EVALUATION OF VECTOR POTENTIAL OF *RHIPICEPHALUS APPENDICULATUS, AMBLYOMMA HEBRAEUM* AND *RHIPICEPHALUS DECOLORATUS* TICKS FOR LUMPY SKIN DISEASE VIRUS

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ACADEMIC DISSERTATION

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ABSTRACT

Lumpy skin disease (LSD) is a high-impact pox disease of cattle caused by a lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus* within the family *Poxviridae.* The disease is characterized by skin nodules accompanied with high fever, lymphadenopathy, and ulcerative lesions in the mucous membranes of mouth and nasal cavities. Currently LSD is widespread throughout the African continent and in many parts of the Middle East. Between 2013 and 2014, new outbreaks of LSDV were reported from previously disease-free countries such as Turkey, Iraq, Iran, Azerbaijan and Northern Cyprus, raising concerns of further spread of the disease to the Thrace region, Caucasus and the rest of Asia.

The disease is categorized as a notifiable disease by the World Animal Health Organization (Office International des Epizooties, OIE). In the event of an outbreak, notification, control measures, as well as trade of live animals and their products, are regulated by the OIE and the European Union. The disease is of substantial economic impact in endemic countries due to decreased milk and meat production, abortions, temporary or permanent infertility of males and females and damaged hides and skins. Indirect losses are caused by international trade restrictions of live cattle and their products from affected countries, high costs of vaccination campaigns and compulsory limitation of animal movements.

The main mode of transmission of LSDV is mechanical by blood-feeding vectors. Transmission occurs to a lesser extent through the consumption of contaminated feed or water, direct contact with infected animals or via natural mating or artificial insemination. In general, LSDV causes a clinical disease only in cattle and Asian water buffalo while some strains may replicate in sheep and goats. Although some wild ruminants are known to be susceptible for the virus, the role of wildlife in the epidemiology of the disease is not yet known.

This thesis presents the first investigation of the vector potential of hard (ixodid) ticks for LSDV. The general aim of the pilot study was to obtain a proof-of-concept of the hypothesis that hard (ixodid) ticks are able to transmit LSDV via either mechanical, intra/transstadial or transovarial routes. Three common sub-Saharan tick species, *Rhipicephalus appendiculatus*, *Amblyomma hebraeum* and *Rhipicephalus* (originally known as *Boophilus*) *decoloratus* were selected for the study. After tentative evidence of transmission of the virus by tick vectors was obtained, further funding was granted for the CIDLID project, that allowed for a more detailed investigation of the vector capacity and the potential route of transmission of the these ticks. The CIDLID study was conducted as a collaboration project between the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria, South Africa

and The Pirbright Institute, United Kingdom. In the CIDLID project the specific aim of the Pirbright partner was to investigate if mechanical transmission of LSDV occurs by *R. appendiculatus* males and transovarial by *R. decoloratus* females by monitoring viraemia, seroconversion and clinical signs in recipient cattle whereas the DTVD partner investigated the persistence of the virus in tick vectors in detail. An additional objective of the study was to investigate if feeding directly on top of the skin lesions was required or whether feeding on only viraemic animals was sufficient for successful mechanical transmission. The final objective was to investigate if the virus was able to grow *in vitro* in *Rhipicephalus spp.* tick cell lines. In addition, the presence of the virus or viral DNA in ticks collected from naturally infected animals in the field was investigated.

Two animal experiments were included in this study. Fully susceptible, seronegative, donor cattle were experimentally infected with LSDV and laboratory-reared *R. appendiculatus* and *A. hebraeum* nymphs and adults and *R. decoloratus* larvae were placed to feed on the skin of donor animals during the viraemic stage. Semi-engorged *R. appendiculatus* and *A. hebraeum* males were then transferred to feed on naïve recipient animals while nymphs were allowed to moult and then were either tested or placed to feed on recipient cattle. *Rhipicephalus decoloratus* larvae were allowed to develop on donor animals until fully-fed females, which were then harvested for oviposition. Subsequent eggs and larvae were tested and some larvae were placed to feed on naïve recipient cattle. The development of clinical signs, viraemia and seroconversion was closely monitored in recipient animals.

The objectives set for the project were fully obtained. For the first time, transmission of LSDV or any pox virus by hard ticks was demonstrated to occur mechanically by *R. appendiculatus* males and vertically by *R. decoloratus* females. Feeding directly on skin lesions was not necessary for successful transmission of the virus between infected and naïve cattle. However, no evidence of replication of the virus *in vitro* in *Rhipicephalus* tick cell lines was obtained. In addition, the presence of the viral DNA was detected in *Rhipicaphalus*, *Amblyomma* and *Hyalomma* ticks collected during natural LSDV outbreaks in South Africa and Egypt.

