	2011	CVRL/Sudan	SL	Cattle	82.14	LSDV
8	LSDV Sundus/2012	Sudan	2012	CVRL/Sudan	SL	Cattle	82.14	LSDV
9	LSDV_Toke/B6/2008	Ethiopia	2008	NAHDIC	SL	Cattle	82.27	LSDV
10	LSDV Asella/B2/2011	Ethiopia	2011	NAHDIC	DNA	Cattle	82.21	LSDV
11	LSDV Adama/B4/2011	Ethiopia	2011	NAHDIC	DNA	Cattle	82.21	LSDV
12	LSDV Ambo/B8/2008	Ethiopia	2008	NAHDIC	DNA	Cattle	82.27	LSDV
13	LSDV chilimo/B11/2008	Ethiopia	2008	NAHDIC	DNA	Cattle	82.27	LSDV
14	LSDV Galesa/B12/2008	Ethiopia	2008	NAHDIC	DNA	Cattle	82.27	LSDV
15	LSDV Humbo/B23/2010	Ethiopia	2010	NAHDIC	DNA	Cattle	82.21	LSDV
16	LSDV Sodo/B24/2010	Ethiopia	2010	NAHDIC	DNA	Cattle	82.27	LSDV
17	LSDV Sennar/12	Sudan	2012	CVRL	SL	Cattle	82.45	LSDV
18	LSDV BG24/2010	Kenya	2010	Kenya	SS	Cattle	82.29	LSDV
19	LSDV B291 /2007	Kenya	2007	Kenya	SL	Cattle	82.45	LSDV
20	LSDV B338 / 2011	Kenya	2011	Kenya	SL	Cattle	82.29	LSDV
21	LSDV Massalamia P4	Sudan	1971	Sudan	CC	Cattle	82.45	LSDV
22	LSDV Massalamia P66	Sudan	NA	Sudan	CC	Cattle	82.29	LSDV
23	LSDV MB597 LN AMB C1	Ethiopia	2014	NVI	LN	Cattle	82.45	LSDV
24	LSDV MB575 /14 NS K24	Ethiopia	2014	NVI	NS	Cattle	82.45	LSDV
25	LSDV Mojo BG cattle	Ethiopia	2011	NVI	SL	Cattle	82.45	LSDV
26	LSDV MB581 TS Fic 7715	Ethiopia	2014	NVI	SL	Cattle	82.29	LSDV
27	LSDV Wonji S Cattle	Ethiopia	2011	NVI	SL	Cattle	82.29	LSDV
28	LSDV MB589 TS Qur C1	Ethiopia	2014	NVI	SL	Cattle	82.29	LSDV
29	LSDV Akaki TS C-1	Ethiopia	2014	NVI	SL	Cattle	82.29	LSDV
30	LSDV MB581 TS Fic 7758	Ethiopia	2014	NVI	SL	Cattle	82.45	LSDV
31	LSDV MB607 TS Tig C3	Ethiopia	2014	NVI	SL	Cattle	82.29	LSDV
32	LSDV MB597 NS Am C9	Ethiopia	2014	NVI	NS	Cattle	82.29	LSDV
33	GTPV Iraq/61_Gorgan	Iraq	1961	Pirbright	CC	Goat	81.79	GTPV
34	GTPV Mongolia/C3/08	Mongolia	2008	Mongolia	SL	Goat	81.86	GTPV
35	GTPV Oman/84	Oman	1984	Pirbright	CC	Goat	81.86	GTPV
36	GTPV Awi/O13/2011	Ethiopia	2011	NAHDIC	SL	sheep	81.66	GTPV
37	GTPV Bale/O14/2007	Ethiopia	2007	NAHDIC	SL	sheep	81.66	GTPV
38	GTPV Ghana	Ghana	2011	Pirbright	CC	Goat	81.18	GTPV
39	GTPV Kitengela/O58/2011	Kenya	2011	CVL	SL	sheep	81.79	GTPV
40	GTPV Yemen/83	Yemen	1983	Pirbright	CC	Goat	81.45	GTPV
41	SPPV Oman/84	Oman	1984	Pirbright	CC	Goat	81.45	GTPV

42	GTPV Kitengela/O59/2011	Kenya	2011	CVL	SL	sheep	81.66	GTPV
43	Mog/ GP/T5/2008	Mongolia	2008	Mongolia	SS	Goat	81.86	GTPV
44	SPPV Mongolia /O2/07	Mongolia	2007	Mongolia	SL	sheep	79.88	SPPV
45	SPPV Mauritania/85 Gorgol	Mauritania	1985	Mauritania	SL	Sheep	80.18	SPPV
46	SPPV Mongolia/T1/06	Mongolia	2006	Mongolia	SL	sheep	79.88	SPPV
47	SPPV Turkey/98 Van 2	Turkey	1998	VCRI-Pendik	CC	sheep	80.23	SPPV
48	SPPV Turkey/98 Corum	Turkey	1998	VCRI-Pendik	CC	Sheep	79.88	SPPV
49	GTPV Saudi Arabia/93	Saudi Arabia	1993	Pirbright	CC	Goat	80.01	SPPV
50	SPPV Turkey/98 Darica	Turkey	1998	VCRI-Pendik	CC	Sheep	80.01	SPPV
51	SPPV Turkey/98 Denizli	Turkey	1998	VCRI-Pendik	CC	Sheep	80.23	SPPV
52	SPPV Algeria/93 Djelfa	Algeria	1993	INMV-LCV	CC	Sheep	79.81	SPPV
53	SPPV HSL	Unknown	NA	HSL-AGES	SL	sheep	80.23	SPPV
54	SPPV Algeria/05 Illizi	Algeria	2005	INMV-LCV	CC	Sheep	80.18	SPPV
55	SPPV Niger/88	Niger	1988	CIRAD	CC	Sheep	80.01	SPPV
56	Mog/SP/T3/2007	Mongolia	2007	Mongolia	SS	sheep	79.81	SPPV
57	SPPV Turkey/98 Sivas	Turkey	1998	VCRI-Pendik	CC	Sheep	79.88	SPPV
58	SPPV PANVAC-6 Vaccine	Egypt	NA	PANVAC	CC	sheep	76.27	SPPV vaccine
59	SPPV Algeria vaccine Lot_7	Algeria	2009	INMV-LCV	CC	Sheep	76.67	SPPV vaccine
60	SPPV Senegal vaccine	Senegal	NA	CIRAD	CC	Goat	76.89	SPPV vaccine
61	SPPV Morocco vaccine	Morocco	NA	Biopharma	CC	Sheep	77.1	SPPV vaccine

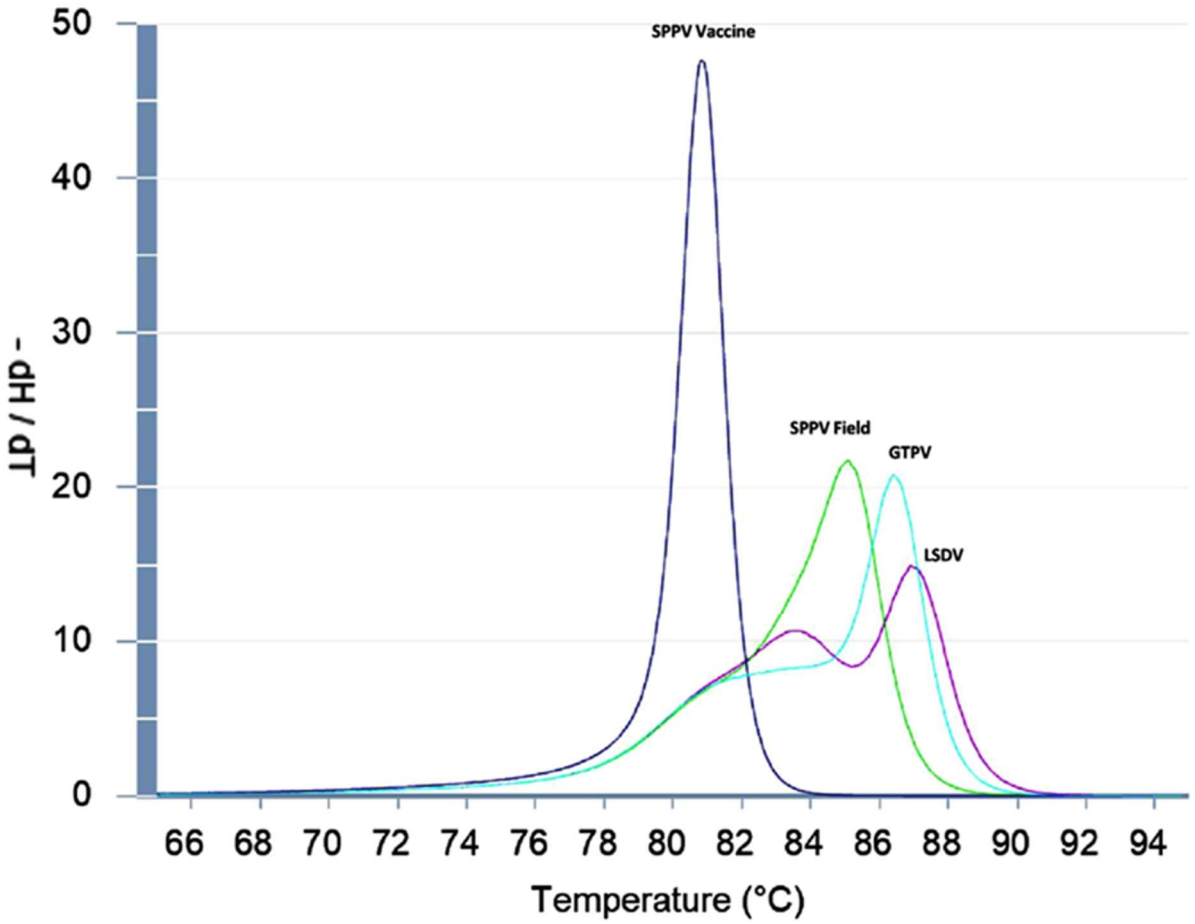
Abbreviations: VCRI = Veterinary Control and Research Institute; LNERV-ISRA = Laboratoire National de l'Elevage et de RecherchesVétérinaires, InstitutSénégalais de RecherchesAgricoles ; INMV-LCV = Institut National de la MédecineVétérinaire, Laboratoire Central Vétérinaire; IVM = Institute of Veterinary Medicine; Pirbright = The Pirbright Institute; PANVAC = Pan African Veterinary Vaccine Centre; NAHDIC = National Animal Health Diagnostic and Investigation Center; HSL-AGES = High Security Laboratory, Austrian Agency for Health and Food Safety; CVL: Central Veterinary Laboratories. NA = Not Applicable; CC = Cell culture; SL = Skin lesions; SC = Skin scraping; NS = Nasal swab; LN = Lymph node

Supplementary table S2. Non-Capripoxvirus samples used for this study.

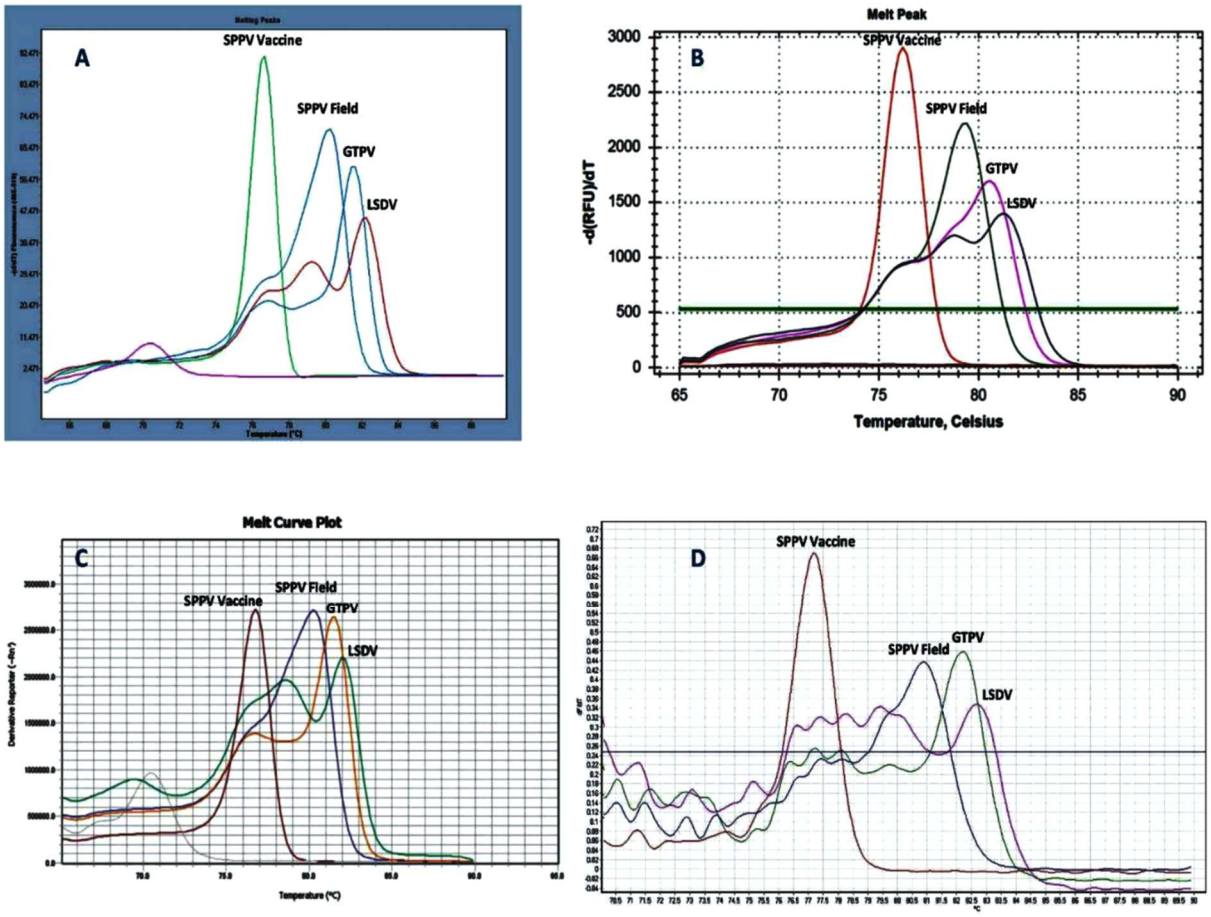
No.	Strain name	Source	Country	Sample type	Host
1	ORFV MB38/13 C-2	NVI/Ethiopia	Ethiopia	Skin scraping	Sheep
2	ORFV MB38/13 C-3	NVI/Ethiopia	Ethiopia	Skin scraping	Sheep
3	ORFV MB38/13 C-4	NVI/Ethiopia	Ethiopia	Skin scraping	Sheep
4	ORFV MB38/13 C-5	NVI/Ethiopia	Ethiopia	Skin scraping	Goat
5	ORFV MB38/13 C-6	NVI/Ethiopia	Ethiopia	Skin scraping	Goat
6	ORFV MB38/13 C-7	NVI/Ethiopia	Ethiopia	Skin scraping	Goat
7	PPRV (cDNA)	NVI/Ethiopia	Ethiopia	Nasal swab	Goat
8	PPRV (cDNA)	NVI/Ethiopia	Ethiopia	Nasal swab	Goat
9	PPRV (cDNA)	NVI/Ethiopia	Ethiopia	Nasal swab	Goat
10	Mccp	NVI/Ethiopia	Ethiopia	Pathological lesions	Goat
11	Mccp	NVI/Ethiopia	Ethiopia	Pathological lesions	Goat
12	BOHV-1 404/2018	HSL-AGES/Austria	Austria	Cell culture	Cattle
13	BOHV-2 95-5/2016	HSL-AGES/Austria	Austria	Cell culture	Cattle

Abbreviations: BOHV = Bovine herpes virus; Mccp= Mycoplasma capricolum ssp. Capri pneumonia; NVI = National Veterinary Institute; HSL-AGES = High Security Laboratory, Austrian Agency for Health and Food Safety; PPRV (cDNA) = Peste des petits ruminants' virus complementary deoxyribonucleic acid; ORFV= Orf Virus

Supplementary Figure S1. Multiple sequence alignments of the partial B22R gene sequences of 37 Capripoxviruses from this study and representative Capripoxviruses from GenBank



Supplementary Figure S2. Derivative melt curves, using the uMELT BATCH2.2, for SPPV vaccine, SPPV field isolate, GTPV and LSDV. Sugimoto (Nucleic Acids, 1996) thermodynamic library was used, with a temperature range from 65 to 95°C. Resolution setting was 0.1°C and salt concentrations were 20 mM for Mono+ and 3 mM for Mg++. The results show 4 distinct melting peaks for SPPV vaccine (Tm = 80.8°C), SPPV field isolate (Tm = 85.0°C), GTPV (Tm = 86.4°C) and LSDV (Tm = 87.1°C)



Supplementary Figure S3. Cross-platform testing of the HRM assay. The melting curves of SPPV vaccine, SPPV field isolate, GTPV and LSDV are shown for:(A) the Light Cycler 480 II (Roche), (B) the CFX96 Real Time PCR system (Bio-Rad), (C) the Quant Studio 6 Flex Real Time PCR system (Thermo Fisher Scientific Inc) and (D) the Rotor Gene Q Real Time PCR cycler (Qiagen).

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Chapter III

Molecular characterization of Lumpy skin disease viruses in East Africa

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Manuscript ready for submission

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Molecular Characterization of Lumpy Skin Disease Viruses in East Africa

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Abstract

Lumpy skin disease (LSD) is an economically significant disease of cattle, caused by Lumpy skin disease virus (LSDV), and is endemic in nearly all of Africa. Since 2012, LSDV has emerged as a significant epizootic pathogen given its rapid geographic range expansion out of Africa and into the Middle East, Eastern Europe, and Russia. To assess the genetic diversity of LSDV in Eastern Africa, we retrospectively sequenced and analyzed the RPO30 and GPCR genes of 22 LSDV isolates. The isolates were collected in Ethiopia, Kenya and Sudan, prior to the wave of LSD in the Middle East and its incursion in Europe and were compared to publicly available sequences of LSDVs from the same region and those collected elsewhere. The results showed that the Eastern African field isolates of this study were highly similar to each other and previously sequenced field isolates of LSDV, for the RPO30 and the GPCR genes. The only exception was LSDV Embu/B338/2011, a field isolate collected in Kenya which displayed mixed features between LSDV Neethling vaccine and field isolates. This isolate contained the 12-nucleotide insertion found in LSDV Neethling and KS-1 vaccines. Further analysis of the partial EEV glycoprotein and B22R genes showed that LSDV Embu/B338/2011 differs from previously described LSDV variants with by a 12-nucleotide insertion in the GPCR gene. These findings highlight the importance of constant monitoring of genetic variation among the LSDV isolates.

Keyword: LSDV, GPCR gene, RPO30 gene, EEV glycoprotein and B22R genes

Introduction

Lumpy skin disease (LSD) is an important contagious disease of cattle characterized by the appearance of nodules on the skin and enlarged superficial lymph nodes. It is caused by Lumpy skin disease virus (LSDV) of the *Capripoxvirus genus* within the family Poxviridae (Babiuk et al. 2008; Diallo and Viljoen 2007). LSDV has a double-stranded DNA genome of approximately 151 kb. It is closely related to sheep poxvirus (SPPV) and goat poxvirus (GTPV), two other members of the *Capripoxvirus genus*. However, there exists a distinct genetic variability causing differences in the virulence and host range of capripoxviruses. These differences make LSDV a host-specific pathogen for cattle (Tulman et al. 2001), although LSDV DNA was reported to be found in springbok antelopes in South Africa (Le Goff et al. 2009).

As LSD has a considerable economic impact on the cattle industry, the World Organization for Animal Health (OIE) has listed it as a notifiable disease (OIE 2017). In East Africa, Kenya was the first country to report LSD in 1957 (Davies 1976), followed by Sudan in 1971 (Ali 1977), and Ethiopia and Somalia in 1983 (Mebratu et al. 1984). Currently, LSD is endemic in most African countries except, Tunisia, Morocco, and Libya. The disease has also spread into most of the Middle East, Central Asia, Eastern Europe and Russia, posing a direct threat to the rest of the European countries (Babiuk et al. 2008; Tuppurainen and Oura, 2012). East Africa has a notable cattle population with important cross-border trade along the boundaries of Kenya, Ethiopia, Somalia, and Sudan. As this region is also a significant livestock export market for North Africa and the Arabian Peninsula, it was suggested that LSDV was re-introduced in Egypt in 2006 via infected cattle imported from the Horn of Africa. This seems to have been the starting point before the disease spread further in the Middle East (Alkhamis and VanderWaal 2016).

Therefore, this work focused on the molecular characterization of LSDV isolates collected in Kenya, Ethiopia, and Sudan, before the wave of LSD in the Middle East and Europe.

Molecular epidemiological studies of LSDV rely on the analysis of various regions of its genome such as the GPCR, the RPO30, and the EEV genes (Le Goff et al., 2009; Lamien et al., 2011; Gelaye et al., 2015; Menasherow et al., 2016).

Although Lumpy skin disease is endemic in East Africa, reports on molecular characterization of LSDV in the region are inadequate. Previously, the GPCR and the RPO30 of LSDV field isolates collected between 2008 and 2012 from several regions in Ethiopia were analyzed(Gelaye et al. 2015). . Our study further expands this analysis by investigating new

outbreaks in Ethiopia and further looking at isolates from Kenya and Sudan where little insight was available on LSDV's genomic data from post-2000 outbreaks. Because of the large size of the poxvirus genome, multiple genes or whole genome analysis is necessary to supplement the available molecular data. The present study describes the comprehensive molecular characterization of LSDV isolates from Eastern Africa.

Material and Methods

Samples and DNA Extraction

This study included 22 isolates (Table 1) of lumpy skin disease virus collected at various geographical locations in Ethiopia, Kenya and Sudan (Figure 1). The total DNA was extracted from clinical samples and cell culture supernatants using the All Prep DNA/RNA extraction kit (Qiagen) according to manufacturers' instructions. The DNA was eluted using 80 µl elution buffer and stored at -20 °C until used.

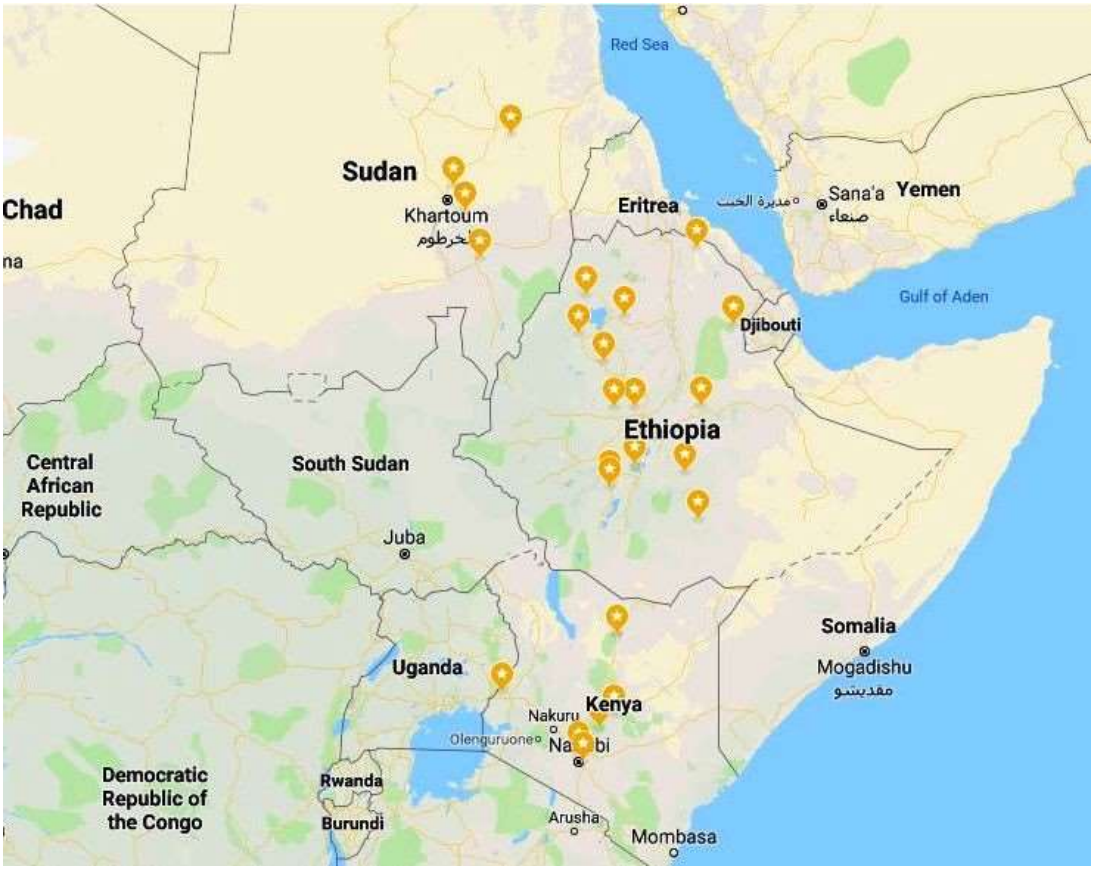

Figure 1. Map of East Africa showing Sudan, Kenya and Ethiopia, and the approximate geographical origins (highlighted in orange) of LSDV isolates included in this study.

Amplification of RPO30, GPCR, EEV glycoprotein and B22R genes

For all 22 samples, the RPO30 and the GPCR were amplified as previously described (Gelaye et al. 2015). A pair of primers; EEVGly F- 5'- ATGGGAATAGTATCTGTTGTATACG-3' and EEVGly R-5'- CGAACCCCTATTTACTTGAGAA-3' were designed for the amplification of fragments containing the partial EEV glycoprotein (encoded by ORF LSDV126) and hypothetical protein LSDV 127 gene.

The PCR reaction was performed in a reaction volume of 20 µl containing 500nM of each of the forward and reverse primers, 0.2 mM of dNTPs, 1x buffer (Qiagen), 2.5 U of Taq DNA polymerase (Qiagen) and 2 µl template DNA. The thermal cycler (Bio-Rad, USA) parameters were: initial denaturation at 95 ºC for 4 minutes followed by 35 cycles of 95 ºC for 40 sec, 55 ºC for 30 sec and 72 ºC for 1 minute, and a final extension step at 72 ºC for 7 minutes.

The partial B22R gene was amplified as described by (Chibssa et al. 2019).

The amplified PCR amplicons were separated by electrophoresis on a 1.5 % agarose gel at 100 V for 60 min and visualized using a Gel Documentation System (Bio-Rad, USA).

Sequencing and Phylogenetic analysis

The positive PCR products were purified using the Wizard SV Gel and PCR clean-up system kit (Promega) according to the manufacturer's instructions. The purified PCR products were sequenced at LGC Genomics (Germany). The sequence data were assembled using Vector NTI 11.5 software (Invitrogen, USA).

Nucleotide sequences and deduced amino acid sequences were aligned using the ClustalW algorithm implemented in BioEdit 7.1.7 and (Kumar et al., 2016). The complete RPO30 and GPCR gene sequences of eleven additional LSDVs from eastern Africa (Table 1), ten LSDVs from other regions, nine GTPVs and ten SPPVs retrieved from Genbank, were included for comparative analyses.

For phylogenetic reconstructions, the RPO30 and GPCR nucleotides sequences were aligned using the Muscle algorithm (codon option) implemented in MEGA 7. The aligned sequences in FASTA format were converted to nexus format using Seaview. The Bayesian phylogenetic inference was performed with BEAST v1.8.4 (Drummond et al. 2012). First, the BEAUti module was used to generate BEAST files from the sequence alignments in nexus format. The HKY substitution +G nucleotide substitution was used with a UPGMA starting tree. The Markov Chain Monte Carlo method was run with BEAST, for 10,000,000 generations with a sample taken each 10,000 generations. The TRACER programme was used to inspect the log files and determine the optimum number of burn-in based on the Effective Sample Sizes (ESS

> 200). Tree Annotator was used to generate the Maximum Clade Credibility (MCC) after discarding the 2% burn-in. The tree was visualized using the ggtree package in R (Yu et al. 2017). The read.beast and the get.fields functions were used to import the tree and associated meta-data. Additionally, for the GPCR tree, the multiple sequence alignment file of the nucleotide sequences was imported using the read.fasta function and a specific slice of the alignment, between position 80 and 120, was visualized together with the tree using the msaplot function (Yu et al. 2017).

Table 2. LSDV isolates from East Africa included in this study and previous isolates

No	Strain name	Host	Origin	Sample type	Vaccination history	Year	Accession number	
							RPO30	GPCR
1	LSDV Marsabit/B291/2007	Cattle	Kenya	Skin lesion	Non-vaccinated	2007	This study	This study
2	LSDV Embu/B338 /2011	Cattle	Kenya	Skin lesion	Vaccinated	2011	This study	This study
3	LSDV Bungoma/B624/2010	Cattle	Kenya	Skin lesion	Non-vaccinated	2010	This study	This study
4	LSDV Arsi/B1/2011	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2011	This study	This study
5	LSDV Asella/B2/2011	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2011	This study	This study
6	LSDV Ziway/B3/2011	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2011	This study	This study
7	LSDV Adama/B4/2011	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2011	This study	This study
8	LSDV Guder/B5/2008	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2008	This study	This study
9	LSDV Sodo/B22/2010	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2010	This study	This study
10	LSDV Humbo/B23/2010	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2010	This study	This study
11	LSDV Sodo/B24/2010	Cattle	Ethiopia	Skin lesion	Non-vaccinated	2010	This study	This study
12	LSDV Sundus/1971	Cattle	Sudan	Skin lesion	Non-vaccinated	1971	This study	This study
13	LSDV Sudan North//2008	Cattle	Sudan	Skin lesion	Non-vaccinated	2008	This study	This study

14	LSDV Sinnar/2006	Cattle	Sudan	Skin lesion	Non-vaccinated	2006	This study	This study
15	LSDV Toke/B6/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
16	LSDV Gindo/B7/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
17	LSDV Ambo/B8/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
18	LSDV Holeta/ B9/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
19	LSDV Ginchi/B10/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
20	LSDV Chilimo/B11/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
21	LSDV Galesa/B12/2008	Cattle	Ethiopia	Swab samples	Non-vaccinated	2008	This study	This study
22	LSDV Masalamia P04	Cattle	Sudan	Cell culture	Non-vaccinated	1971	This study	This study
23	LSDV Sudan/99-Atbara	Cattle	Sudan			1999	GU119944	FJ869367
24	LSDV Sudan/06-Obied	Cattle	Sudan			2006	GU119938	FJ869369
25	LSDV SGP_O-240	Sheep	Kenya			**1976**	KJ818288	KJ818281
26	LSDV KSGP 0-240	Sheep	Kenya			1976	KJ818289	KJ818282
27	LSDV KS-1	Sheep	Kenya			1976	KJ818290	KJ818283
28	LSDV NI-2490	Cattle	Kenya			1958	AF325528	AF325528
30	LSDV Adama/B01/2011	Cattle	Ethiopia			2011	KP663667	KP663690
31	LSDV Andassa/B04/2012	Cattle	Ethiopia			2012	KP663671	KP663694
32	LSDV Kadjima /B01/2009	Cattle	Ethiopia			2009	KP663681	KP663704
33	LSDV Mojo/B01/2011	Cattle	Ethiopia			2011	KP663683	KP663706
34	LSDV Wenji/B01/2011	Cattle	Ethiopia			2011	KP663687	KP663710

Results

PCR amplification and sequencing

For each of the 22 samples, we have successfully amplified and sequenced two fragments for the RPO30 gene (554 bp and 520 bp) and three for the GPCR gene (617 bp, 603 bp and 684 bp), and assembled them to produce the complete RPO30 and the complete GPCR gene sequences. Additionally, we amplified and sequenced the partial EEV glycoprotein and the B22R genes of LSDV Embu/B338/2011. All sequences generated in this work were submitted to Genbank database under accession numbers MK302070 to MK302091 and MK302092 to MK302113 for RPO30 and GPCR genes, respectively.

Analysis of the RPO30 gene

The phylogenetic tree based on the RPO30 gene showed that all 22 isolates of this study belong to LSDV. The phylogenetic reconstructions produced three subgroups for LSDVs with strong posterior probabilities (Figure 2). Subgroup I included all the isolates of this study and LSDV field isolates from East Africa, South Africa, North Africa, West Africa and Europe; subgroup II included mainly isolates from Kenya such as LSDV NI-2490 (AF325528) and LSDV KS-1 (KJ818290) and subgroup III included LSDV Neethling vaccine LW-1959 (AF409138) and RSA/54 Haden (FJ869376), both from South Africa and recovered from outbreaks that occurred before 1960 (Figure 2).

Multiple sequence alignments of the RPO30 gene showed 100% identity among the Eastern African isolates of this study, at both the nucleotide and amino acid level, except for the isolate LSDV Bungoma/B624/2010 from Kenya which presented a non-synonymous mutation (A/G) leading to an E to G amino acid change.

The comparison of the newly sequenced RPO30 genes to publicly available RPO30 sequences from Eastern Africa showed high similarities between the sequences. LSDV KS-1 and similar vaccines, as well as LSDV NI-2490 (AF325528) a field isolate from Kenya, each presented a non-synonymous mutation, T/C, leading to an S to P change in their amino acid sequences. LSDV Sudan/06-Obied (GU119938) and LSDV Sudan/99-Atbara (GU119944), two previously sequenced isolates from Sudan both presented one non-synonymous mutation, C/A and A/G, respectively, leading to T to N and D to G amino acid changes.

Analysis of the GPCR gene

As observed for the RPO30 gene, the phylogenetic tree of the GPCR genes also confirmed all 22 isolates of this study to be LSDVs. In contrast to the RPO30 tree, the GPCR tree produced only two subgroups with strong posterior probability values: Twenty-one out of 22 LSDVs of this study clustered with subgroup I, along with previously sequenced LSDV isolates from East Africa, other Africa countries, and European countries. Subgroup II contained the isolate LSDV Embu/B338/2011, from Kenya, together with the two historical isolates, LSDV Neethling-vaccine LW-1959 (AF409138) and LSDV RSA/54 Haden (FJ869376) from South Africa (Figure 3).

Furthermore, a close inspection of the multiple sequence alignments of GPCR nucleotide sequences showed that the GPCR genes of the isolates of this study were almost 100% similar to each other at both nucleotide, and amino acids levels, except for the isolate LSDV Embu/B338/2011. Expanding this comparison to previously sequenced LSDV isolates of East Africa, we observed that the GPCR gene sequences shared 100% identity except for isolates LSDV collected from Sudan, LSDV Wenji/B01/2011 (KP663710) and LSDV Mojo/B02/2011 (KP663707) from Ethiopia and LSDV KS-1 (KJ818290) and LSDV NI-2490 (AF325528) a historical field isolate both from Kenya.

LSDV Wenji/B01/2011 differed from other Eastern Africa field isolate LSDVs with a T/C mutation leading to F to P change in the amino acid sequence. There was also one synonymous nucleotide change in each of LSDV Sudan/06-Obied (FJ869369), LSDV Wenji/B01/2011 (KP663710) and LSDV Mojo/B02/2011 (KP663707). LSDV Embu/B338/2011 contained an insertion of 12 nucleotides (Figure 3), corresponding to amino acids ILST and was 100% identical to LSDV Neethling-vaccine LW-1959 (AF409138) in the GPCR gene. The 12-nucleotide insertion was also present in LSDV KS-1 (KJ818290) and LSDV NI-2490 (AF325528), a historical field isolate from Kenya (Figure 3). Nevertheless, they differed from LSDV Embu/B338/2011 and LSDV Neethling-vaccine by 23 additional nucleotides leading to four additional amino acid changes (S/N, M/I, L/I, M/T).

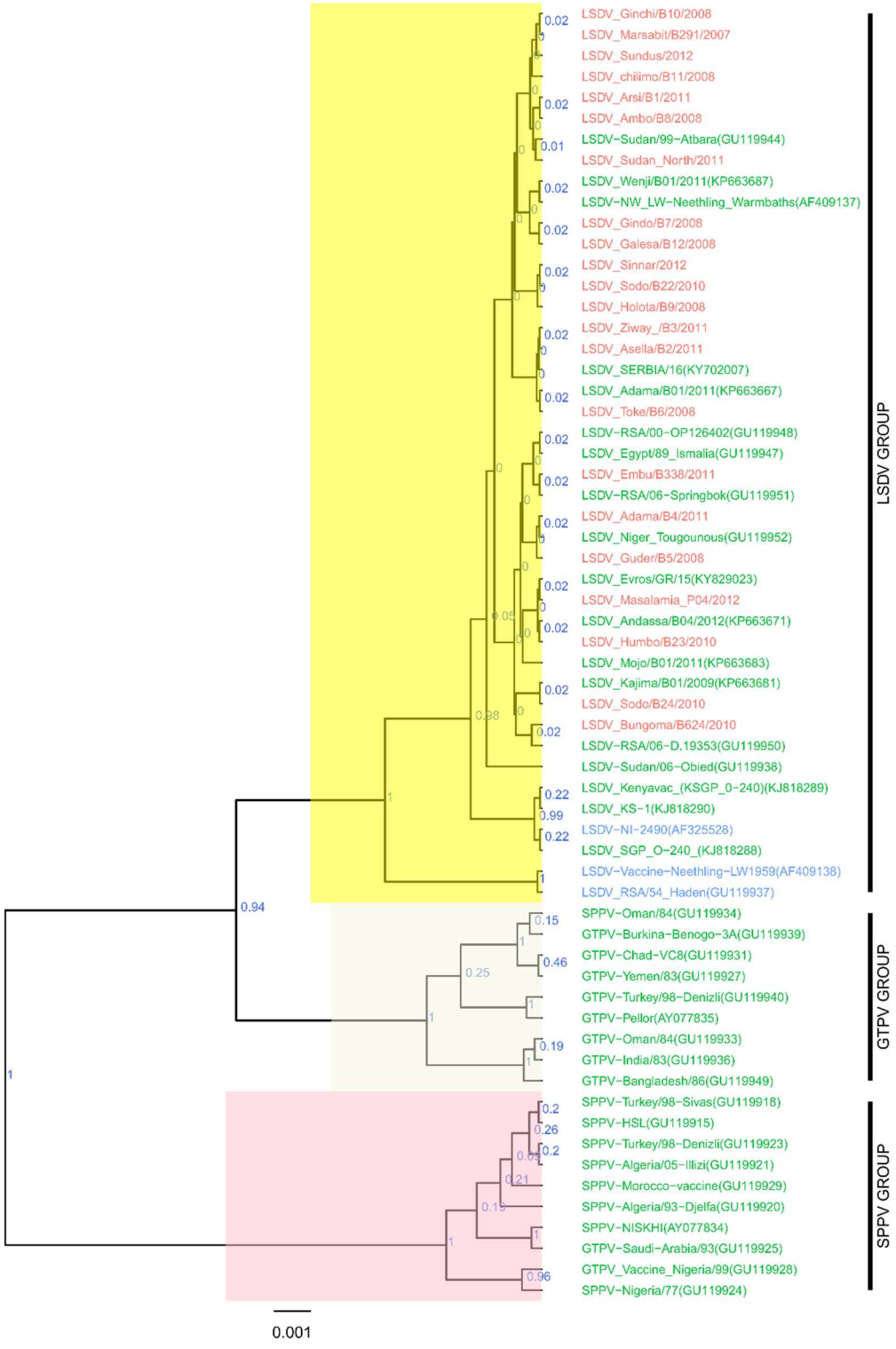

Figure 2. Maximum clade credibility (MCC) tree based on of the complete RPO30 complete gene sequences of capripoxvirus. The posterior probabilities are plotted as respective nodes labels. The sequences of this study are highlighted in red and reference sequences are represented with their accession numbers.