In 2014 LSDV seems to be spreading globally at a scale never seen before. In order to be able to curb the spread of the disease, it is essential to understand the role of different arthropod vectors and their importance in the field. Close contact between cattle enhances the possibility of infected male ticks to spread the disease by swapping hosts. The presence of infected tick eggs or different instars in soil and vegetation contaminates the environment and provides a source of infection to domestic cattle and possibly to wild bovines if using same grazing areas.

Treatment of cattle with tick repellents together with vaccination campaigns, using efficient vaccine and sufficient vaccination coverage, form the basis of the control of the disease in the affected regions. The trade conditions set for importation of domestic or wild bovines from infected regions to disease-free countries need to be adjusted to cover potential transmission of the virus from viraemic cattle with subclinical disease by arthropod vectors. Whether the virus actually infects the tick cells and replicates within those cells requires further investigation.

Lumpy skin disease is currently exotic in the United Kingdom and Finland. As importation of live bovines from infected countries is heavily restricted or prohibited, the incursion of LSD is unlikely to occur in either country. As such, LSDV is considered as a potential bioterrorism agent and in theory deliberate release of the virus could occur although this possibility seems negligible. The results obtained in this study will underpin future investigations into the role various arthropod vectors may play for other zoonotic pox diseases, currently occurring in the UK and Finland, such as cowpox, contagious echtyma (orf) or parapox infections of reindeer.

LIST OF ORIGINAL PUBLICATIONS

I.   Tuppurainen E.S.M., Stoltsz W.H., Troskie M., Wallace D.B., Oura C.A.L., Mellor P.S., Coetzer J.A.W. and Venter E.H. (2010). A potential role for ixodid (hard) tick vectors in the transmission of lumpy skin disease virus in cattle. *Transboundary and Emerging Diseases*, 58 (2), 93-104

II. Tuppurainen E.S.M., Lubinga J.C., Stoltsz W.H., Troskie M., Carpenter S.T., Coetzer J.A.W., Venter E.H. and Oura C.A.L. (2013). Mechanical transmission of lumpy skin disease virus by *Rhipicephalus appendiculatus* male ticks. *Epidemiology and Infection*, 141, 2, 425-430

III. Tuppurainen E.S.M., Lubinga J.C., Stoltsz W.H., Troskie M., S.T. Carpenter, Coetzer J.A.W., Venter E.H. and Oura C.A.L. (2013). Evidence of vertical transmission of lumpy skin disease virus in *Rhipicephalus (Boophilus) decoloratus* ticks. *Ticks and Tick-borne Diseases*, 4, 329-333

IV. Tuppurainen E.S.M., Venter E.H., Coetzer J.A.W and Bell-Sakyi L. (2015). Lumpy skin disease: Attempted propagation in tick cell lines and presence of viral DNA in ticks collected from naturally-infected cattle. *Ticks and Tick-borne Diseases*, 6, 134-140

ABBREVIATIONS

CAM	Chorio-allantoic membrane
CIDLID	Combating Infectious Diseases of Livestock for International Development
CPE	Cytopathic effect
CCHFV	Crimean-Congo haemorrhagic fever virus
DIVA	Differentiating Infected from Vaccinated Animals
DPA	Days post-attachment
DPI	Days post-infection
DMEM	Dulbecco's Modified Eagle's Medium
DVTD	Department of Veterinary Tropical Diseases
EV	Extracellular virus
EEV	Extracellular enveloped virion
FBS	Foetal bovine serum
GPCR	G-protein-coupled chemokine receptor
GTPV	Goatpox virus
IEV	Intracellular enveloped virion
EU	European Union
ID	Intradermal
IEIB	Intracytoplasmic eosinophilic inclusion bodies
IMV	Intracellular mature virion
IU	International units
IV	Intravenous
Kbp	Kilo base pair
LSD	Lumpy skin disease
LSDV	Lumpy skin disease virus
LT	Lamb testis
MV	Mature virion
OIE	Office International des Epizooties
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PI	Post-infection
qPCR	Quantitative polymerase chain reaction
RH	Relative humidity

SAT	Saliva–assisted transmission
SNT	Serum neutralisation test
SPPV	Sheeppox virus
TCID$_{50}$	Median tissue culture infective dose
UPBRC	University of Pretoria's Biological Research Unit
VI	Virus isolation

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1. INTRODUCTION

1.1. GENERAL DESCRIPTION OF LUMPY SKIN DISEASE

Lumpy skin disease (LSD) is an economically important poxvirus disease of cattle, occurring across Africa and in the Middle and Near East. The disease is caused by the lumpy skin disease virus (LSDV) which belongs to the genus *Capripoxvirus* within the family *Poxviridae* (Buller et al., 2005). The disease is characterised by pyrexia, generalized skin lesions, internal ulcerative lesions and lymphadenopathy (Haig, 1957, MacOwan, 1959, Weiss, 1968, Woods, 1988). Transmission of LSDV is considered to be mainly mechanical, via insect vectors (Kitching and Mellor, 1986, Weiss, 1968, Chihota et al., 2001). However, transmission may also occur by direct or indirect contact, via contaminated food or water, or via artificial insemination or natural mating (Annandale et al., 2013, Haig, 1957, Weiss, 1968). The disease is classified as a Notifiable Disease by the World Organization for Animal Health (OIE) due to its ability to spread rapidly and internationally, causing substantial economic losses to the cattle farming industry.

The OIE provides recommendations for international trade standards, in their Terrestrial Code (Chapter 11.12) and for diagnostic assays and vaccines, in their Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Chapter 2.4.14). Within the European Union (EU) several legislative acts apply to LSD, covering notification of the disease (82/894/EEC of 21 December 1982), intra-community trade in live animals and their products (90/425/EEC of 26 June 1990) as well as control and eradication measures applied in case of an outbreak within the EU (92/119/EEC of 17 December 1992).

1.2. CLINICAL SIGNS

The clinical signs of the disease have been described in detail by several authors (Babiuk et al., 2008b, Carn and Kitching, 1995a, Coetzer, 2004, Haig, 1957, Prozesky and Barnard, 1982, Tuppurainen et al., 2005, Weiss, 1968). The incubation period in experimentally infected animals varies between 4 and 7 days, while in naturally infected animals it may be up to five weeks (Haig, 1957). The disease starts with lachrymation and nasal discharge. Subscapular and precrural lymph nodes become markedly enlarged. High fever accompanies the appearance of highly characteristic skin lesions of 10-50 mm in diameter. The number of the lesions may vary from a few in mild cases, to multiple lesions, covering the entire body in severely infected individuals (Fig. 1). Necrotic plaques may appear in the mucous membranes of the oral and nasal cavities, causing purulent or mucopurulent nasal discharge and

excessive salivation. Painful ulcerative lesions may appear in the cornea of one or both eyes, leading to blindness in some cases. Pox lesions are found throughout the entire digestive and respiratory tracts and on the surface of almost any internal organ.

Necrotic skin lesions in the legs and on top of the joints may lead to deep subcutaneous infections complicated with secondary bacterial infections and lameness. Pneumonia caused by the virus itself or secondary bacterial infection, is a common complication. Silent subclinical infections are common in the field (Weiss, 1968). In experimentally infected animals approximately one third of the cattle did not show any clinical signs, although all of the infected animals became viraemic (Annandale et al., 2013, Osuagwuh et al., 2007, Tuppurainen et al., 2005, Weiss, 1968). The existence of subclinically infected viraemic animals can complicate control and eradication measures, particularly in countries with limited resources where slaughter of all infected and in-contact animals is not feasible.

Figure 1. Severely infected breeding bull with multiple skin lesions (E. Tuppurainen, The Pirbright Institute)

1.3.   ECONOMIC IMPORTANCE

Lumpy skin disease is a high-impact cattle disease, causing substantial production losses for the cattle industry as a whole. Unfortunately, the poorest small-scale farmers and rural communities, whose livelihood is totally dependent on cattle, bear the heaviest burden during outbreaks. In addition, the disease is a major constraint to international trade of cattle and their products in endemic countries. Costly control and eradication measures, such as vaccination campaigns, as well as the indirect costs due to compulsory limitation of animal movements, cause significant financial losses on a national level (Babiuk et al., 2008a, Tuppurainen and Oura, 2012). General emaciation and a long

convalescence time can cause decreased weight gain in beef cattle. A sharp drop in milk yield is often observed in infected and vaccinated dairy cattle (Abutarbush et al., 2013, Gari et al., 2011, Somasundaram, 2011, Weiss, 1968). Pregnant females may abort due to the viral infection or high fever. Temporary or permanent decrease in infertility in male and female stock has been reported (Ahmed and Zaher, 2008). Sterility may be temporary or permanent in severely infected breeding bulls (Coetzer, 2004). Deep skin lesions can involve all of the layers of the dermis and subcutis, leading to permanent scarring and decreased value of the hides (Green, 1959). Skin lesions and lameness affect the use of oxen for traction power in rural Africa (Gari et al., 2011). Financial costs of an outbreak due to production losses, vaccinations and veterinary treatments were estimated to be USD 6.43 for Zebu and USD 58 per head for Holstein-Friesian cross-bred cattle in Ethiopia (Gari et al., 2011). In a closed 3200 head Holstein dairy cattle farm in the Sultanate of Oman, 40-65% loss of milk production in the herd was reported due to an LSDV outbreak in 2009 (Somasundaram, 2011).