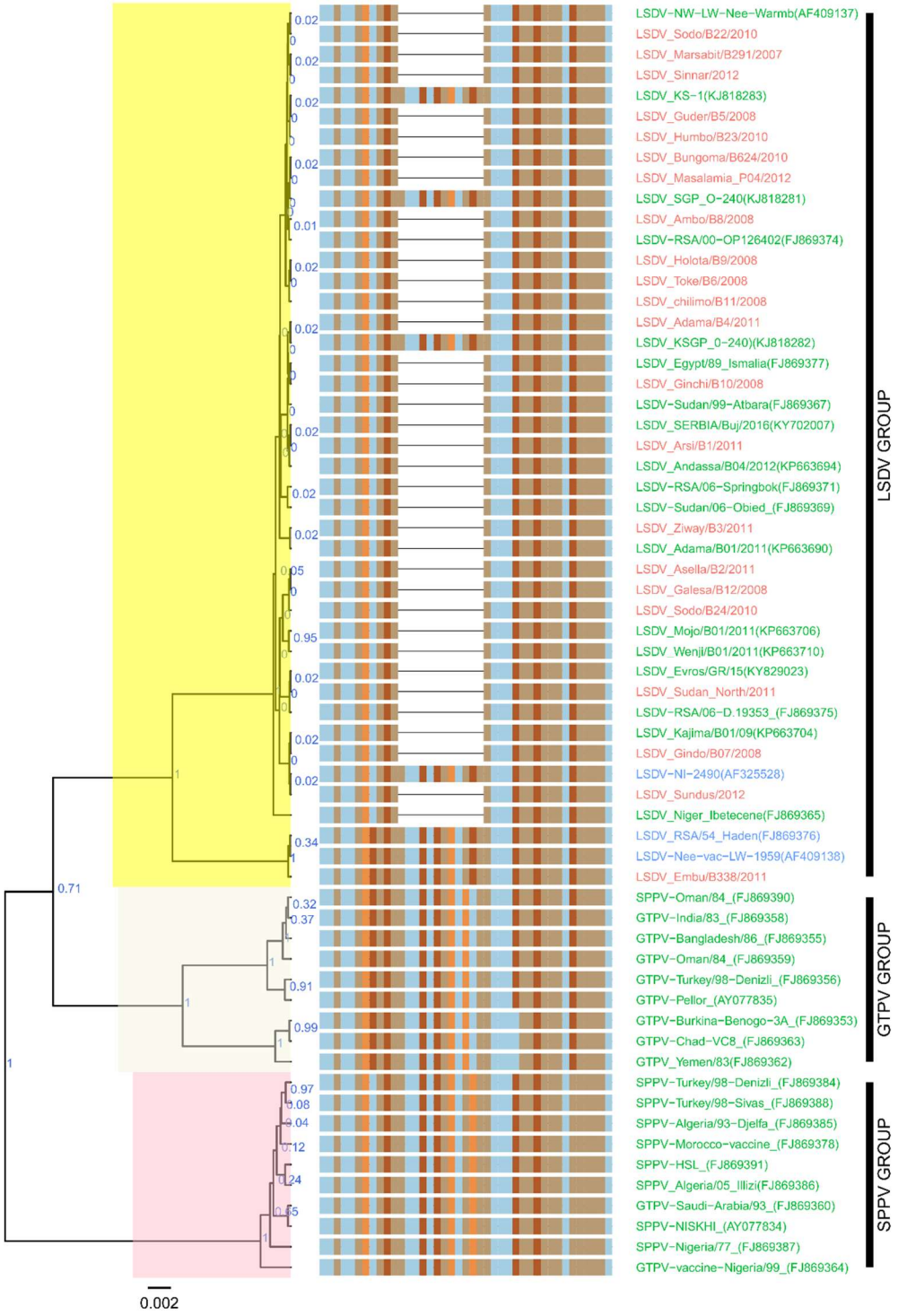

Figure 3. Maximum clade credibility (MCC) tree based on of the complete GPCR gene sequences of capripoxviruses, plotted together with multiple sequence alignment. Only the portion of the alignment, between position 80 and 120 is shown. The posterior probabilities are plotted as respective nodes labels. Study sequences are highlighted in red and reference sequences are represented with their accession numbers.

Analysis of the EEV glycoprotein and the B22R genes of LSDV Embu/B338/2011

As the LSDV Embu/B338/2011 showed great similarity to LSDV vaccines on the GPCR gene and LSDV field isolates on the RPO30 gene, we sequenced the EEV glycoprotein gene and compared it to the publicly available sequences for further elucidation. The EEV glycoprotein gene sequence of LSDV Embu/B338/2011 contained an insertion of 27 nucleotides (9 amino acids) as previously reported in LSDV field isolates as well as the LSDV KS-1 vaccine. This fragment was absent in LSDV Neethling vaccine-like viruses (Figure 4).

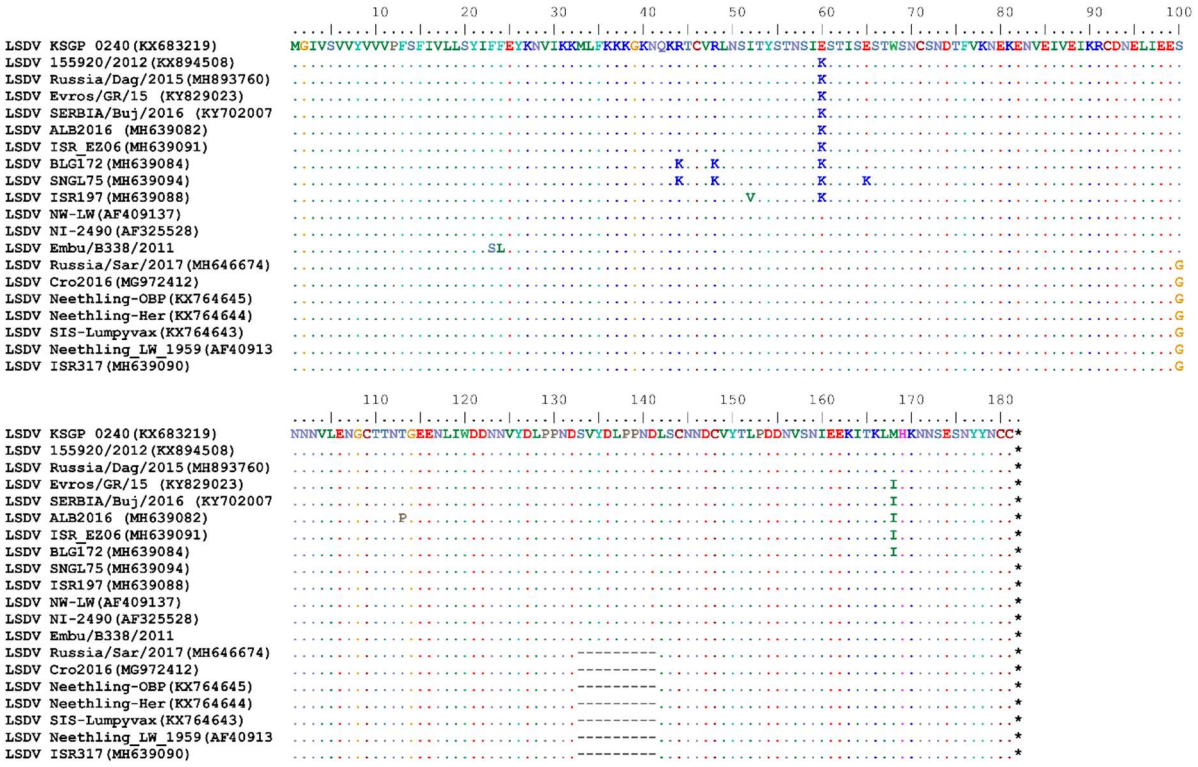

Figure 4. Multiple sequence alignments of the partial of EEV glycoprotein amino acids sequence of LSDV Embu/B338/2011 with 19 additional LSDVs' sequences retrieved from GenBank. A unique sequence signature of 9 amino acids only in LSDV Neethling like viruses is highlighted in the box. Identical nucleotides are indicated with dots.

This result confirmed that Embu/ B338/2011 was different from the LSDV Neethling vaccine. We also sequenced the B22R gene of LSDV Embu/B338/2011 and compared it to publicly available LSDV sequences. The results showed that part of the B22R gene of LSDV Embu/B338/2011 was identical to that of field isolates and contained four mutations (A/G, A/G, C/T, A/G) as compared to LSDV Neethling vaccine and LSDV RUSSIA/Saratov/2017 a recombinant-like virus from Russia. (Figure 5).

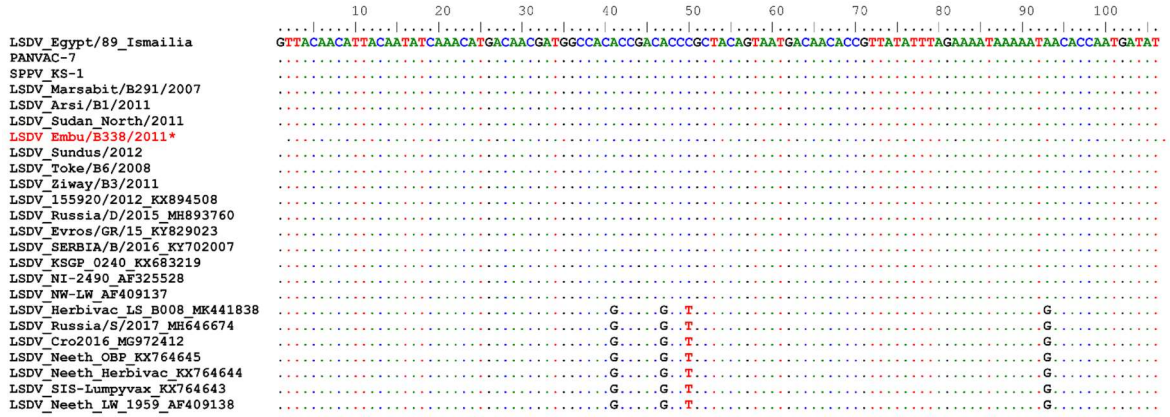

Figure 5. Multiple sequence alignments of the partial B22R gene sequences of LSDV Embu/B338/2011 and 23 additional LSDVs retrieved from GenBank. The variable site appears as a letter while identical nucleotides are indicated with dots.

Discussion

In this study, we have analysed LSDV isolates collected in Eastern Africa before the introduction of LSD in the Middle East and the incursion in Europe. The Eastern African LSDV field isolates covered in this study (newly sequenced and recovered from public databases) were collected between 1958 and 2012.Overall, except for LSDV Embu/B338/2011, the Eastern African field isolates of this study were highly similar to each other and the previously sequenced Eastern African field isolates of LSDV, for the RPO30 and the GPCR genes. Likewise, the Eastern African isolates were also highly similar to LSDV field isolates encountered elsewhere including the Middle Eastern and European isolates. As the LSDV genome is stable, limited variability is likely to occur following the escape of the virus from Africa to the Middle East, then to Europe.

The only exceptions were LSDV Embu/B338/2011, collected in 2011 in Kenya which differed from other field isolates by an insertion of 12 nucleotides in its GPCR gene, as previously described for some historical isolates, namely LSDV Neethling-vaccine LW-1959 (AF409138), LSDV RSA/54 Haden (FJ869376), LSDV KS-1(KJ818290), LSDV NI-2490 (AF325528) (Tulman et al. 2001) and recent isolate LSDV RUSSIA/Saratov/2017 from Russia (Kononov et al. 2019; Sprygin et al. 2018).

In contrast to our findings on the GPCR gene, LSDV Embu/B338/2011 was identical to LSDV field isolates and LSDV KS-1 (KJ818290) for the full RP30 gene, the partial EEV glycoprotein gene and the partial B22R gene. Altogether, the analysis of these four genes showed that LSDV

Embu/B338/2011 differs from the two commonly used LSDV vaccines in Eastern Africa: the LSDV KS-1 and the LSDV Neethling vaccine.

Based on the full GPCR and RPO30 genes, and the partial EEV glycoprotein and B22R genes LSDV Embu/B338/2011 appears to be a hybrid between an LSDV field isolate and the LSDV Neethling vaccine. A previous study, based on the comparative analysis of detailed physical maps of the genomes of four capripoxvirus isolates, suggested a similar recombination of CaPV field strain, Kenya cattle-1 and a vaccine strain, Iraq goat-1, creating the isolate Yemen goat-1 (Gershon et al. 1989). More recently, a report highlighted a similar type of recombination in the field in Russia (Sprygin et al. 2018). Analysing the full genome of LSDV RUSSIA/Saratov/2017 collected at the Russian border with Khazaktan, the authors suggested that this virus was a recombinant escape of one of the LSDV vaccines derived from the Neethling vaccine strain and a field isolate (Sprygin et al. 2018). Interestingly, the comparison of the EEV glycoprotein and the B22R genes of LSDV Embu/B338/2011 to that of the Russian recombinant-like isolate LSDV RUSSIA/Saratov/2017 suggests that they are different. While LSDV RUSSIA/Saratov/2017 resembled LSDV Neethling vaccine on the B22R gene, LSDV Embu/B338/2011 was more related to field isolates on the same gene.

The epidemiological data further supports the hypothesis of recombination, leading to LSDV Embu/B338/2011, showing that this isolate was from a previously vaccinated herd.

It is also possible that such an isolate was circulating before the vaccination. For instance, LSDV NI-2490, a historical field isolate collected in 1958 in Kenya, also contains a 12 nucleotide insertion in the GPCR gene (Agianniotaki et al. 2017; Le Goff et al. 2009).

Several reports have suggested that the occurrences of LSDV in vaccinated flocks were due to vaccination failures or the potential residual virulence of vaccines such as LSDV KS1 (Yeruham et al., 1994;Tuppurainen et al., 2014; Gelaye et al., 2015; Abutarbush et al., 2016). However, our data indicate that that LSDV KS-1 did not contribute to the appearance of this recombinant like Embu/B338/2011 isolate as it is genetically different from LSDV Embu/B338/2011 based on the analysis of the four genes. However, the escape mechanism to vaccinal response following vaccination with common LSD vaccines such as LSDV KS-1 and LSDV Neethling-vaccine remains unclear. The occurrence of LSDV in previously vaccinated herds usually shows that the vaccine used did not protect the animals.

It is also interesting to note, that LSDV isolates carrying the 12-nucleotide insertion in their GPCR gene are rarely detected, with long gaps between the reporting of these viruses. For instance, the LSDV RSA/54 Haden occurred in 1954, followed by LSDV NI-2490 and LSDV

Neethling-vaccine LW-1959 in 1958 and 1959, LSDV KS-1 in 1976, LSDV Embu/B338/2011 of this study in 2011 and LSDV RUSSIA/Saratov/2017 in 2017.

The low frequency of LSDVs with this 12-nucleotide insertion in their GPCR suggests that those variants are of weak virulence, circulating without being readily noticed, or not very viable. It may also suggest the inability of such viruses to transmit efficiently between infected and naïve animals, explaining why two of these variants (LSDV KS-1 and LSDV Neethling-vaccine) are applicable as vaccines against LSD.

These findings on LSDV Embu/B338/2011 highlight the importance of constant monitoring of LSDV genetics in the field. It also shows the importance of analyzing multiple genes while addressing the differentiation between vaccines and field isolates

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: CEL, TRC. Performed the experiments: TRC, MS, Analyzed the data: TRC, CEL, RG, AL, and TBKS. Contributed reagents/materials/analysis tools: AL, NN , KT , HM , MD , ET .Wrote the paper: TRC, CEL , RG , AD, Supervised the study: CEL, RG, AD, GC. Edited the final manuscript: FJB, MSF, AD, RG, GC and TBKS. All authors read and approved the final manuscript.

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Chapter IV

Use of an Alignment-Free Method for the Geographical Discrimination of GTPVs Based on the GPCR Sequences

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Use of an Alignment-Free Method for the Geographical Discrimination of GTPVs Based on the GPCR Sequences

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Abstract

Goatpox virus (GTPV), together with SPPV and LSDV comprise the three known members of the genus Capripoxvirus. Some strains of GTPV can equally infect both sheep and goats, as does SPPV, while LSDV can only affect cattle and some wild ruminants such as springbok antelope. Although LSDV and SPPV have emerged on several occasions, outside their endemic areas, GTPVs remained confined to endemic regions of Africa and Asia, causing severe damage to small ruminants' production systems. The discovery of geographical signatures in the GTPV genome can help trace the source of infection during GTPV incursion into a new geographic area and detect the circulation of a new strain in disease-endemic areas. Previous research has shown three different nucleotide sequence profiles in the G-protein-coupled chemokine receptor (GPCR) gene of GTPVs. However, this study only included a small number of GTPV GPCR sequences and used conventional multiple sequence alignments followed by phylogenetic reconstructions to compare the sequences which limited the ability to provide a definite conclusion linking these profiles to their geographical origins. Therefore, we sequenced the GPCR genes of GTPVs from Ethiopia, Kenya, Ghana and Mongolia, and analyzed them together with GTPV sequences from public databases. We used multiple sequence alignment-based methods, and an alignment-free method exploiting k-mer frequencies. The results show that using the alignment-free method to compare the GPCR gene sequences, GTPVs can be segregated based on their geographical origin: the African GTPVs and Asian GTPVs which further split into Western and Central Asian (WCA) GTPVs, and Eastern and Southern Asian (ESA) GTPVs. This approach will help determine the source of introduction in case of GTPV emergence in disease-free regions and detect the importation of new strains in GTPV endemic areas.

Keyword: *GTPV*, *Alignment-free algorithm, k-mer, GPCR*

Introduction

Goatpox virus (GTPV) is a DNA virus belonging to the genus Capripoxvirus within the family Poxviridae, together with sheeppox virus (SPPV) and lumpy disease virus (LSDV) (Tulman et al. 2002). GTPV affects goats, sheep and small wild ruminants (Dutta et al. 2019; Le Goff et al. 2009).

Although goatpox is a disease of goats, the causative agent can be GTPV or SPPV. Similarly, both GTPV and SPPV can cause sheeppox and, unfortunately, it is not possible to differentiate infections of small ruminants by GTPV or SPPV either clinically or serologically (Kitching and Taylor 1985) due to cross-reactivity between capripoxviruses (CaPVs). Hence, molecular methods are increasingly being used to genotype and classify CaPVs, and undertake molecular epidemiological studies (Le Goff et al. 2009; Lamien et al. 2011a; Lamien et al. 2011b).

Together, GTPV and SPPV cause significant economic losses in small ruminants in endemic regions of Africa and Asia, and together they make up one of the major impediments for the development of intensive production units in the affected areas (Tuppurainen et al. 2017).

Though GTPV is currently confined to Africa and Asia, recent outbreaks of LSDV and SPPV (Tuppurainen et al. 2017) showed that CaPVs could escape their traditional geographical confinements into new areas and cause severe outbreaks.

During an incursion of GTPV into a new geographical area, it would be of great importance to identify the source of introduction in order to implement proper control measures.

The identification of a suitable molecular target for discriminating GTPV strains based on their geographical origins can complement field investigations to determine a potential route of introduction during GTPV outbreaks in disease-free areas and detect the introduction of new strains in disease-endemic areas.

Previous studies showed that the G-protein-coupled chemokine receptor (GPCR gene), a host range gene, was suitable to classify CaPVs into one of the tree species by real-time PCR (Lamien et al. 2011b), and by phylogenetic reconstruction (Gelaye et al. 2015; Le Goff et al. 2009; Santhamani et al. 2014, 2015; Zhou et al. 2012).

Using the GPCR gene, Le Goff et al., 2009 could distinguish three GTPV sub-clusters comprising isolates from different geographical locations, without being able to define the geographical boundaries clearly. The limited number of samples and the absence of isolates from some geographical locations made it impossible to draw definite conclusions.

The GPCR later became a widespread target for molecular characterization of CaPVs (Gelaye et al. 2015; Santhamani et al. 2014, 2015; Zhou et al. 2012). Thus, a large number of sequences were produced by several research groups and made available in public databases.

Here, we have sequenced the GPCR genes of twelve additional GTPVs and analyzed them together with GTPV sequences available in public databases.

Previous analyses using the GPCR relied on conventional sequence alignments and statistical methods for phylogenetic reconstructions. However, those methods usually exclude gap positions or poorly perform when gaps are present in the sequence alignments(Bonham-Carter et al.,2014; Zielezinski et al. 2017). In many cases, these gaps could represent essential information to enable the classification of viruses. For instance, the GPCR sequences alignments showed the presence of a 21-nucleotide deletion in all SPPVs and a number of GTPVs, as well as a 12-nucleotide deletion in a few isolates of LSDV (Le Goff et al. 2009). The removal of gaps during analysis can result in the loss of information that is essential for comparison.

To avoid this loss of information, alignment-free methods were proposed, including those using the k-mer frequencies to compute the pairwise distances between sequences (Babicki et al. 2016; Bonham-Carter et al. 2014).

Here, we have used the k-mer frequencies-based distance calculation and heatmaps (Babicki et al. 2016), to compare the GPCR sequences of GTPVs from various geographical locations and classify them according to their geographical origins.

Methods

Sequencing of new isolates

Twelve isolates (Table 1) of GTPVs from Ethiopia, Kenya, Ghana and Mongolia were newly sequenced and analyzed with publicly available GTPVs sequences. For sequencing, total DNA was extracted from clinical samples, and cell culture supernatants, and the GPCR gene was amplified as previously described (Gelaye et al. 2015). All pathological samples and viral isolates were handled within the biosafety level 3 facility of the Institute for Veterinary Disease Control, Austrian Agency for Health and Food Safety, Austria.

The positive PCR products were purified and sequenced at LGC Genomics (Germany). The sequence data were assembled using Vector NTI 11.5 software (Invitrogen, USA).

Collection of GTPVs for alignment-free method

Nucleotide sequences and deduced amino acid sequences were aligned using the ClustalW algorithm implemented in BioEdit 7.1.7 and MEGA7 (Kumaret al. 2016; Dhami and Kumarasinghe 2014). The complete GPCR gene sequences of 37 additional GTPVs from various regions were retrieved from GenBank.

For phylogenetic reconstructions, 13 LSDV and 19 SPPV sequences were also retrieved from Genbank and included in the analysis. The GPCR nucleotides sequences were aligned using the ClustalW algorithm (codon option) implemented in MEGA 7. The aligned sequences in FASTA format were converted to nexus format using Seaview. The Bayesian phylogenetic inference was performed with BEAST v1.8.4 (Drummond et al. 2012). First, the BEAUti module was used to generate BEAST files from the sequence alignments in nexus format. The HKY substitution +G nucleotide substitution was used with a UPGMA starting tree. The Markov Chain Monte Carlo method was run with BEAST, for 10000000 generations with a sample taken each 10000 generations. The TRACER programme was used to inspect the log files and determine the optimum number of burn-in based on the Effective Sample Sizes (ESS >200).

TreeAnnotator was used to generate the Maximum Clade Credibility (MCC) after discarding the 10 % burn-in. The tree was visualized using the ggtree package in R version 3.5.2 (Yu et al. 2017). The read.beast() and the get.fields() functions were used to import the tree and associated meta-data (Yu et al. 2017).

Distances calculation and visualization

Only the sequences that clustered within GTPVs group in the phylogenetic analysis were used in the alignment-free analysis. The multi-fasta file with the GPCR gene sequences of 49 GTPVs was loaded into the pairwise analysis interface of Heatmapper (http://www.heatmapper.ca/pairwise/). Heatmapper (Babicki et al. 2016) was used to compute k-mer frequencies for each sequence and produce the distance matrix table using an alignment-free algorithm. Briefly, k-mer size of 3 was used to compute the k-mer frequencies for each sequence; then the Euclidian distance measurement method was used to calculate the pairwise distances between each pair of sequences. The distance matrix was visualized as a heat map with “gplots” package in R version 3.5.2, and the Complete-linkage clustering method was implemented to re-order the sequences.

Additionally, three alignment-based algorithms were also used to generate distance matrices (p-distance, Maximum Composite Likelihood (MCL), and Kimura 2-parameter, all implemented by MEGA software) and produce heatmaps. To examine the influence of the gaps,

we manually removed the positions corresponding to the 21-nucleotide deletion of East and South Asian GTPVs and the pattern of distance matrix with alignment-free algorithm was analyzed.

Results

Sequencing and phylogenetic reconstructions.

We have successfully sequenced the full GPCR gene for all 12 samples collected from both sheep and goats from Ethiopia, Ghana, Kenya and Mongolia and compared that to GTPVs retrieved from GenBank.

The maximum credibility tree of the GPCR sequences showed that all 12 sequences of this study belonged to GTPVs (Figure 1). In the phylogenetic tree, 37 additional sequences retrieved from GenBank clustered with GTPVs. GTPVs formed two distinct groups: one with African isolates and a second one with Asian isolates (Figure 1). The Asian GTPVs produced two sub-clusters: sub-cluster 1 contained GTPVs from Eastern and Southern Asia (ESA) mainly, and sub-cluster 2 consisted of isolates of Western and Central Asia (WCA). Among the newly sequenced GTPVs, one isolate from Ghana, five isolates from Ethiopia and three from Kenya, clustered with African GTPVs, while the three GTPVs from Mongolia, clustered with ESA GTPVs (Figure 1).

Some isolates retrieved from GenBank clustered outside the groups matching their geographical origin. SPPV Oman/84 (FJ869390), GTPV Oman/84 (FJ869359) and GTPV SA2/2017 (MG232389), from Western Asia, clustered with ESA GTPVs, while GTPV Yemen/83(FJ869362) also from Western Asia, clustered with African GTPVs (Figure 1).

To further determine whether the GPCR gene sequences could help classify GTPVs based on their geographical origins, we selected all 49 GTPVs (37 from GenBank and 12 from this study) for alignment-free sequence comparison.

Distances calculation and visualization using alignment-free methods

In the heatmap, based on the Euclidian distances calculated from the k-mer frequencies of each pair of sequences, we observed two mains clusters: cluster 1 comprised isolates from Asia and cluster 2 comprised isolates from Africa (Figure 2). Cluster 1 with Asian GTPVs further splitted into two sub-clusters: sub-cluster 1.1 comprising ESA GTPVs and sub-cluster 1.2 containing WCA GTPVs (Figure 2). In general, all GTPVs clustered according to their geographical origins, except GTPV Yemen/83 (FJ869390) from Western Asia, located within cluster 2 of

African GTPVs, and GTPV Oman /84(FJ869359) and SPPV Oman/84 (FJ869390), also from Western Asia, located in sub-cluster 1.1 of ESA GTPVs (Figure 2). Contrasting with the result of the phylogenetic reconstruction, GTPV SA2/2017 (MG232389) clustered within sub-cluster 1.1 of WCA GTPVs (Figure 2).

Heatmaps based on distances calculated from multiple sequence alignments using various substitution models (p-distance, MCL and k2P) produced similar patterns, however with lower resolution. While the two blocks of Asian and African GTPV were apparent, the separation between the two sub-groups of Asian isolates was less prominent, and the GTPV SA2/2017 (MG232389) isolate clustered within the ESA GTPVs sub-block 2.1 (Supplement Figure S1). Likewise, after removing the positions containing gaps before calculating the Euclidian distances from the k-mer frequencies, the resulting heatmap, appeared to have a lower resolution, with GTPV SA2/2017 (MG232389) clustering within the ESA GTPVs sub-block 1.2 (Supplement Figure S2).

Amino acids profiles of GTPVs

The comparative analysis of the amino acid sequence alignments showed 10 conserved amino acid differences between African and WCA GTPVs, and 17 aa (7 aa deletion and 10 aa substitution) between African and ESA GTPVs out of which, nine were common to all Asian GTPVs. The WCA GTPVs differed from ESA GTPVs by seven amino acids, missing in the ESA GTPVs (Supplement Figure S3). In summary, based on the amino acid profile, we can distinguish three different clusters of GTPVs: the African GTPVs, the WCA GTPVs and the ESA GTPVs (Supplement Figure S3). Most of the 49 GTPVs of this study had a GTPV profile that matched the geographical origin except SPPV Oman/84 (FJ869390), and GTPV Oman/84 (FJ869359), which presented the ESA GTPV profile, and GTPV Yemen/83 (FJ869390), with the African GTPV profile. Based on the amino acid sequence, GTPV SA2/2017 (MG232389) clustered within the WCA GTPVs sub-group, like the result obtained using the heatmap based on the Euclidian distances calculated from the k-mer frequencies. GTPV SA2/2017 was the only Western Asian isolate sharing an additional amino acid similarity with ESA GTPVs: H was present at position 375 instead of R.

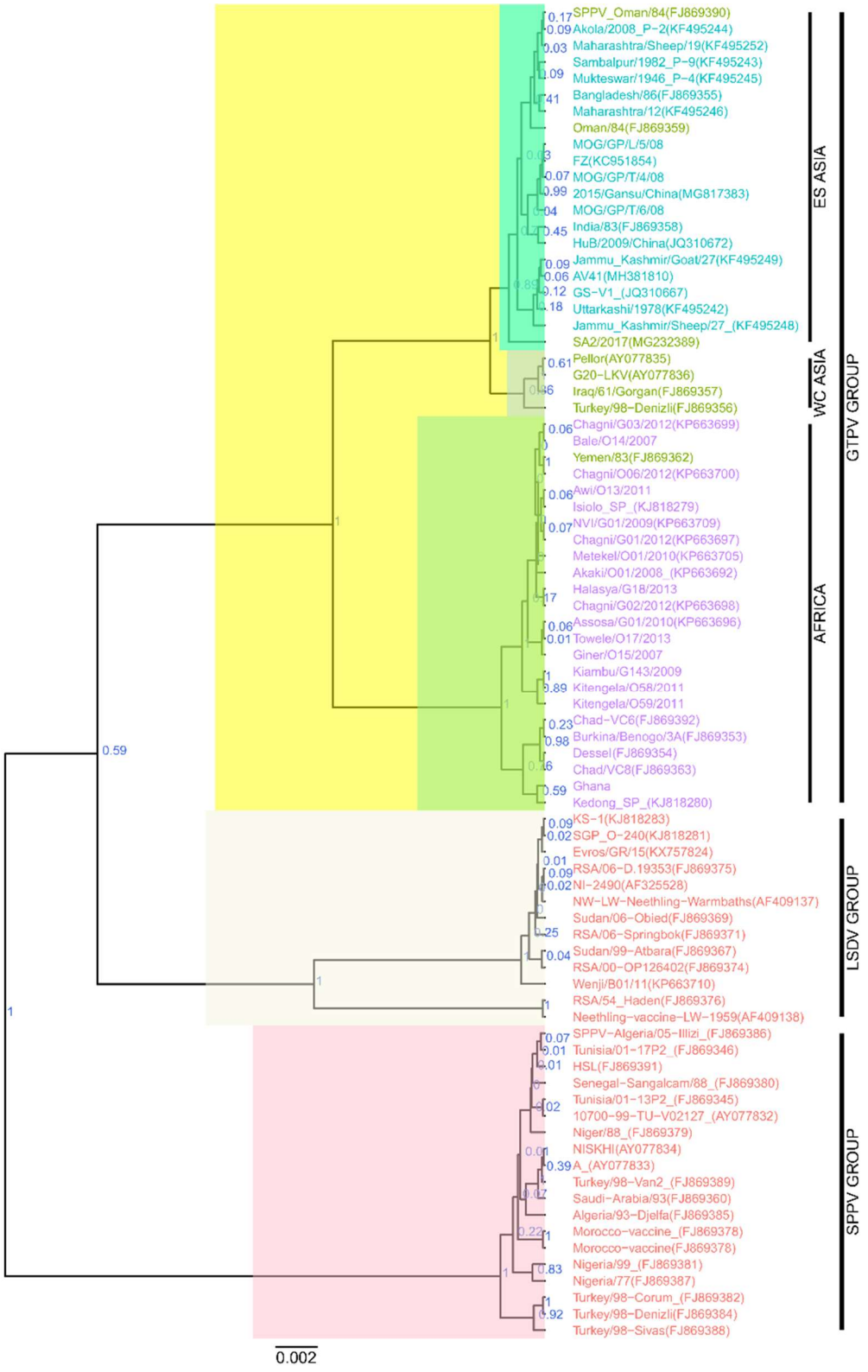

Figure 1. Maximum clade credibility (MCC) tree based on of the complete GPCR complete gene sequences of capripoxviruses. The posterior probabilities are plotted as respective nodes labels. The GTPV sequences are highlighted based their geographical origins.

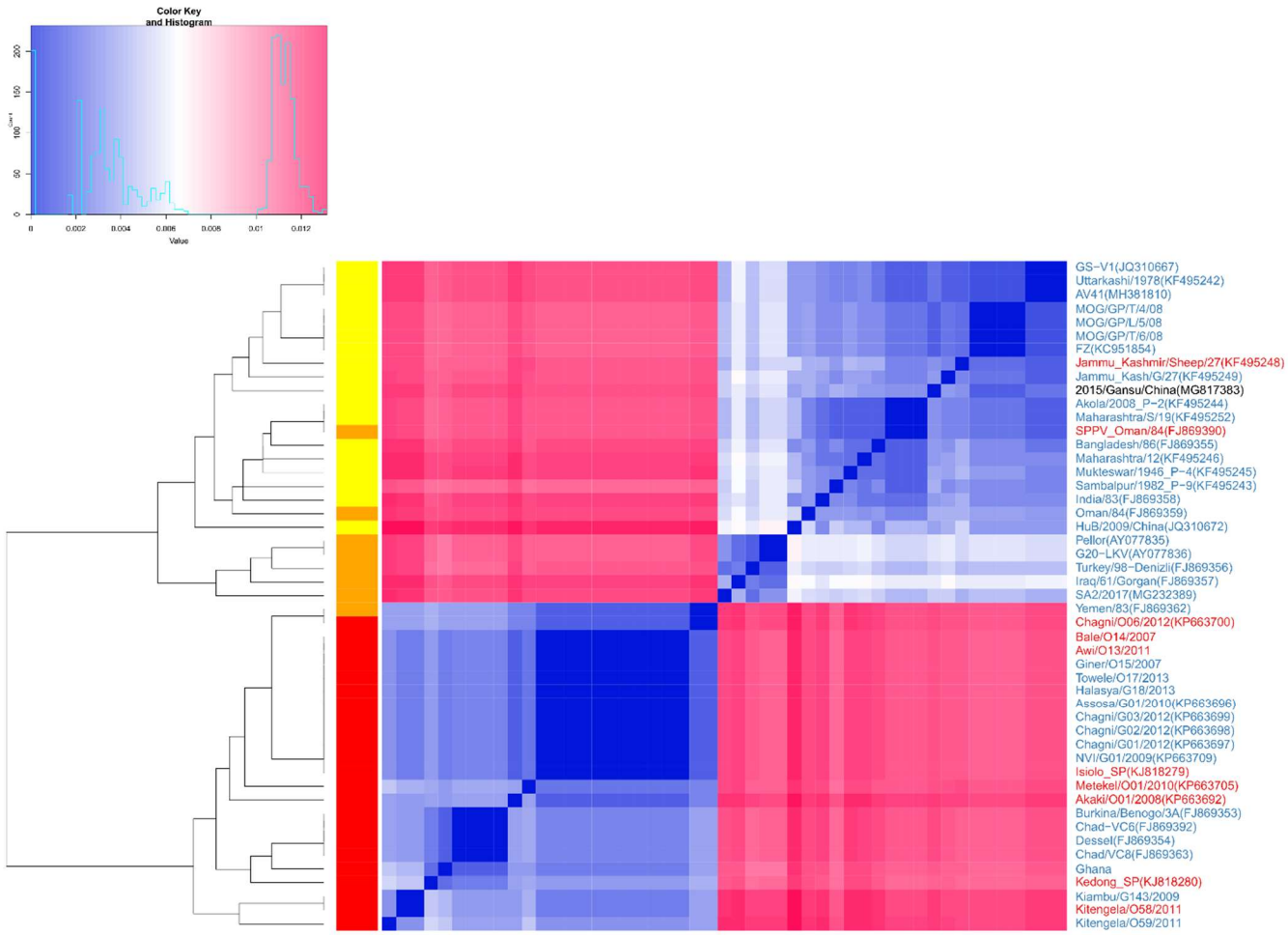

Figure 2. The heatmap for the nucleotides k-mers frequencies variations of 49 GTPVs. The Complete-linkage clustering method was used to re-order the sequences. The vertical side colors indicate the origin of the samples (red for Africa, orange for West and Central Asia, yellow for East and South Asia).

Discussion

We analyzed the GPCR sequences of GTPVs from various locations, based on multiple sequence alignments and alignment-free methods.

The analysis of the maximum credibility tree of the GPCR gene sequences confirmed that the newly sequenced isolates belong to GTPVs and enabled the identification of 37 additional GTPVs included for alignment-free analysis. Based on the phylogenetic analysis, the alignment-free sequence comparisons and the information attached to the sequences, we identified two mains clusters: the African GTPVs and the Asian GTPVs, which further subdivided into ESA GTPVs, and WCA GTPVs.

In the phylogenetic reconstruction, four isolates, SPPV Oman/84, GTPV Oman/84, SPPV Yemen/83 and GTPV SA2/2017, all from Western Asia, clustered outside the groups corresponding to their geographical origin. Contrasting with the phylogenetic analysis, the alignment-free method based on the Euclidian distances calculated from the k-mer frequencies (Babicki et al. 2016), correctly classified GTPV SA2/2017 within the WCA GTPVs, consistently with the results of the comparative analysis of the amino acid profiles of the GTPVs. The failure to correctly classify GTPV SA2/2017 using conventional sequence comparison methods is likely due to the mishandling of the 21-nucleotide deletion observed in ESA GTPVs. Indeed, the heatmaps produced using distance calculated based on conventional methods such as MCL, K2P and p-distance, and the one based on k-mer frequencies after removal of the positions with gaps, also failed to classify GTPV SA2/2017 in the correct cluster. Our study further highlights the power of the alignment-free methods in handling useful information such as gaps. Contrasting with global and local alignment algorithms which work base-by-base, frequency-based algorithms can better deal with complexities caused by mismatches, gaps and sequence inversions often found while comparing sequences (Zielezinski et al. 2017)

In a previous study, using thirteen GTPVs GPCR sequences, Le Goff et al., 2009, observed three groups: however, due to the limited number of sequences and the absence of sequences from Eastern Asia and Eastern Africa, they were unable to draw any definite conclusion for the classification of GTPVs based on their geographical origins.

Though the heatmap based on the alignment-free method provided a better classification of GTPVs according to their geographical origins, this method identified GTPV Yemen/83 as an African GTPV, and GTPV Oman/84 and SPPV Oman/84 as ESA GTPVs.

This misclassification could also suggest that GTPV Yemen/83 is an African GTPV imported to Yemen, and GTPV Oman and SPPV Oman are two ESA GTPVs imported to Oman. In the 1980s, when GTPV Yemen/83 was collected, Yemen imported sheep and goats from Eastern Africa, mainly, Somalia, Sudan, Eritrea and Sudan, before the outbreaks (Kitching et al., 1986). Kitching et al. 1986 suggested that new GTPV strains, imported via Eastern Africa, could have caused these outbreaks. Similarly, Oman imported sheep and goats from Africa, other countries in the Middle East and India, during the same period, with the risk of introducing new GTPVs in the country (Kitching et al. 1986). The introduction of foreign GTPV strains from Africa to Yemen and or South Asia to Oman via trade or illegal imports is also consistent with livestock trading routes between African and Asian countries (Di Nardo et al., 2011).