1.4. GEOGRAPHICAL DISTRIBUTION

A cattle disease called pseudo-urticaria, lumpy disease or Ngamiland disease, was known to exist in the central African territories for many years (Thomas and Mare, 1945). The disease was reported for the first time in Northern Rhodesia (Zambia) during 1929 (MacOwan, 1959, Thomas, 1945, Thomas and Mare, 1945). By the 1940s the disease had spread across the southern African countries, affecting large numbers of livestock (Thomas and Mare, 1945). During the following decades, LSD spread slowly northwards and it is currently present throughout virtually the entire African continent, including Madagascar (World Animal Health Information database, OIE WAHID Interface). The only African countries that are still disease-free are Libya, Algeria, Morocco and Tunisia (Fig 2).

The first LSD outbreak in Egypt was reported in May 1988 (Ali and Obeid, 1977) and in August 1989 the disease spread for the first time out of Africa, into Israel (Yeruham et al., 1995). Many Middle Eastern countries import live cattle and frozen meat from the Horn of Africa, where LSD is endemic (Shimshony and Economides, 2006). In 2006-2007 after an apparent absence of 17 years, LSD re-occurred in Egypt and Israel, after being re-introduced into Egypt in cattle imported from the Horn of Africa (El-Kholy et al., 2008).

According to the OIE Wahid and Handistatus II Databases, LSD has been reported in Kuwait (1991 and 2014), Yemen (1995), United Arab Emirates (2000), Bahrain (2009) and Oman (2013). The presence of LSDV in Saudi Arabia (1992) was never confirmed with certainty (Greth et al., 1992). Between 2012 and 2013 several outbreaks of LSD occurred in Israel (ProMed 20120728.1218484), Lebanon (ProMed

20130118.1505118), Jordan (ProMed 20130612.1768278) and the West Bank (ProMed 20130311.1581763). LSD is already endemic in parts of the Middle East (Tageldin et al., 2014). No data is available from Syria, but due to the LSDV outbreaks in the Golan Heights in Israel and southern Lebanon, in close proximity of the Syrian borders, it is highly likely that the disease is also present in the country. Since 2012 control of the disease has been hampered by political unrest and conflicts in parts of the Middle East, followed by increased movement of refugees and livestock, a lack of veterinary infrastructure, under-reporting and a shortage of effective vaccines (Abutarbush et al., 2013). Incursions of the disease into Turkey (ProMed 20130831.1915595) and Iraq (ProMed 20130718.1831781) were reported in late 2013. In July 2014 the disease spread further to Iran (ProMed 20140623.2561202) and Azerbaijan (ProMed 20140719.2621294). In November 2014 LSDV cases were for the first time reported in northern part of Cyprus (ProMed 20141205.3012426). Rapid spread of the disease indicates how challenging it is to effectively control LSD without extensive vaccination campaigns throughout the region. The threat of incursions of the disease into the EU, the Caucasus region and Asia is increasing.

Figure 2. Geographical distribution of lumpy skin disease in November 2014 (OIE Wahid and Handistatus II databases)

1.5.   LUMPY SKIN DISEASE VIRUS

1.5.1.   TAXONOMY

Lumpy skin disease virus belongs to the family *Poxviridae* which is divided into two subfamilies: poxviruses affecting insects (*Entomopoxvirinae*) and vertebrates (*Chordopoxvirinae*) and several genera (Table 1). Within the *Chordopoxvirinae* the genus *Capripoxvirus,* comprises LSDV, sheeppox virus (SPPV) and goatpox virus (GTPV). The prototype of LSDV is Neethling strain which was first isolated in South Africa (Alexander et al., 1957).

Table 1. Genera within the family *Poxviridae* and the subfamily *Chordopoxvirinae* (The International Committee on Taxonomy of Viruses, 2013)

Subfamily *Chordopoxvirinae*	
Genus	Species
Orthopoxvirus	Variola, monkeypox, vaccinia, cowpox, buffalopox, camelpox, ectromelia, raccoonpox, skunkpox, taterapox, and volepox viruses
Capripoxvirus	Sheeppox, goatpox, lumpy skin disease viruses
Parapoxvirus	Pseudocowpox, bovine papular stomatitis, orf virus, parapoxvirus of red deer in New Zealand
Suipoxvirus	Swinepox virus
Avipoxvirus	Fowlpox, canarypox, juncopox, pigeonpox, quailpox, sparrowpox, starlingpox, turkeypox, mynahpox and pcittacinepox viruses
Leporipoxvirus	Hare fibroma, myxoma virus, rabbit fibroma and squirrel fibroma viruses
Molluscipoxvirus	*Molluscum contagiosum* virus
Yatapoxvirusvirus	Tanapox and Yaba monkey tumor viruses
Cervidpoxvirus	Mule deerpox virus
Crocodylidpoxvirus	Nile crocodilepox virus
Unassigned	Squirrelpox virus