The spread of foot and mouth disease (FMD) in the Middle East ,which better illustrates the association between trade and introducing transboundary diseases, was associated with the importation of animals from Asia and Africa leading to epidemics involving new FMD virus strains (Di Nardo et al. 2011). The presence of all three GTPV profiles in the Western Asia further supports the hypothesis of GTPVs importation in the region via livestock trade.

These geographical signatures in the GPCR were highly conserved, with no change observed for each region over 50 years. For instance, the WCA GTPVs included in this study were from outbreaks that occurred between 1961 and 2017, the ESA GTPVs, between 1946 and 2009, and the African GTPVs, between 1955 and 2013, implying a great adaptation of these profiles for each region.

The CaPVs' GPCR gene enabled the discrimination of GTPVs based on their geographical origins. While the GPCR gene encodes for a host factor protein, the link between this adaptation of affected breeds in each region remains unclear. It is also worth noting that some ESA and Africa GTPVs, including six newly sequenced African GTPVs, were from sheep, showing that these isolates can affect both small ruminant species. In contrast, though only a few GTPVs were available from WCA, we noticed that all of them were exclusively from goats.

In conclusion, this study showed that, using alignment-free sequence comparison methods, the GPCR gene sequences enable the classification of GTPVs based on geographical origin. The alignment-free approach based on k-mer frequencies enabled a higher resolution as compared to standard approaches using alignment to calculate distance matrix. The result of the alignment-free approach correlates better with the specific features found in the amino acid sequence comparison. The approach described here can help support investigations to determine GTPV's origin of introduction whenever it emerges in a disease-free region and determine the origin of contamination with a new strain in GTPV endemic areas.

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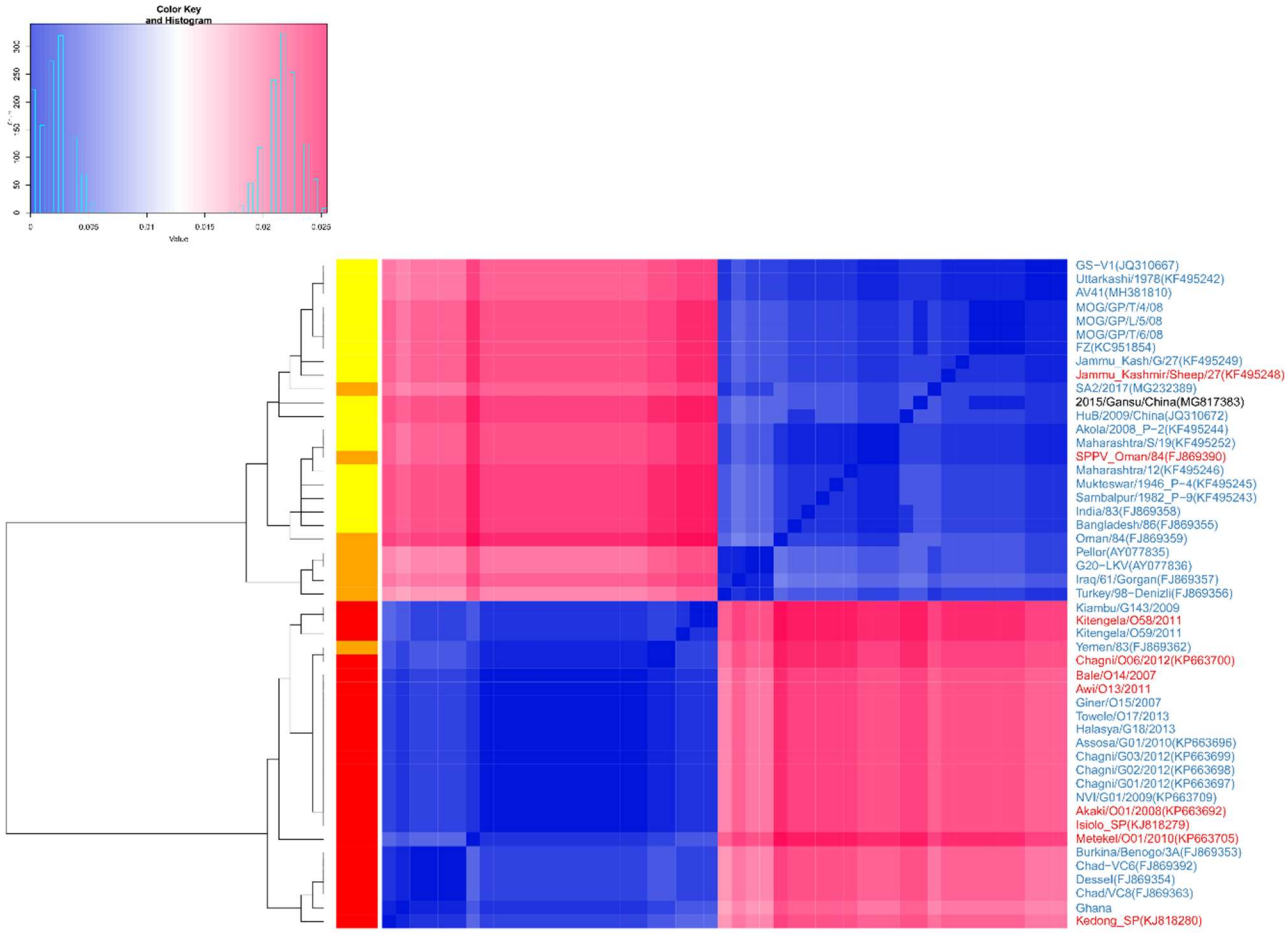
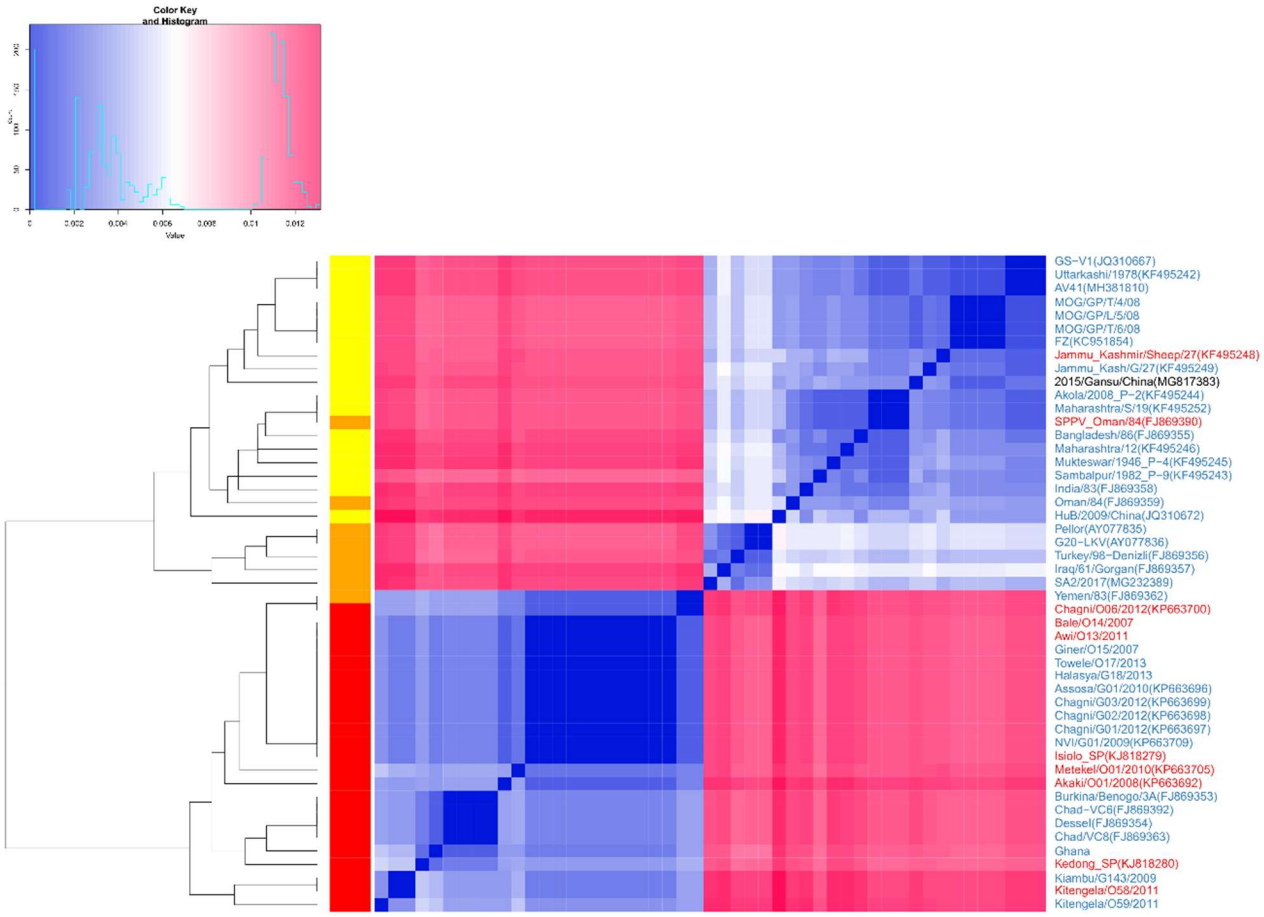
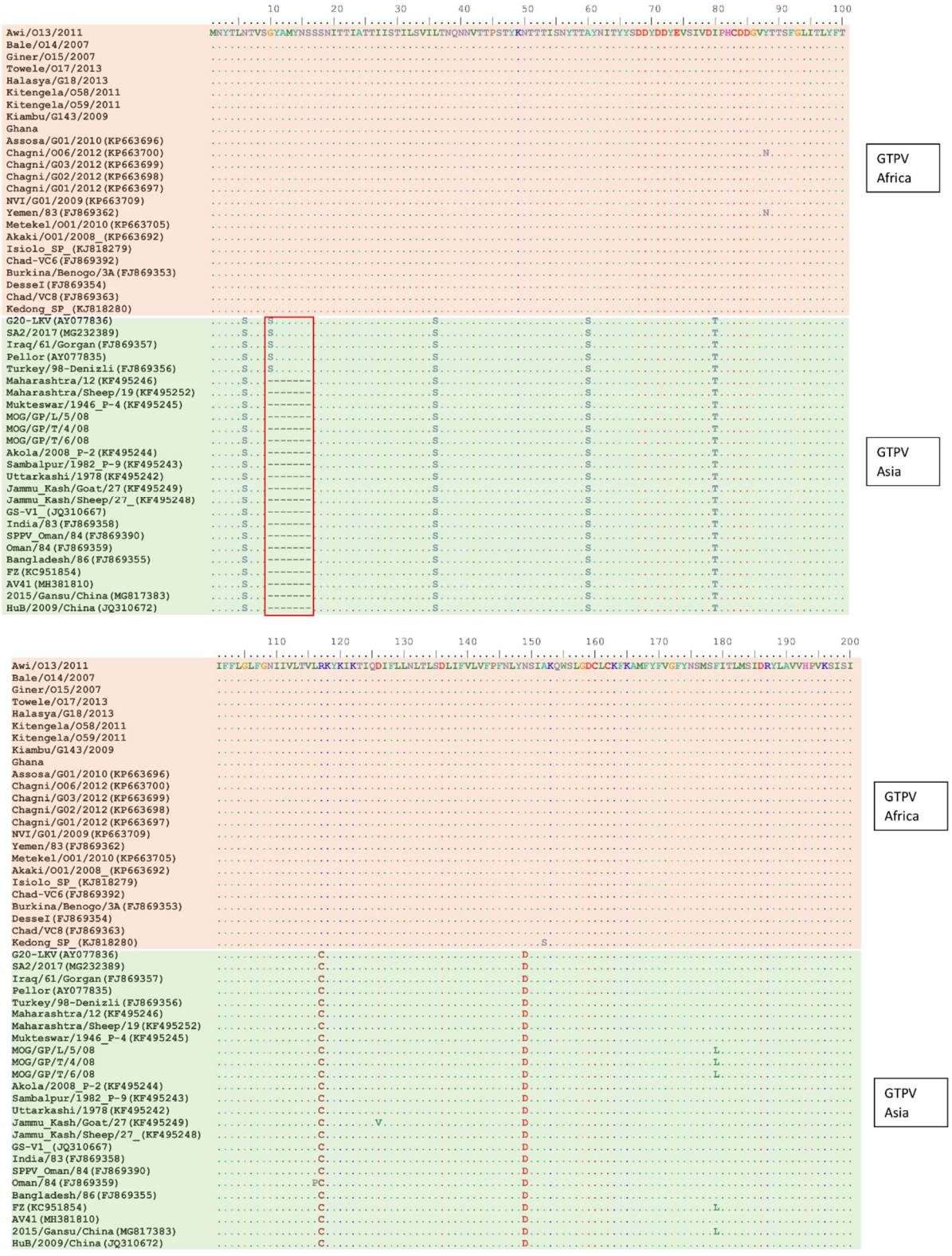
Supplementary Information



Supplementary Figure 1. The heatmap for the nucleotide variations of 49 GTPVs based on the MLC distances. The Complete-linkage clustering method was used to re-order the sequences. The vertical side colors indicate the origin of the samples (red for Africa, orange for West and Central Asia, yellow for East and South Asia).

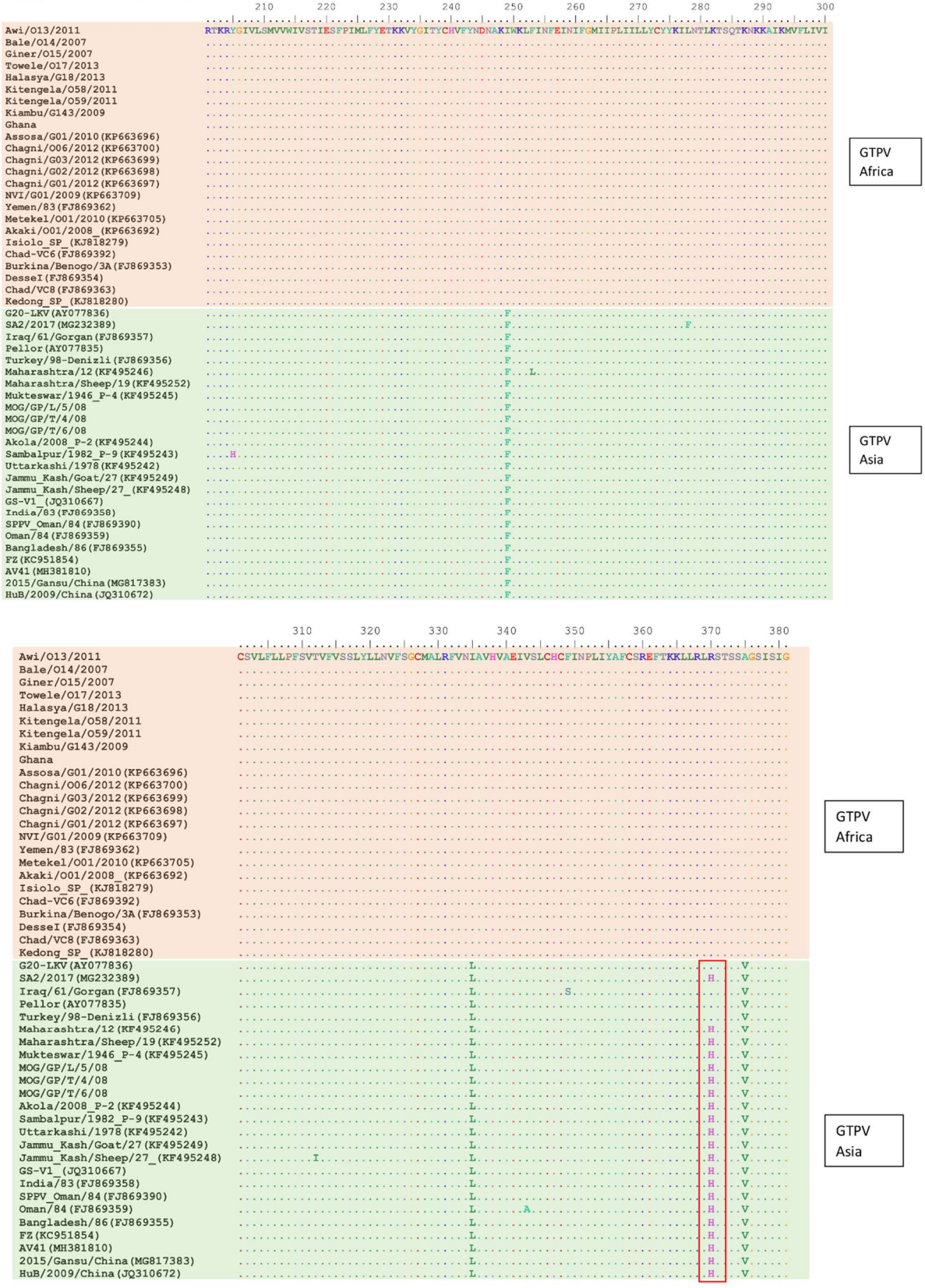


Supplementary Figure 2. The heatmap for the nucleotides k-mers frequencies variations of 49 GTPVs after manual removal of 21 nucleotides from the GPCR genes of WCA and African GTPV. The 21 nucleotides correspond to the gap observed on the multiple sequence alignment in ESA GTPV. The Complete-linkage clustering method was used to re-order the sequences. The vertical side colors indicate the origin of the samples (red for Africa, orange for West and Central Asia, yellow for East and South Asia).



Supplementary Figure 3. Multiple sequence alignment of the complete amino acid sequences of the GPCR of 49 GTPVs from various geographical locations.

(Supplementary Figure 3 continued.)



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Chapter V

Analysis of Sheeppox virus-host innate immune interactions in-vitro

Tesfaye Rufael CHIBSSA, Richard Thiga KANGETHE, Tirumala Bharani K SETTYPALLI, Yang Liu, Reingard GRABHERR, Angelika LOITSCH, Elena Lucia SASSU, Rudolf PICHLER, Giovanni CATTOLI, Adama DIALLO, Viskam WIJEWARDANA, Charles Euloge LAMIEN

Manuscript ready for submission

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Analysis of Sheeppox virus-host innate immune interactions in-vitro

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Abstract

Sheeppox (SPP) is a highly contagious disease of small ruminants that predominantly occur in Asia and Africa, causing massive economic losses. The causative virus, Sheeppox virus (SPPV), belongs to the genus Capripoxvirus (CaPV) of the family Poxviridae.

SPPV can also infect goats, and usually, SPPV live attenuated vaccines (LAVs) are used for vaccination against SPP and goatpox, because of cross-protection between CaPVs. Although adaptive immunity is responsible for long-term immune memory, innate immune responses induced by LAVs is a mechanism that leads to better protection. The mechanisms related to the innate immune response elicited by LAV SPPVs are unknown.

We analyzed the relative expression of thirteen selected genes that included pattern recognition receptors (PRRs), Nuclear factor-κB p65 (NF-κB), and cytokines to better understand the interaction between SPPV and its host. The transcripts of targeted genes in sheep PBMC infected with either wild type (WT) SPPV or LAV SPPV were analyzed by quantitative PCR.

Among the pattern recognition receptors (PRRs), we observed a significantly higher expression of RIG-1 in PBMC infected with both WT and LAV, with WT SPPV producing the highest expression level.

There was high inter-individual variability in the cytokine transcripts levels among different donors, though, the expression of TNFA, IL-15, and IL-10 were significantly higher in both PBMC infected with either WT or LAV as compared to control PBMC.

Our correlation studies revealed a strong significant correlation between RIG-1 and IL-10, between TLR4, TNFA and NF-κB, between IL-18 and IL-15 and between NF-κB and IL-10.

There was also a significant negative correlation between RIG-1 and IFNG, between TLR3 and IL-1 β, and between TLR4 and IL-15 ($P< 0.05$) in PBMC.

There was an absence of activation downstream signalling of RIG-1, probably because of the ability of SPPV to produce immunomodulatory molecules.

This study identified RIG-1 as a signalling pathway of innate immune activation in SPPV infection, possibly through dsRNA. These findings advance our knowledge on SPPV induction of a protective immune response and will further help to develop safer and more potent vaccines against SPP and GTP.

Keywords: Gene expression, PRRs, cytokine, RIG-1, LAV, SPPV

Introduction

Sheep Pox (SPP) is a highly contagious viral disease in domestic and wild small ruminants, causing great economic losses in sheep and goat productivity (Carn, 1993). Sheeppox virus (SPPV), the causative agent of the disease, and two other viruses, goatpox virus (GTPV) and lumpy skin disease virus (LSDV), are the three members of the genus *Capripoxvirus* to within family Poxviridae (Tulman et al. 2002). Both SPPV and GTPV cause SPP in sheep, and GTP in goats, while LSDV is restricted to cattle (Diallo and Viljoen 2007). Given their economic relevance and severity, SPP and GTP are listed as notifiable diseases by the world organization for animal health (OIE) (www.oie.int/animal-health-in-the-world/oie-listed-diseases-2019) .

Live attenuated vaccine (LAV) derived from various SPPV strains are used for the control of SPP, GTP and even LSD in endemic areas and recently have been used in countries that we newly infected by LSDV (Tuppurainen et al. 2014; McFadden 2005).

Several reports have recognised LAVs as effective vaccines against CaPV infections, however, field data on vaccination suggest some efficacy and safety concern, as they do show some cases of vaccination failure and adverse reactions (Ahangran et al., 2016; Chibssa et al., 2018). Most LAV SPPVs were developed by serial passages of virulent or low virulent SPPVs.

Immune responses and host interactions with viruses, including poxviruses, are widely studied *in vitro* using peripheral blood mononuclear cells (PBMC) (Rubins et al. 2004). In addition to fibroblasts, PBMC also include target cells for SPPV such as monocytes and macrophages (Gulbahar, 2006). The cytokine expression of PBMC collected from animals vaccinated with SPPV (Romanian vaccine strains) and Goatpox virus (Gorgan strain) was previously studied. However, this study uses PBMC stimulated with inactivated viruses to evaluate the immune responses to those vaccines by using real-time PCR assays (Norian et al., 2018).

SPPV infection of sheep PBMC can also induce an innate antiviral response that leads to the recruitment of adaptive immune cells. While adaptive immunity is responsible for the long-term memory, innate immune responses induced by LAVs is a mechanism that leads to better protection by SPPV vaccines. Innate immune response is responsible for recognizing pathogens at the initial encounter through pattern-recognition receptors (PRRs) such as Toll-like receptors (TLRs) and RIG-1-like receptors (RLRs). This recognition of viral pathogens creates the activation a cascade of events leading to induction of downstream signalling molecules such as transcription factors as well as activation of inflammatory cytokines and chemokines (Takeuchi and Akira 2007). Therefore, the investigation of innate immune

responses provide information about virus-host interactions. However, little is known on mechanisms related to the innate immune response elicited by both wild type (WT) and LAV SPPVs.

In this study, we assesesd the differences in the innate immune responses related to LAV and WT SPPV infections in sheep PBMC by assaying the expression levels of thirteen targeted genes that included pattern recognition receptors (PRRs), Nuclear factor-kB p65 (NF-κB), and cytokines in the sheep PBMC exposed to SPPV and compared between WT or LAV in vitro.

Material and Methods

Viruses

Wild type isolate SPPV Algeria/93 Djelfa, obtained from the Institut National de la Médecine Vétérinaire, Algiers, Algeria, and Roumanian SPPV vaccine, obtained from BioPharma, Morocco, were used for this study. Both viruses were propagated on embryonic skin cell line from sheep (ESH-L cells) in Hank's Minimum Essential Medium (MEM) supplemented with 10% fetal calf serum and 1% antibiotics (Lamien et al. 2011). The virus titers (TCID50) of both strains were calculated according to the Reed-Muench method (REED and MUENCH, 1938). The viral suspensions were stored at −80 °C until further use. All procedures were performed in the BSL-3 laboratory facilities at AGES, Austria.

PBMC isolation and viral infection

Blood samples were collected from healthy local Austrian sheep breed via jugular vein using heparinized vacutainer tubes and needles. The samples were collected by a certified veterinary service according to the guidelines of the Austrian Agency for Health and Food Safety (AGES). The PBMC were separated by density-gradient centrifugation using Ficoll-Paque (density, 1.077 g/ml; GE health care, Sweden) and washed twice with RPMI 1640 medium (Gibco, Carlsbad, CA, USA). The resultant pellet was re-suspended in complete media (RPMI 1640 media containing 10% FBS and 100 IU/mL penicillin and 100 µg/mL streptomycin). The cell number was determined by counting with a hemocytometer.

PBMC derived from the donor sheep (n=5), were suspended in complete media and placed in either 24 well plates containing 5 x 10^6 cells in 1 ml per well (for flow cytometry) or 6 well plates containing 20 x 10^6 cells in 5 ml per well (for real-time PCR analysis). PBMC were then cultured either with 100µl of WT or LAV strain of SPPV at a concentration of 1x10^4 TCID50/ml

per well. Uninfected controls consisting of 100µl of PBS added to the PBMC suspensions were also prepared. The plates were incubated at 37^0C in a humidified atmosphere containing 5% CO_2 for 3 days (for real-time PCR analysis) or 5 days (for flow cytometry).

RNA extraction and cDNA synthesis

After incubation periods of 3 days, the PBMC were harvested and total RNA extracted using the RNeasy mini kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The total RNA was resuspended in 30µl of RNase free water and quantified using a Nanodrop ND-1000 spectrophotometer.

Total RNA extracted from PBMC incubated either with WT or LAV as well as control PBMC was converted into cDNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA) as described by the manufacturer. Approximately, 1µg of each RNA sample was reverse transcribed in a final volume of 10µl of a reaction mixture containing 1µl random hexamers (50ngµ/l) and 1µl dNTPs (10mM). Following 5 min of incubation at 65°C, the reaction mixture was quick chilled on ice for one minute and reverse transcription was started by addition of 10µl/well of freshly prepared cDNA synthesis mix containing 2µl of 10X RT buffer, 4µl of 25mM $MgCl_2$, 1µl of 0.1M DTT, 1µl of RNase inhibitor and 1µl SuperScript III RT making to a final volume of 20µl. The reverse transcription reaction was then carried out at 25°C for 10 min and 50 min at 50°C, with finally termination by heating at 85°C for 5 min and chilled on ice. The mixture was then incubated at 37°C for 20 min in the presence of 1µl of RNase H to remove the original RNA templates. The resulting cDNA was stored at -20°C until further use.

Quantitative real-time PCR

The cDNA diluted at 1: 100 was used as a template for in-house real-time PCR using specific primers presented in Table1. GAPDH was used as a housekeeping gene. The real-time PCR was performed in a total reaction mixture volume of 10 µl containing 2 µl of cDNA as template, 1 µl of forward and reverse primers (Table 1), and 5 µl of iQ SYBR Green Supermix 2X (Bio-Rad, USA) and 1 µl of water for each reaction. All samples were tested in triplicate. Real-time PCR was performed in a CFX96 real-time PCR Detection System (Bio-Rad, Hercules, USA). PCR conditions for all the targeted genes of different cytokines consisted of initial denaturation of 3 min 95 °C, followed by 40 cycles of PCR with denaturation at 95 °C for 10 sec, annealing temperature at 59 °C for 20 sec, and extension at 72 °C for 20 sec while recording the fluorescent data at elongation step. The amplicons were denatured at 95 °C (held for 15 sec)

following by cooling at 60 °C for 5 sec before melting through a ramping back to 95 °C in 0.5 °C increments with 5 sec hold time for each acquisition step. The specificity of amplification was checked using a melting curve analysis of real-time PCR products. The quantification cycles (Cq) of each sample run in triplicates were used for calculating the relative gene expression.

The efficiencies of the PCRs for the targeted and reference (GAPDH) genes were estimated by amplifying serially diluted cDNA templates using the respective primers. A standard curve was prepared and the slope of each line, obtained from the regression equation was used to calculate efficiencies of the target and reference genes based on the equation: Efficiency (E)=[$10^{(1/slope)}$](Sreedharan et al., 2018).

Surface and intracellular cytokine staining

After 4 days of incubation of PBMC with SPPV (WT or LAV) or PBS, the protein transport in the cells was blocked by adding GolgiStop (BD Biosciences) overnight. A positive control for cytokine production was generated by adding a cell stimulation cocktail (Leukocyte Activation Cocktail with GolgiStop (BD Bioscience) to a third well and incubated overnight. On day 5, PBMC were harvested, washed in PBS and transferred to microcentrifuge tubes and stained for flow cytometry. Surface staining master mix containing mouse anti-sheep CD4 antibody (clone CC8: Bio-Rad) and mouse anti-bovine CD8 (clone CC63, cross reacts with sheep: Bio-Rad) were added to each tube and samples were incubated at 4ºC for 30 min. Following surface staining, dead cell exclusion staining was done with a fixable viability stain (BD Biosciences) before cell fixation.

The cells were washed, fixed and permeabilized with a saponin-based buffer (Fixation/Permeabilization Solution Kit BD Biosciences) according to manufacturer's protocol, and intracellular cytokine staining was done with mouse anti-bovine interferon gamma antibody (clone CC302, cross reacts with sheep: Bio-Rad) for 30 min at room temperature in dark. Cells were washed twice in 1x Perm/Wash buffer and resuspended in FACS buffer (PBS containing 2% FBS and 2mM ethylenediaminetetraacetic acid).

Flow cytometry data were acquired using the Gallios flow cytometer (Becton Dickinson Beckman Coulter) and analyzed with Kaluza software (Becton Dickinson, Beckman Coulter). Cell populations were gated by forward and side-light-scatter parameters as shown in Figure 1. Intracellular cytokine expression was calculated as a percentage of the parent population.

Table 3. Primer sequences for real-time PCR amplification of sheep target genes

Target gene	Primer sequences (5'-3')	Primer conc.	Amplicon size (bp)	Tm (°C)
TNFA	F-CCAGAGGGAAGAGCAGTCC R-GGCTACAACGTGGGCTACC	1.25	112	84
IFNG	F-CAGAGCCAAATTGTCTCCTTC R-ATCCACCGGAATTTGAATCAG	1.25	168	81
IFNA	F-GTGAGGAAATACTTCCACAGAGTCACT R-TGA RGA AGA GAA GGC TCT CAT GA	1.25	107	81.50
NF-κB p65	F-CGGGGACTACGACCTGAATG R-GCCTGGTCCCGTGAAATACA	2.5	250	88.50
IL-1β	F-ATTGCCCAGGTTTCTGAAACA R-CTCGTCACTGTAGTAAGCCATCAT TT	2.5	78	79
IL-6	F-CCGCTTCACAAGCGCCTTC R-CCAGTGTCTCCTTGCTGCTT	2.5	248	82.50
IL-10	F-CTTTAAGGGTTACCTGGGTTGC R-CTCACTCATGGCTTTGTAGACAC	1.25	262	87.50
IL-15	F-TTCATGTCTTCATTTTGGGCTGT R-AAGCACTGCATCGCTGTTAC	2.5	181	80.50
IL-18	F-GCTGCCGTCTTCTGTAAGGA R-TCCAGGTCTTCATCATTTTCAGC	1.25	191	81.50
TLR3	F-CTCCCCAATGGAGGACGAAG R-GCTTGCTGAACTGCATGGTG	2.5	195	80.50
TLR4	F-TCCCCGACAACATCCCCATA R-AAAGGCTCCCCAGGCTAAAC	1.25	224	83
TLR8	F-CCCTGACCCAACTTCGCTAC R-TAAGGAGGGCATTTCGTCCA	2.5	137	82.50
RIG-1	F-GCCTTAAAGAACTGGATTGA R-ATACCCATTGTCTGATTTGTT	1.25	95	82.50
GAPDH	F-CCTGGAGAAACCTGCCAAGT R-GCCAAATTCATTGTCGTACCA	2.5	214	85.50

RIG-1 = retinoic acid-inducible gene I; TLR3 = Toll-like receptor 3; TLR4 = Toll-like receptor 4; TLR8 = Toll-like receptor 8; IL-18 = Interleukin 18; IL-15 = Interleukin 15; IL-10 = Interleukin 10; IL-6 = Interleukin 6; IFNG = Interferon Gamma; IFNA = Interferon alpha, TNFA = Tumor Necrosis Factor Alpha; NF-κB p65 = nuclear factor kappa-light-chain-enhancer of activated B cells; IL-1β = Interleukin 1 beta GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; Tm = melting temperature

Data analysis and presentation

In the present study, gene expression was analysed using the efficiency-corrected calculation models, based on multiple samples, to estimate the relative changes in the gene expressions (normalized with the GAPDH housekeeping genes) (Pfaffl 2006). Boxplots of the $\log_2$ (FC) values and the percentage of the parent population for flow cytometry data were generated using ggplot2 package in R (Wickham, 2016). One sample t-test and two sample paired t-test were

performed in R to compare the differences in gene expression between the infected and control groups. The significance level was set at P-value ($p<0.05$*, $p<0.001$**; $p<0.0001$***).

The heatmap.2 function of the R gplots package was used to create both expression and correlation heatmaps. The Pearson correlations were computed in R. Hierarchical clustering was performed using the complete linkage method to compute the distance between clusters.

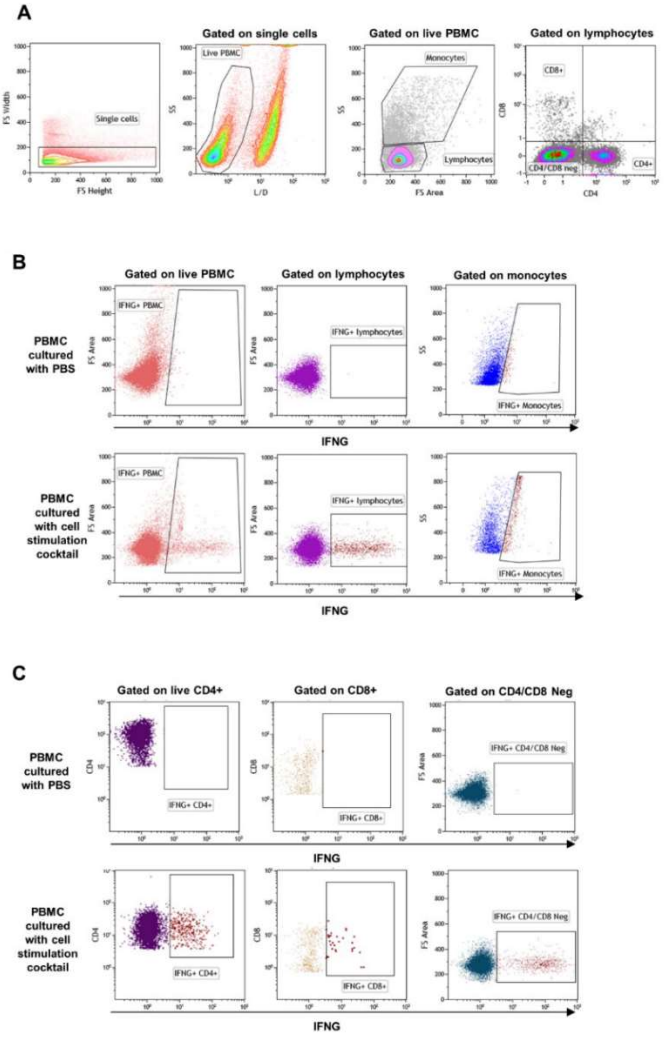

Figure 1. Gate represents gating strategy for Flow cytometry. Analytic gating of flow cytometry showed a representative graph of the change in the mean fluorescence intensity of IFNG in PBMC subpopulation. A) Single cells were selected in the forward scatter-height (FSC-A) versus forwarding scatter-width (FSC-A) plot, then, live/ dead was gated to identify live cells. Next, monocytes and lymphocytes were gated from the live cells on FSC-A versus SSC-A plot. Gating of lymphocyte subset was performed as CD4+, CD8+ and CD4/CD8 double negative (DN). B) Expression of IFNG by PBMC, monocytes and lymphocytes: IFNG positive populations were gated using PBMC cultured with PBS (negative control) and PBMC cultured with cell stimulation cocktail (positive control). C) Expression of IFNG by CD4+, CD8+ and DN: IFNG positive populations were gated using PBMC cultured with PBS (negative control) and PBMC cultured with cell stimulation cocktail (positive control).

Results

The relative fold-change for thirteen selected genes in PBMC infected either with WT or LAV compared to PBMC control were analyzed by real-time PCR. A panel of specific primers compatible with a same thermal cycling program was used. The specificity of each primer pair were evaluated by melting curve analysis, agarose gel electrophoresis and sequencing. All amplified PCR products showed a distinct and unique Tm.

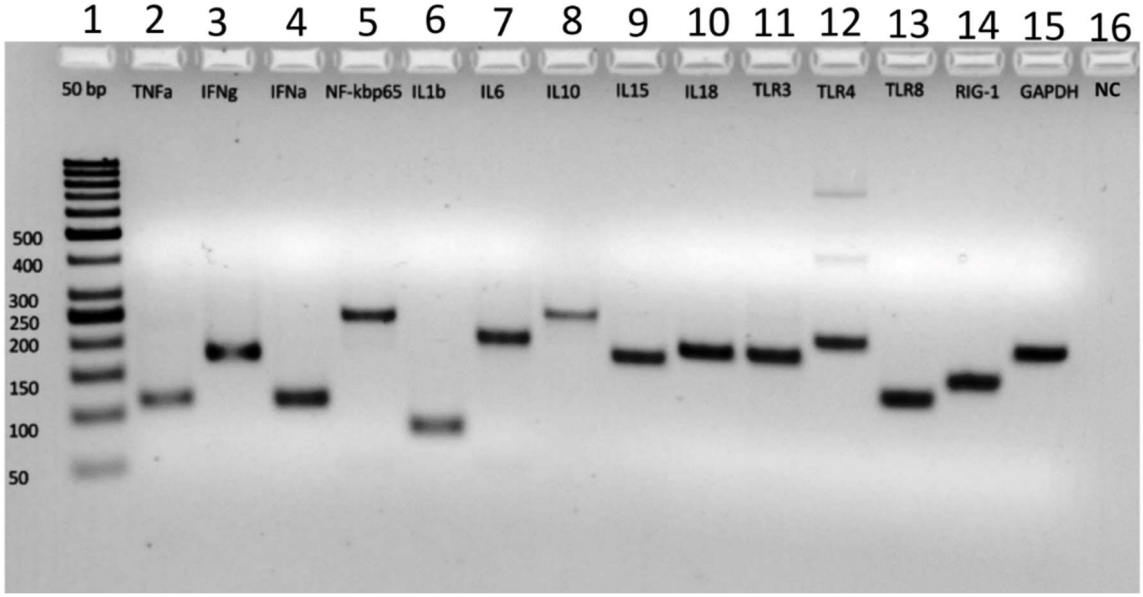

Figure 2. Agarose gel electrophoresis image showing PCR product of Target gene. Amplicon obtained from PCR products of the target genes amplification were visualized by Gel documentation system (Bio-Rad) after agarose gel electrophoresis through a 2.0 % agarose gel at 100 V for 60 min. Lanes were labeled with the numbers, Lane 1: 50bp DNA ladder, Lane 2 to 15: indicated with different target and reference genes, Land 16: Negative control.

The result of gel electrophoresis showed a single band of the expected size for all the thirteen targeted genes except, TLR4 which presented two additional light bands (Figure 2). Despite the presence of the two light bands, we proceeded with the TLR4 primer set as the amplicon showed a single Tm. The relative quantities of the target genes were normalized against the relative quantities of the internal standard (GAPDH). Cq values of the amplified templates in sheep PBMC cultured with either WT or LAV were used for the calculation of gene expression. Differences in expression levels were given as fold-change (FC) using the sheep GAPDH gene for normalization and compared with PBMC control.

Analysis of expression level of PRRs transcripts in PBMC cultured with SPPV

The expression level of the transcripts for the PRRs, including TLR3, TLR4, TLR8 and RIG-1, following the infection of sheep PBMC with WT SPPV or LAV SPPV were converted to Log2 FC and presented in Figure 3.