Poxviruses are the largest animal viruses; the average size of LSDV is length 294±20 nm and width 262±22 nm (Kitching and Smale, 1986). Poxvirions are brick- or oval-shaped (Fig 3). The morphology of the viruses of the genus C*hordopoxvirus* are similar with the exception of the parapoxviruses. Within the virion, there are over 100 polypeptides, which are arranged in a core, two lateral bodies, an outer membrane and an envelope. The outer membrane and the envelope interact with the host cell. The core of the virus is dumbbell-shaped and the nature of lateral bodies is unknown. The core of the viruses contains proteins that include a transcriptase and several other enzymes (Fenner et al., 1987).

All vertebrate poxviruses share a group-specific antigen (NP antigen) (Woodroofe and Fenner, 1962). Poxviruses exist in the intracellular space, with or without an envelope and are enveloped in the extracellular space (Fenner et al., 1987). Both forms are infectious and have the same core and genetic material. "Mature virions" (MV) (Moss, 2006) also called "intracellular mature virions" (Fenner et al., 1987) are surrounded by a single lipid membrane with irregular arrangements of tubular proteins on the surface. These are the most abundant form of the virus and are believed to be responsible of host-to-host spread. Intracellular enveloped virions (IEV) (Fenner et al., 1987), more recently referred as "wrapped virions" (Moss, 2006) develop from MV, surrounded by two additional layers of membrane, originating from the trans-Golgi apparatus or endoplasmic network. While budding out, the outmost layer of wrapped virions fuses with the plasma membrane, releasing extracellular enveloped viruses (EV) (Fenner et al., 1987).
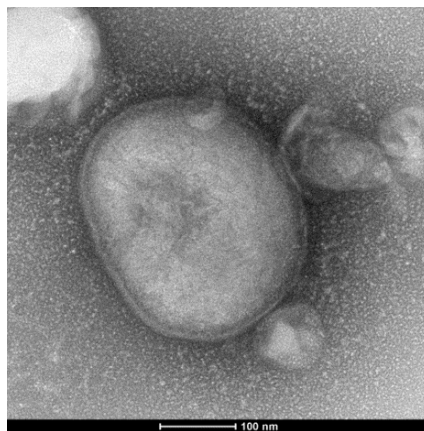
Figure 3. Electron micrograph of a lumpy skin disease virus particle prepared by conventional negative staining method (P. Hawes & E. Tuppurainen, The Pirbright Institute)

1.5.3. GENOME

Lumpy skin disease virus is a double-stranded DNA virus (Weiss, 1968). The size of the LSDV genome is 151 kbp (Tulman et al., 2001). The genome consists of a central coding region with identical 2.4 kbp inverted terminal repeats and 156 putative genes. The genes encoding proteins involved in determination of host range, virulence and immune evasion are located in the near terminal regions of the genome (Tulman et al., 2001). DNA analyses using restriction endonucleases on both field samples and vaccine strains, showed 80 % homology between strains of capripoxviruses (Black et al., 1986). The genomes of SPPV and GTPV are very similar to that of LSDV, sharing 96% nucleotide identity within the genus *Capripoxvirus* (Tulman et al., 2002).