At 72 hours post infection, there was highly significant expression of RIG-1 in both WT (100-FC) and LAV (60 FC) in infected PBMC cultures ($P < 0.001$). Moreover, RIG-1 expression in PBMC that were cultured with WT was significantly higher than in those cultured with LAV ($P < 0.05$).

TLR3 presented various patterns of regulation depending on the donor: it was down regulated in one animal for both LAV and WT, upregulated in two donors and close to the controls in two remaning donors. Consequently, the expression level of TLR3 (WT 2.3 FC, LAV 2.6 FC) was not significantly different from control PBMC. TLR4 was clearly upregulated in one donor for both LAV and WT, however the overall the expression level of TLR4 (WT 2.9, LAV 2.6) were not significantly different from the control PBMC. In contrast, TLR8 was mostly downregulated (in all donors treated with WT and 4 out of 5 donors treated LAV), athough the means expresion levels for WT (-1.7 FC) and LAV (-1.3 FC), were not stastically different from those of control PBMC.

Overall, there was a moderate inter-individual variability for each of the PRRs transcripts levels (2.1 fold for TLR8 to 8.14 f fold or TLR4 for PBMC treated with LAV and 4.13 fold for TLR8 to 7.64 fold for TLR4 for those treated with WT).

Analysis of expression level of NF-κB in PBMC cultured with SPPV

To confirm and expand these results we analysed the expression of NF-κB family transcription factors which are down-stream signalling molecules for the expression of PRRs.

NF-κB p65 was clearly upregulated in two donors for both LAV and WT, howerever, the mean fold changes for PBMC infected with either WT (4.3) or LAV (3.7) were not statistically different from that of the control PBMC ($P > 0.05$; Figure 3).

NF-κB p65 showed the highest inter-individual variability among all the studied transcripts, especially in PBMC treated with WT virus (35.83 fold difference between the individual with highest transcript level and the one with lowest level).

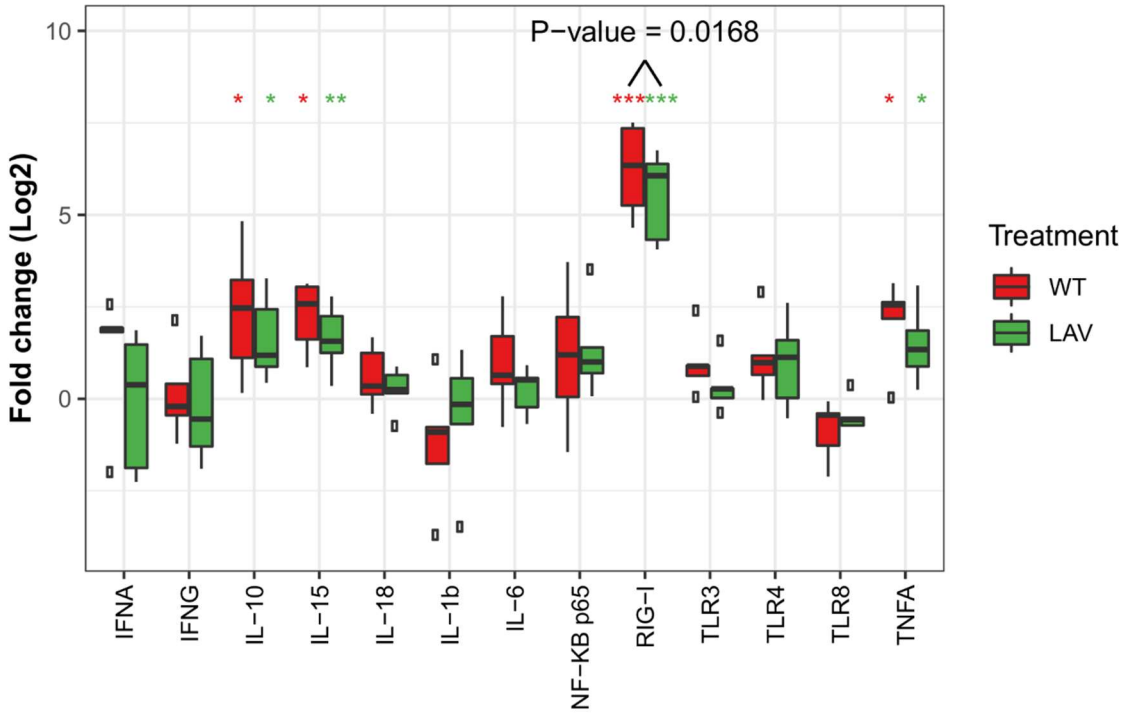

Figure 3. Box plots representing the differential expression of mRNA of thirteen genes. The expression of RIG-1, IL-10, IL-15 and TNFA were significantly upregulated. Note that the expression RIG-1 was significantly higher in WT as compared to LAV. Data represent the Log2 FC of five independent experiments.

Expression analysis of cytokine mRNA in PBMC cultured with SPPV

The expression level of transcripts for IFNA, IFNG, TNFA, IL-1β, IL-6, IL-10, IL-15 and IL-18 at 72 hours post-infection of sheep PBMC with WT or LAV strains were converted to Log2 Foldchange and presented in Figure 3.

There was a high inter-individual variability in the cytokine transcripts levels among different donors: 3.1 fold for IL-18 to 28.14 fold for IL- 1β for PBMC treated with LAV; and 4.22 fold for IL-18 to 27.26 fold for IL-1β for those treated with WT.

The mean fold changes for TNFA (WT = 5.3, LAV = 3.5), IL-15 (WT = 5.6, LAV = 3.6), and IL-10 (WT = 9.3, LAV = 4.1) were significantly higher in both PBMC infected with either WT or LAV as compared control PBMC ($P < 0.05$, Figure 3). The expression level of TNFA, IL-15, and IL-10 were higher in PBMC incubated with WT compared to LAV, though the difference was not significant ($P > 0.05$).

IFNG, IFNA, IL-6, and IL-18 presented each, various patterns of regulation depending on the donor. Consequenlty, the mean fold changes for IFNG (WT = 1.6, LAV = 1.4), IFNA (WT = 3.4, LAV = 1.6) IL-6 (WT = 2.7, LAV = 1.3), and IL-18 (WT = 1.7, LAV = 1.3) were not

significantly different between PBMC infected with WT or LAV and the controls. In contrast, IL-1β was downregulated in 4 donor out of 5 for both WT and LAV. Nevertheless, the mean fold changes (WT = -1.4, FC, LAV = -1.1 FC) not significant ($P > 0.05$, Figure 3). In general, none of the cytokines were differentially expressed between WT and LAV treatments.

Collectively, these data reveal that there is a greater innate immune gene expression in WT infected sheep PBMC cultures, as compared with LAV infection, likely due to increased levels of viral dsRNA within cytoplasm of infected PBMC. Additionally, inter-individual variability was more pronounced with cytokine transcripts levels.

Correlation analysis of an immune gene expression signature

Since many immune markers followed a similar pattern of expression between the two (WT and LAV) treatments, we next investigated which markers correlated in their expression. Indeed, heatmap analysis revealed relationships between receptors, transcription factors and cytokines (Figure 4).

Our results showed a strong significant correlation between the expression of RIG-1, which recognizes dsRNA, and IL-10 ($P< 0.01$), and a significant negative correlation between RIG-1 and IFNG ($P< 0.05$) in PBMC. TLR3, another PRR that can recognize dsRNA moderately correlated with IL-6, IL-15 and IL-18, however, these correlations were not statistically significant. We also observed a highly significant negative correlation between the expression of TLR3 and IL-1 β ($P< 0.01$) in PBMC. Similarly, there was a significant positive correlation between the expression levels of TLR4, TNFA and NF-κB ($P< 0.05$), and a significant negative correlation between TLR4 and IL-15 ($P< 0.05$) in PBMC (Figure 4).

There was a strong and significant positive correlation between the expression level of IL-18 and IL-15 ($P< 0.01$). The expression of NF-κB positively and significantly correlated with the expression of IL-10 ($P< 0.05$).

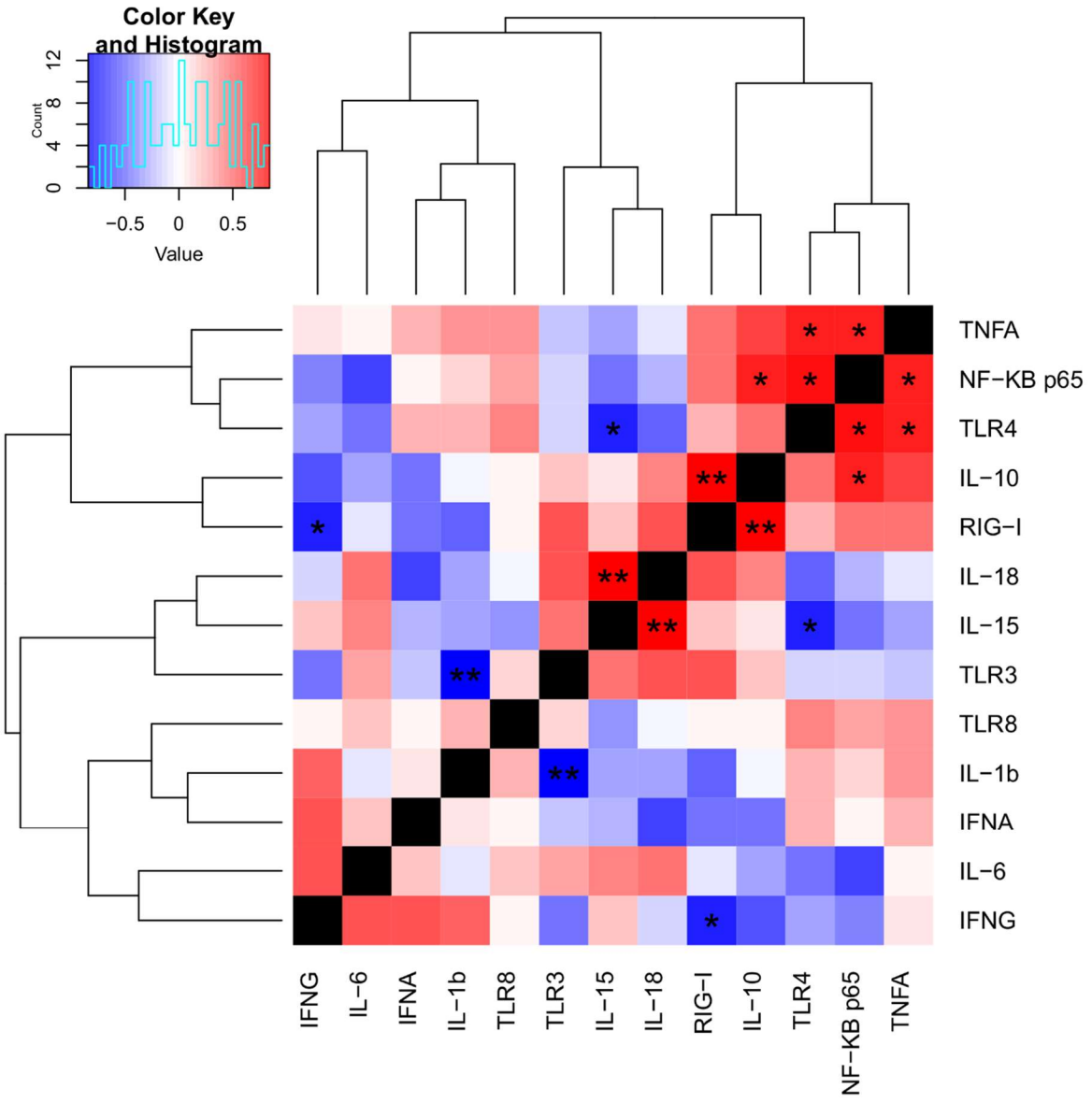

Figure 4. Heat map for correlations of target genes expression FC using qPCR. The correlation heat map describes the combined Pearson correlation coefficient based on distance between the two gene expression value against all target groups. This shows that the heatmap will cluster together, genes that have positively correlated log2 FC value, P value, P<0.05*, P<0.01.**

Flow cytometry analysis of IFNG in sheep PBMC cultured with SPPV

Next, to validate mRNA expressions of immune markers, the protein production by individual cell populations within the PBMC were analyzed through flow cytometry. The IFNG producing cells were identified as a percentage of total live PBMC, monocytes, lymphocytes or CD4+, CD8+ and CD4/CD8 double negative cells within the lymphocyte population (Figure 1). None of the subpopulations had a difference in the percentage of IFNG producing cells in the

identified cell populations between WT or LAV treatments ($P < 0.05$; Figure 5). Collectively, the IFNG production by PBMC detected with flow cytometry were consistent with that determined through quantitative RT-PCR and showed that even among the subpopulation of cells there was no disparity.

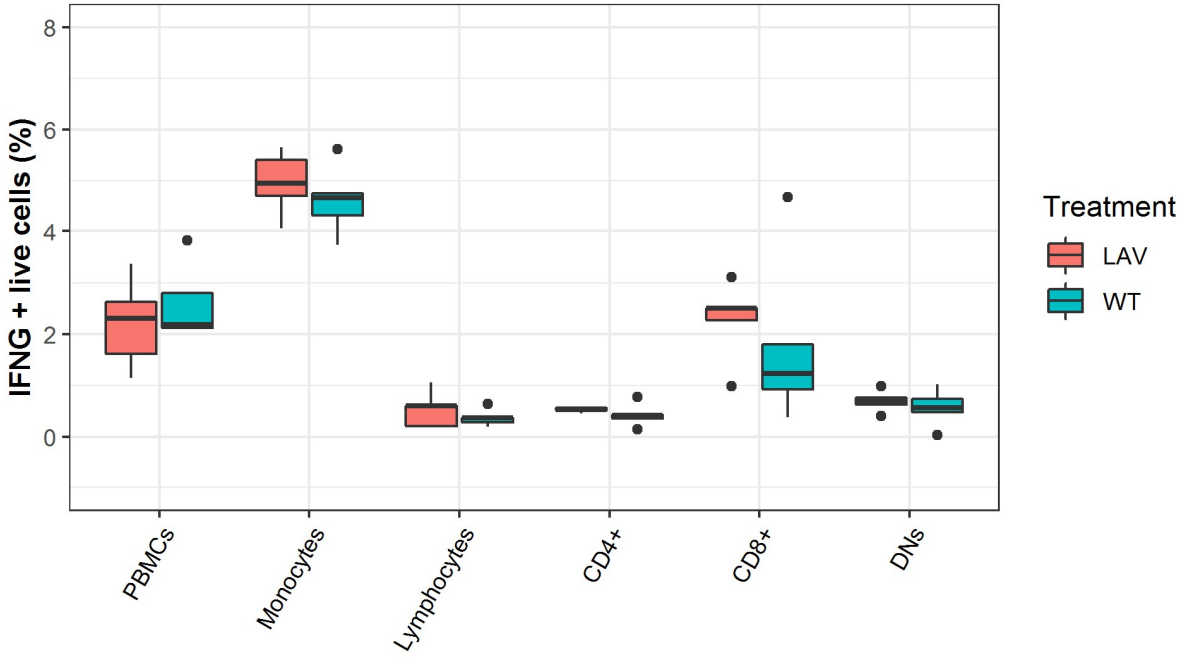

Figure 5. Flow cytometry analysis of the expression of IFNG in PBMC subpopulations. The box plots represent the percentage of IFNG expression in healthy sheep PBMC sub-population infected with either WT SPPV or LAV SPPV.

Discussion

We have analyzed the relative expression of thirteen selected genes, encoding for proteins involved in host innate and adapted response to viral infections, following the infection of Sheep PBMC with WT SPPV and LAV SPPV to better understand the interaction between SPPV and it host.

Among the mRNA of the pattern recognition receptors (PRRs), we have observed that RIG-1 was highly expressed in PBMC infected with both WT and LAV, suggesting that RIG-1 may play an essential role for the innate recognition of SPPV. As RIG-1 is a sensor for dsRNA (Dixit and Kagan 2013), the high expression of this receptor in both WT and LAV suggests that dsRNA intermediates are produced during SPPV infection. Indeed, previous studies showed that some dsDNA viruses, such as poxviruses, that replicate and transcribe their genomes and

assemble infectious particles exclusively in the cytoplasm of cells produce dsRNA intermediates (Moss 2013).

We have also observed that RIG-1 expression was significantly higher in PBMC infected with WT as compared to those infected with LAV, suggesting that WT infection produced more dsRNA intermediates as compared to LAV.

It was interesting to notice that among the tested PRRs, only RIG-1 was significantly positively expressed, suggesting that SPPV sensing of ds RNA intermediates is mostly through RLRs than TLRs.

There was a high inter-individual variability for cytokines as compared to PRRs, though the levels of both cytokines and PPRs appeared to be characteristics of different donors. Indeed, in each donor, the cytokine levels and PRRs levels followed the same trend independently of the SPPV strain used for PBMC infection. Similar inter-individual variabilities in cytokine productions in human PBMCs were previously reported and attributed to polymorphisms in genes that control the expression of cytokines (Yaqoob et al.,1999;ZHANG et al. 2005)).

Among the tested cytokines, the expressions of TNFA, IL-15 and IL-10 were all significantly upregulated, suggesting that they may play an important role in SPPV infections. A similar increase in IL-10 expression following SPPV infection in vivo has been reported (Abu-El-Saad and Abdel-Moneim, 2005). IL-10 is an important ant-inflammatory cytokine secreted by monocytes following their infection with pathogens and an increase production of IL-10 following infection by other poxviruses has been previously reported (Zhang et al. 2005; Wong et al. 2018). Similarly, the increase in TNFA production following poxvirus infection has been reported (Zhang et al. 2005).

It was interesting to note the upregulation of both inflammatory cytokines IL-15 and TNFA in response to PBMC infection with SPPV. Virus infections result in an inflammatory environment with an increased IL-15 and TNFA and the subsequent recruiting of antigen presenting cells to the infection site leading to the induction of an adaptive immune response. However, IL-10 can inhibit those effects. This dichotomy of inflammatory and anti-inflammatory cytokine expressions could also result from the expression of different cytokines from infected and non-infected cells. Therefore, it would be worth to look at cytokine expression after sorting infected and non-infected cells. As a first step, we are currently conducting experiments to further analyze SPPV replication and the accumulation of viral dsRNA in PBMC.

Interestingly, our correlation studies suggested a strong positive correlation between the PRR RIG-1 and IL-10, both positively significantly expressed in WT and LAV SPPV infected PBMC.

The strong positive correlation between RIG-1 and IL-10 suggests that immune evasion of SPPV could be mediated by the anti-inflammatory properties of IL-10. Anti-inflammatory cytokines such as IL-10 create an inhibitory environment (Iyer and Cheng 2012) that does not attract other immune cells and antigen presenting cells. On the other hand, RIG-1 negatively correlated with IFNG, a cytokine that is necessary for driving Th1 type cell mediated immunity to control viral infections.

Curiously, the activation of RIG-1 did not lead to the subsequent expression of other signalling molecules of the RIG-1 pathway. For instance, our data suggested that the production of NF-κB and type I and type II interferon response were comparable between treated and control PBMCs. The flow cytometry results further supported the absence of specific IFNG production following PBMC infection with SPPV.

A possible explanation is that SPPV-encoded virokines inhibited RIG-1 downstream signalling leading to a type 1 interferon response. For instance, SPPV possesses VAVC orthologous genes encoding for E3, A52, and N1L proteins, all known to interfere with the downstream regulation of RIG-1 (Smith et al., 2018). Of interest, previous reports have shown that N1L promotes the virulence of VACV (Symons et al. 2002) through various mechanisms such the inhibition to signalling to NF-κB (DiPerna et al., 2004 ; Zhang et al. 2005; Smith et al. 2018), as well as the IL-1β and TLR4 signalling cascades (Zhang et al. 2005) .