However, molecular studies have demonstrated that LSDV, SPPV and GTPV are phylogenetically distinct (Tulman et al., 2001, Tulman et al., 2002) (Fig 4). Based on these sequencing studies, virus-species-specific molecular assays have recently been developed targeting the host-specific G-protein-coupled chemokine receptor (GPCR), or the 30 kDa RNA polymerase subunit (RPO30) genes, enabling differentiation and phylogenetic grouping of the different capripoxviruses (Lamien et al., 2011a, Lamien et al., 2011b, Le Goff et al., 2005, Le Goff et al., 2009).
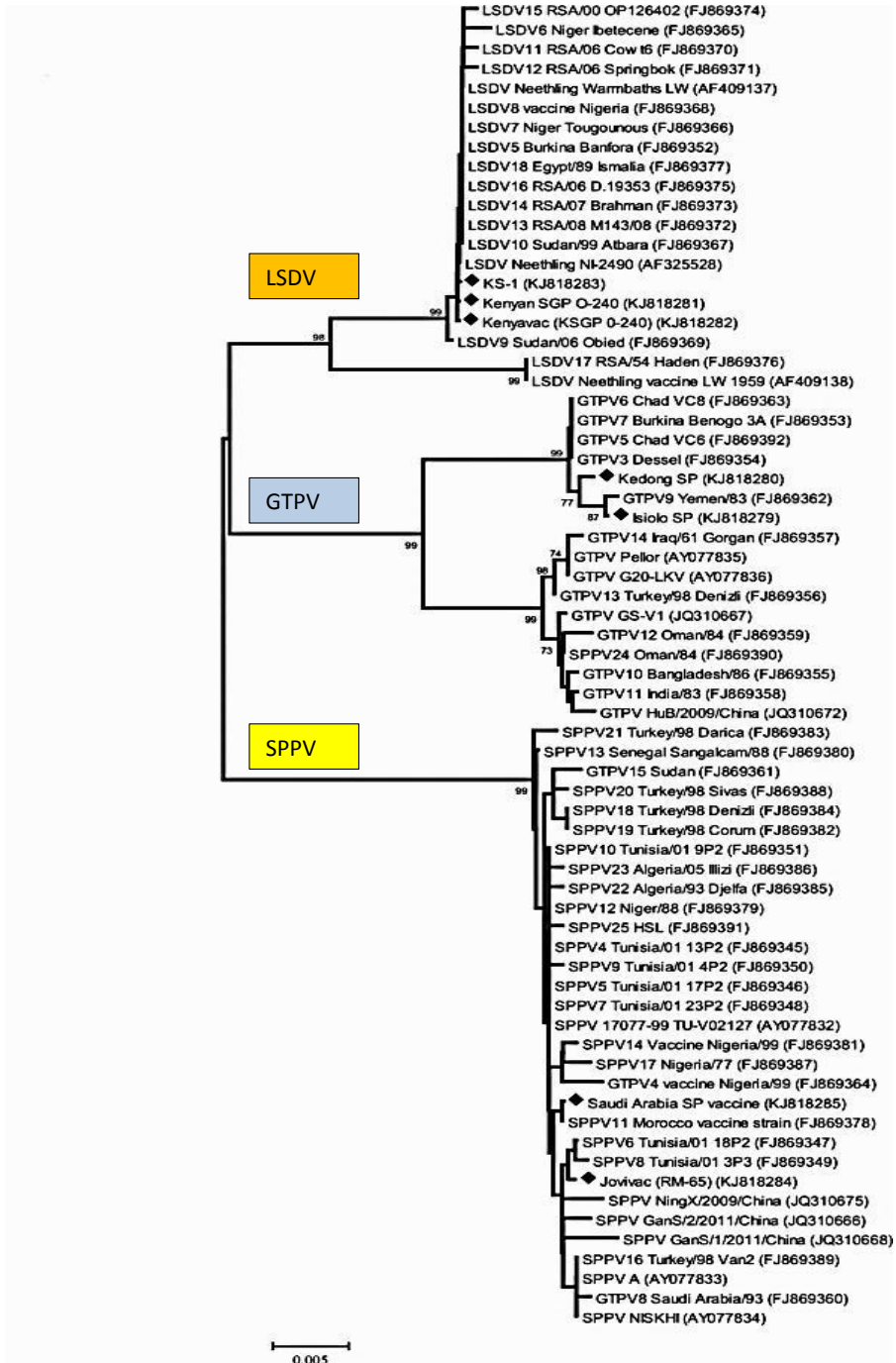
Figure 4. Molecular phylogenetic analysis of capripoxvirus G-protein-coupled chemokine receptor (GPCR) gene (Tuppurainen et al., 2014)

1.5.4.   REPLICATION IN A HOST CELL

The replication of LSDV occurs in the cytoplasm of the host cell, within intracytoplasmic eosinophilic inclusion bodies (IEIB), that can be detected using microscopic examination of haematoxylin and eosin stained infected cell monolayers (Alexander et al., 1957, Prozesky and Barnard, 1982, Prydie and Coackley, 1959). These IEIBs are round or irregularly shaped and there may be one or several within a single cell (Weiss, 1968).

Recent studies have demonstrated that the MV enters the host cell by macropinocytosis. The process resembles the uptake of apoptotic cell debris by phagocytotic cells and is triggered by the viral surface protein phosphatidylserine. Initiation of the process requires activation of the cellular p21-activated kinase1 (PAK-1) by the virus (Mercer and Helenius, 2008). In contrast, enveloped virus enters the host cell by endocytosis. Inside the endocytic vesicle the envelope is lysed, releasing MV. The core of the virus is then released into the cytoplasm of the host cell by the fusion of the outer membrane of MV with the vesicle membrane (Fenner et al., 1987, Moss, 2006).

The replication of a poxvirus occurs in three phases. In the early phase, the replication of the virus is initiated by release of the transcriptase enzyme from the core of the virion into the cell cytoplasm. During the first uncoating, the envelope and outer membrane of the virus are lost and the core is released into the cytoplasm. Then the transcription of viral mRNA starts, resulting in the synthesis of polypeptides for the second uncoating of the core (Buller and Palumbo, 1991). During an 'intermediate' phase the synthesis of viral DNA, copied from the parental virus genome, begins. In the final stage, which starts within 2 to 48 hours after infection, the late viral genes are transcribed, allowing the synthesis of new virus structural proteins and assembly of virus particles (Fenner et al., 1987).

Infective MVs are released from the host cells by cell eruption (Fenner et al., 1987). The majority of extracellular EVs remain attached to the host cell surface and can mediate efficient cell-to cell spread of the virus. The rest of EVs are free in the extracellular space and are responsible of long-range dissemination of the virus within the host (Fenner et al., 1987).

1.5.5.   PROPAGATION OF THE VIRUS *IN VITRO*

Capripoxviruses grow slowly in cell cultures and may require several passages. They grow on a wide variety of bovine and ovine cells, causing easily recognizable cytopathic effects (CPE) on cell monolayers (Alexander et al., 1957, Munz and Owen, 1966, Prydie and Coackley, 1959). Primary lamb

testis (LT) and bovine dermis cells, or a commercially available LT cell line (OA3.Ts), are the most commonly used cells for the propagation of LSDV (Babiuk et al., 2007). In addition, LSDV can be cultured in lamb and calf kidney cells, calf testis cells, sheep kidney cells, lamb and or calf adrenal or thyroid cultures, foetal lamb and calf muscle cells, sheep embryonic kidney or lung cells, rabbit foetal kidney or skin cells, chicken embryo fibroblasts, adult vervet monkey kidney cell line (AVK 58), equine lung and baby hamster kidney cells (BHK/21) (Alexander et al., 1957, Prydie and Coackley, 1959, Weiss, 1968). The virus can also be propagated in the chorioallantoic membranes (CAM) of embryonated chicken eggs, causing macroscopic pock lesions (Alexander et al., 1957, Van Rooyen et al., 1969). Generalized skin lesions can also be detected in LSDV infected rabbits (Alexander et al., 1957).

1.5.6. PERSISTENCE AND STABILITY OF THE VIRUS

Lumpy skin disease virus is remarkably stable (Weiss, 1968). In the environment LSDV is susceptible to direct sunlight, but remains well-protected and viable inside dried scabs which develop on top of the skin lesions and are shed by infected cattle for at least 3 month (Davies and Otema, 1981). The virus persists in unclean shaded pens for as long as 6 months (OIE Manual, LSD Chapter 2.4.14, 2008). The virus survives freezing and thawing well, but infectivity may be reduced (Haig, 1957).

A rise in body temperature indicates the start of the viraemic stage, which may persist for two weeks (Tuppurainen et al., 2005). In infected animals the level of viraemia is usually low and it is difficult to isolate live virus from blood samples. The titre of the virus, particularly in animals showing mild clinical signs is often below the detection level of virus titration method and no data on viral titres in blood samples collected from LSDV viraemic cattle have yet been published.

The first skin lesions start to appear at the onset of fever. High virus titres (5.1-5.3 (Log_{10}) plague forming units mg^{-1}) have been demonstrated in the skin lesions (Babiuk et al., 2008b). Live virus has been isolated for up to 39 days post-infection (dpi) from the skin lesions of convalescent cattle. Using conventional polymerase chain reaction (PCR) methods, viral DNA was detected until 92 dpi (Tuppurainen et al., 2005). The virus was recovered from infected tissue culture fluid kept at 4 °C for 6 months and from intact skin nodules that had been kept at –80 °C degrees for 10 years. (Weiss, 1968). The virus remains viable for 18 days in the skin lesions and superficial epidermal scrapings from air-dried hides kept at room temperature (Weiss, 1968). Live LSDV was demonstrated in saliva and nasal discharge from experimentally infected cattle, 11 days after the onset of fever (Babiuk et al., 2008b). Live virus was also isolated 42 dpi from semen samples from experimentally infected bulls (Irons et al., 2005).

The virus is stable between pH 6.6 and 8.6, but is readily inactivated by the detergent sodium dodecyl-sulphate and it is chloroform and 20% ether sensitive (Plowright and Ferris, 1959, Weiss, 1968). The virus is inactivated at 56°C degrees for 2 hours and at 65°C degrees for 30 minutes. Common disinfectants such as 2% phenol, 2–3% sodium hypochlorite, iodine compounds in 1:33 dilution, 2% Virkon® and 0.5% quaternary ammonium compounds can be used for disinfection against LSDV (OIE, Lumpy skin disease, Technical Disease Card).