In conclusion, it is well-established that innate immunity is essential for an effective host defense response against SPPV infections. Although adaptive immunity provides the long-term protection, innate immunity provides the necessary priming for this to occur. Interestingly, vaccines against viral infections such as classical swine fever provide protection very early on before an adaptive immune response is mounted probably through the activation of innate immune system (Franzoni et al. 2013), indicating a greater need to understand the innate immune responses to vaccine viruses. Consequently, by identifying key innate mechanisms that follows PBMC exposure to SPPV, this study will enable us to compensate for deficiencies in our knowledge of cellular mechanisms that activate innate immunity in sheep PBMC infected with SPPV. The data generated will also aid in developing a killed vaccine for SPPV and other pox viral diseases in small and large animal ruminants by incorporating adjuvants that will yield the desired innate immune activation pathway.

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Discussion and Conclusions

This thesis addressed the development of molecular assays to differentiate LAV SPPV from WT SPPV along with the analysis of capripoxvirus genetic diversity and SPPV interactions with the host immune system, to generate tools and advance our knowledge for a better management of capripoxvirus infections.

Although LAVs are widely used for the control of capripoxvirus infections in sheep, goats, and cattle, rapid methods enabling the differentiation of LAV CaPV from WT CaPV are available for LSDV only. At the beginning of this work, despite the increasing number of reports on SPPV infections in vaccinated flocks, little attention was given to the development of tools to rule out the involvement of LAVs during outbreaks.

The first step of this thesis was to develop molecular assays to differentiate LAV SPPV from WT SPPV (Chapter 1 and Chapter 2).

The first chapter of this thesis focused on the identification of a suitable marker to develop a gel-based PCR assay to differentiate LAV SPPV from WT SPPV. While comparing the genome of Morocco's SPPV vaccine to those of publicly available CaPVs, we discovered an 84 base pair nucleotide deletion in the intergenic region between the DNA ligase gene and the B22R gene in LAV SPPV only. Subsequently, we developed a gel-based PCR to differentiate LAV SPPV from WT SPPV and other CaPVs such as LSDV and GTPV (Chapter 1, Chibssa et al. 2018).

The shorter lengths of PCR amplicons from LAVs as compared to WT SPPV and other CaPVs, enabled the differentiation of LAV SPPV from WT SPPV and other CaPVs.

We confirmed that this marker was unique to LAV SPPV derived from the Yugoslavian RM/65 and Romania strains, and validated the gel-based PCR results by the sequencing the targeted fragment in SPPV vaccine from Morocco, Senegal, Algeria and Egypt, and WT SPPV, LSDVs and GTPVs from various geographical origins. The sequencing data were fully in agreement with the PCR data, showing the deletion only in LAV SPPV. The developed assay showed similar sensitivity and specificity to previously developed gel-based methods (Lamien et al. 2011a) for the detection of CaPVs, and presented the added advantage of being able to differentiate LAV SPPV from WT SPPV and other CaPVs. As the gel-based PCR assay could not differentiate WT SPPV from GTPV and LSDV, we searched for additional markers to develop an assay that could provide both the differentiation of LAV SPPV from WT SPPV and simultaneously genotyping CaPVs into SPPV, GTPV, and LSDV (Chapter 2, Chibssa et al.

2019). In the B22R gene, we identified two genome deletions (21 and 27 nucleotides) in LAV SPPV and species-specific markers for SPPV, GTPV, and LSDV, and developed an HRM assay. The HRM assay enabled the differentiation between LAV SPPV, WT SPPV, GTPV, and LSDV, producing four easily distinguishable melting peaks (Chapter -2, Chibssa et al. 2019). The results of the HRM matched sequencing data and the results of published genotyping assays (Lamien et al. 2011b; Gelaye et al. 2013; Gelaye et al. 2017) .

Besides their ability to differentiate LAV SPPV from WT SPPV, both the gel-based and HRM assays have the advantage of also detecting all CaPVs, with similar sensitivities to previous assays (Heine et al., 1999; Stram et al., 2008; Lamien et al., 2011a).

It is important to refine assays intended to differentiate LAV SPPV from WT SPPV to include the general detection of CaPVs, as both SPPV and GTPV can infect sheep and goats a produce disease (Lamien et al. 2011). An assay specific for LAV SPPV and wild type SPPV only is likely to misdiagnose cases of GTPV infections in a previously vaccinated herd, therefore, not suited for routine diagnostics of CaPVs.

In contrast, we could use the two methods presented here as alternatives to existing detection techniques for the direct screening of clinical samples. The HRM assay can genotype CAPVs and assign them as WT SPPV, GTPV, or LSDV with similar sensitivity, specificities, and accuracy as compared to the previous genotyping assays (Lamien, et al. 2011b; Gelaye et al. 2017). This is an added value, allowing the use of this method as an alternative to previous genotyping approaches, including sequencing. Since this HRM method doesn't use labeled probes, it is inexpensive to implement and therefore suited for laboratories with low resources.

In summary, the two methods, developed during this thesis work, offer cost-effective means for direct screening of clinical samples from both vaccinated and non-vaccinated herds. They present the potential to improve SPP and GTP diagnosis and surveillance and facilitate epidemiological investigations in countries using live attenuated SPP vaccines.

Molecular tests can help rule out the involvement of LAVs when a CaPV outbreak occurs in a vaccinated herd. When such assays produce a positive result for WT CaPVs, it becomes essential to know whether the isolate recovered from the vaccinated animal differs from commonly circulating CaPVs. Hence, besides the use of molecular tests to differentiated LAV CaPV from WT CaPVs, a continuous characterization of CaPV isolates can help monitor the evolution of the virus.

LSD, GTP, and SPP have been endemic for many decades in Eastern Africa despite the extensive used of LAVs to control the disease (Tuppurainen and Oura 2012). As the continuous use of LAVs can also contribute to the emergence of new isolates, a comprehensive molecular

characterization of CaPVs in Eastern Africa, where little information is available, can help to discover new CaPV strains.

We addressed the molecular characterization of CaPVs, focusing on LSDV collected in Eastern Africa, before LSDV introduction in Central Asia and Europe (Chapter 3-Chibssa et al., ready for submission).

Our comparative analysis of LSDVs from Sudan, Kenya, and Ethiopia suggested that LSDV genome is very stable. Nevertheless, we discovered that LSDV Embu/B338/2011, an LSDV field isolate collected from Kenya in a previously vaccinated herd had mixed features of LSDV Neethling vaccine and WT LSDV (Chapter 3-Chibssa et al., ready for submission). The isolate presented a 12-nucleotide insertion in the GPCR gene, which is characteristic of LAVs such as LSDV Neethling and KS-1 vaccines, however, its RPO30 resembled that of WT LSDVs. A similar variant, LSDV RUSSIA/Saratov/2017, has been reported recently in Russia, based on scanning signals for recombination in the full genome of the virus (Sprygin et al. 2018). As LSDV RUSSIA/Saratov/2017 was collected at the Russian border with Kazakhstan, the authors suggested that this virus was a recombinant escape of a LAV derived from the Neethling vaccine strain and a WT LSDV (Sprygin et al. 2018).

Here we used an approach based on the sequencing of fragments from four genes (the GPCR, RPO30, EEV glycoprotein and the B22R genes) to show that LSDV Embu/B338/2011 presents mixed features of the LSDV Neethling vaccine strain and a WT LSDV, and that the virus differed from any other previously described variants of LSDVs with a 12-nucleotide insertion in their GPCR gene. Our epidemiological data suggest that LSDV Embu/B338/2011 could have emerged by recombination as this isolate was collected from a previously vaccinated herd.

This approach based on sequencing of four targets in the LSDV genome is much easier to implement in laboratories with limited resources.

As a future direction, we suggest that PCR or HRM based assays should be developed for the parallel testing of multiple markers as barcodes for CaPV, based on the targets we have analyzed in Chapter 3. Such assays will provide a cheaper alternative to sequencing for a better characterization of LSDV and facilitate the monitoring of the virus.

Our results also suggest that the insertion of 12 nucleotides in the GPCR of LSDV might account for some level of attenuation as, LSDV variants with the 12-nucleotide insertion are only seen occasionally and do not seem to sustain and keep the chain of transmission.

These findings highlight the importance of constant monitoring of genetic variation among the CaPV isolates.

The recent LSD crisis in Western and Central Asia, and Europe, and the incursion of SPP in Bulgaria and Greece (Beard 2016) demonstrated the potential of CaPVs to spread outside their usual endemic regions. When LSD emerged in Europe, there was no mean to trace the source of LSDV infection and, only data on the movement of animals (especially illegal movements) could serve as a mean to understand the origin of the infection as there was no previous attempts to classify the CaPVs based on their geographical origins.

In this work, we have used GTPV as an example, and analyzed their GPCR genes sequences using conventional approaches based on multiple sequence alignments and evaluated an alternative alignment-free method approach for sequence comparison, to classify the virus based on their geographical origin (Chapter 4- Chibssa et al. submitted). We sequenced the GPCR genes of additional GTPVs from Ethiopia, Kenya, Ghana and Mongolia, and analyzed them together with GTPV sequences from public databases.

As previously reported (Le Goff et al. 2009), we observed three groups using multiple sequence alignment (MSA) followed by phylogenetic reconstruction; however, we were unable to draw any definite conclusion for the classification of GTPVs based on their geographical origins. In contrast with this finding, the alignment-free method exploiting k-mer frequencies separated GTPVs into three different groups according to their geographical origin: the African GTPVs and the Asian GTPVs which further split into Western and Central Asian (WCA) GTPVs, and Eastern and Southern Asian (ESA) GTPVs (Chapter 4- Chibssa et al. ready for submission). Our study further highlighted that the power of the alignment-free methods resides in its ability to consider the differences in sequence length which are often translated into gaps and discarded by global and local alignment-based algorithms (Zielezinski et al. 2017).

The alignment-free approach classified three isolates: GTPV Yemen/83, collected in western Asia as an African GTPV and GTPV Oman and SPPV Oman both from western Asia as ESA GTPVs. Interestingly, the epidemiological data associated with these isolates suggested that they were imported from abroad (Kitching et al., 1986), confirming the power of the alignment-free method to detect sources of GTPV introduction.

We have shown, by using an alignment-free method, that the GPCR gene was suitable for classifying GTPVs, but not LSDV and SPPV, based on their geographical origin. The study provides a proof of concept to develop similar approach for other CAPVs such as LSDV and SPPV. Furthermore, we suggest that, based on specific markers on the GPCR of GPTVs, simple molecular assays such as PCR, qPCR or HRM could be developed for the classification of GTPV according to their geographical origin and trace the introduction of new isolates within a given region.

Although LAVs are available for the control SPP and GTP, the increasing number of reports of disease emergence in previously vaccinated herds raises concerns on their efficacy and safety. Hence, studies are necessary to understand the immunology of SPPV infections and the host response to LAV SPPVs.

In the last part of this thesis, we infected sheep PBMC with either WT SPPV or LAV SPPV and analyzed the relative expression of thirteen selected genes by real-time PCR to understand the interaction between SPPV and its host (Chapter 5- Chibssa et al. ready for submission). The targeted genes included pattern recognition receptors (PRRs), Nuclear factor-kB p65 (NF-κB), and cytokines genes.

We discovered that among the PRRs, RIG-1 was highly expressed in both PBMC infected with WT (100-fold) and LAV (60-fold) compared to control PBMC. The expression of RIG-1 in PBMC infected with WT was significantly higher than PBMC infected with LAV. We have also found a significant upregulation of the mRNA expression levels for TNF-α, IL-15, and IL-10 in PBMC infected with WT or LAV, compared with matching controls. A similar increase in production of IL-10 following monocytes infections by other poxviruses, including SPPV, has been previously reported (Abu-EL-Saad and Abdel-Moneim 2005 ; (Zhang et al. 2005; Wong et al. 2018). A previous study also showed an increase in TNFA production following poxvirus infections (Zhang et al. 2005) .

However, to the best of our knowledge, this is the first report on the involvement of RIG-1 in PBMC response to SPPV infection.

Since many immune markers followed a similar pattern of expression between the two (WT and LAV) treatments, we next investigated the correlations between the expression of these markers. Our results showed that the expression of RIG-1, which recognizes dsRNA, was significantly and positively correlated with IL-10 and significantly negatively correlated with IFN-A in PBMC. We also observed a highly significant negative correlation of expression between TLR3 and IL-1β level in PBMC. Similarly, the expression level of TLR4 signalling pathway correlated significantly and positively with TNFA and NF-κB, and significantly and negatively correlated with IL-15 in PBMC. These results suggest the key modulating effects of RIG-1 in the induction of innate immune mechanisms against SPPV.

Interestingly, we have noticed that the activation of RIG-1 did not lead to the consecutive expression of other signalling molecules of the RIG-1 pathway such as NF-κB type I and type II interferon responses were comparable between treated and control PBMCs. We, therefore, discussed our findings in light of poxviruses immunomodulatory mechanisms. SPPV possess orthologous genes of vaccinia virus encoding for the E3, A52, and the N1L proteins, known to

interfere with the downstream regulation of RIG-1 (Smith et al., 2018). For instance, previous studies showed that the vaccinia virus N1L inhibits the signalling to NF-κB (DiPerna et al., 2004; Zhang et al., 2005; Smith et al 2018).

In conclusion, we have identified some key innate mechanisms that follow SPPV exposure to host PBMC.

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Appendix

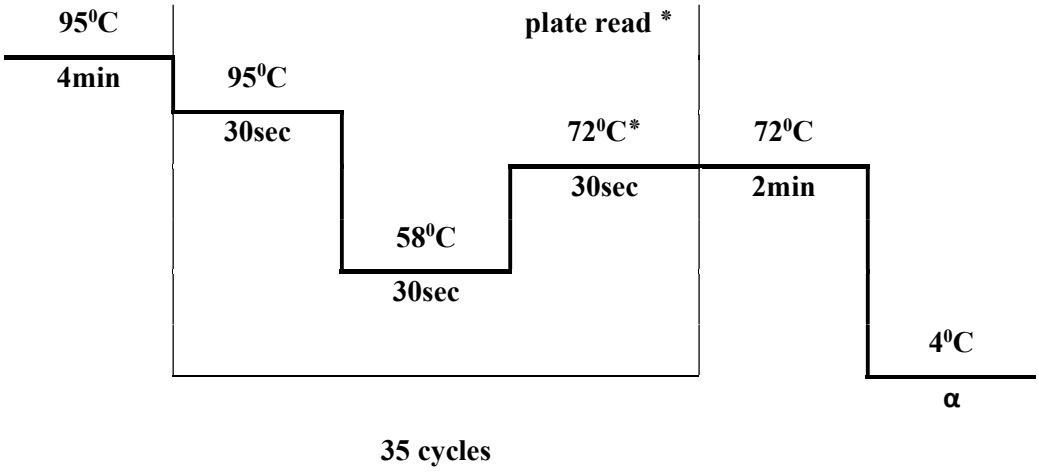
Annex-1. Procedure for gel-based PCR assay to differentiation of SPPV virus field isolates from vaccine strains (Chibssa et al., 2018)

1. Preparation of the master mix for total 20ul volume PCR reaction.

Reagent	Final concentration	Volume per reaction(µl)
SPPV_DIV_For (5pmole/ µl)	500 nM	2 µl
SPPV_DIV_Rev (5pmole/ µl)	500 nM	2 µl
dNTPs	0.2 mM	2 µl
PCR buffer	1×	2 µl
Water		9.75
Taq DNA polymerase		0.25
Template DNA		2

2. Add 18 µl of the master mix to each well in the PCR strip or plate
3. Add 2 µl of Template DNA or water for NTC control to the wells as per layout
4. Seal the plate and spin down the contents
5. Place the plate in real-time PCR machine and run the thermal cycler profile

The cycling conditions were as follow:



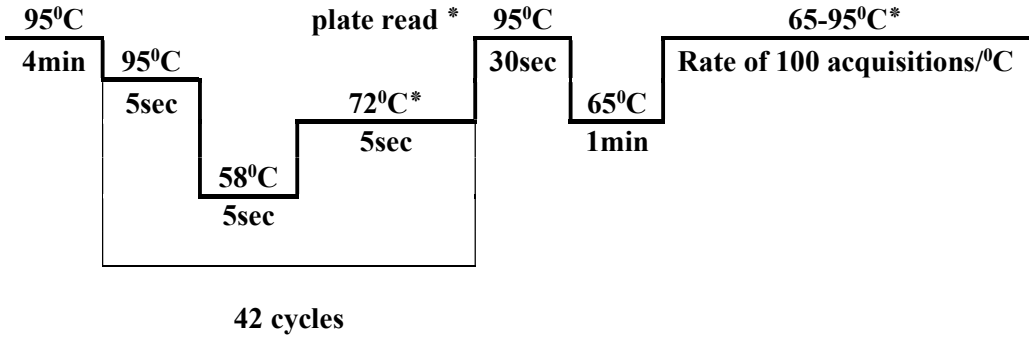
Interpretation of the result: The assay result shows a 218bp product size for SPPV vaccine strain, whereas 302bp product is marked as field isolate of SPPV.

Annex-2. Procedure for HRM assay to differentiate Sheeppox virus vaccine strains from Sheeppox virus field isolates and other Capripoxvirus Species (Chibssa et al., 2019).

1. Master mix preparation for total 10 µl volume PCR reaction.

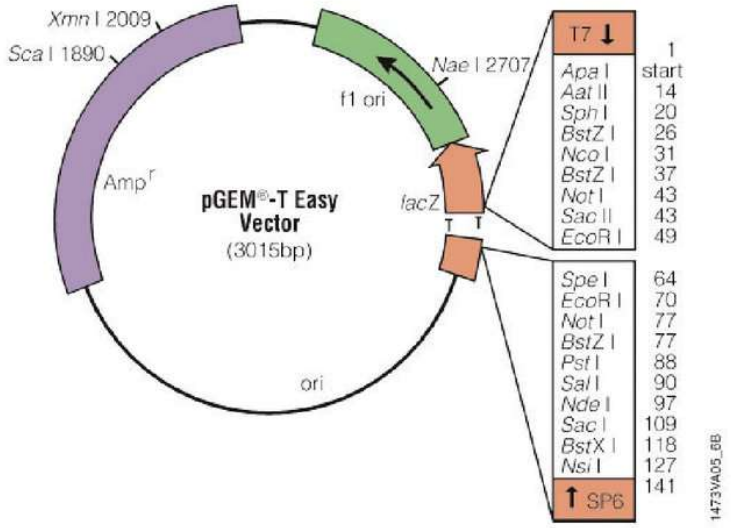
Reagent	Final concentration	Volume per reaction (µl)
Light scanner master mix	1X	4.0 µl
Cap_ B22RDIV_For	250 nM	0.5 µl
Cap_ B22RDIV_Rev	250 nM	0.5 µl
Water		3.0 µl
Template DNA		2

1. Add 8 µl of the master mix to each well in the PCR plate
2. Add 2 µl of template DNA or water for NTC control to the wells as per layout
3. Seal the plate and spin down the contents
4. Place the plate in real-time PCR machine and run the thermal cycler profile



Interpretation of the results: The assay can help to differentiate SPPV vaccine strain from SPPV field isolates and as the same time discriminate CaPVs in to species based on difference in Melting temperature (Tm). Each CaPVs species and vaccine strains acquired Tm values; SPPV vaccine: 76.59–76.88 Tm, SPPV field: 79.87–80.26 Tm, GTPV: 81.39–81.52 Tm and LSDV: 82.07–82.16 Tm values.

Annex-3. pGEM-T Easy vector Circle map and sequence reference points. Used for ligation of a PCR product into the plasmid.



pGEM®-T Easy Vector Sequence reference points:

T7 RNA Polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA Polymerase promoter (–17 to +3)	139–158
SP6 RNA Polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA Polymerase promoter (–17 to +3)	2999-3

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PROFESSIONAL EXPERIENCE

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Asella Regional Veterinary Laboratory, Oromia, Ethiopia

1995- 1999: Team leader of Animal production and health section

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SCIENTIFIC PUBLICATIONS

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