1.6. IMMUNITY

Immunity against LSDV depends on the virulence of the virus, as well as the immune status, age and breed of the host. A natural resistance to infection, not associated with immunity, is known to occur in cattle (Weiss, 1968). Only 40-50 % of cattle, experimentally infected with LSDV are likely to develop generalized skin lesions. The remaining animals either show only a localized and circumscribed painful swelling at the inoculation site of LSD virus, or no clinical signs apart from a mild fever reaction (Annandale et al., 2010, Osuagwuh et al., 2007, Tuppurainen et al., 2005, Weiss, 1968).

Immunity against capripoxvirus infections is predominantly cell-mediated and requires a replicating agent in order to be effectively stimulated (Carn, 1993). Most progeny viruses remain inside infected cells, with the exception of the enveloped poxviruses, which are released into the blood (Boulter and Appleyard, 1973). By spreading directly from cell to cell, the virus is out of reach of circulating antibodies, which are able to limit the spread of the virus in experimental animals, but do not prevent replication of the virus at the site of infection (Kitching, 1986b).

Due to cross-neutralization between different LSDV isolates, it is believed that there is only one immunological type of the virus (Weiss, 1968). The capripox virion contains numerous antigens, most of which are shared by all the members of the genus *Capripoxvirus* (Fenner et al., 1987). There are morphological and antigenic similarities between SPPV, GTPV and LSDV (Kitching and Smale, 1986). All these viruses share a common major antigen for neutralizing antibodies and animals recovered from infection by one capripoxvirus are believed to be protected from infection by another (Coackley and Capstick, 1961). However, current experience obtained from the most recent outbreaks of LSDV in the Middle East and the Horn of Africa indicate that cross-protection is only partial (Ayelet et al., 2013, Khalafalla et al., 1993, Somasundaram, 2011, Tageldin et al., 2014, Yeruham et al., 1994).

Animals recovered from apparent or inapparent natural infection with LSDV, develop antibodies capable of neutralizing up to 3 log TCID50/ml of the virus and are resistant to reinfection (Weiss, 1968).

Animals that have been vaccinated or showed mild disease may develop only low levels of neutralizing antibodies (Kitching and Hammond, 1992) which may be below the detection limits of currently available serological tests, even though these animals are still resistant to challenge (Weiss, 1968). In addition, different SPPV, GTPV and LSDV strains are not distinguishable by serological assays, that include serum/virus neutralization (SNT), fluorescent antibody, indirect fluorescent antibody or agar gel immunodiffusion tests (Davies and Otema, 1981).

In general, the immune status of a previously infected or vaccinated animal cannot be related directly to serum levels of neutralizing antibodies (Kitching et al., 1986). After vaccination, immunity against LSDV may persist for at least 2-3 years although annual vaccinations are recommended by the vaccine manufacturers. Antibodies appear 15 days after vaccination and reach the highest level 30 days post-inoculation. Calves born to immunized cows will have passive immunity that persists for about 6 months (Weiss, 1968).

1.7. EPIDEMIOLOGY

The morbidity (5 to 45%) and mortality (< 10%) rates in endemic countries are usually relatively low, but they can be considerably higher (morbidity up to 100%) during incursions of the disease into previously disease-free areas with highly susceptible European breed of cattle (Coetzer, 2004). Typically, in endemic regions LSD outbreaks occur in epidemics with the quiescent periods between outbreaks lasting several years (Davies, 1991b). The presence of high numbers of susceptible animals, in combination with an abundance of arthropod vectors and uncontrolled animal movements, creates optimal conditions for the spread of LSDV. During the first LSD outbreaks in southern Africa it was observed that isolated outbreaks occurred in widely-scattered herds in the absence of cattle movements. These outbreaks were associated with wet and warm weather conditions, with an abundance of blood-feeding arthropod populations, and it was not possible to control the spread of the disease effectively by quarantine measures (Thomas and Mare, 1945, Weiss, 1968).

Thin-skinned *Bos taurus* breeds are highly susceptible against LSDV, whereas indigenous (*Bos indicus*) breeds such as zebu and zebu hybrids are likely to have some innate immunity against the virus (Davies, 1991b, Gari et al., 2011, Tageldin et al., 2014). The disease affects cattle of both sexes and all ages but it tends to be more severe in milking cows, during the peak of lactation and in young animals (Gari et al., 2011, Tageldin et al., 2014). The disease is a major impediment to the development of intensive dairy and meat production in Africa, due to the high susceptibility of high-producing European dairy and beef cattle.

In general, capripoxviruses are host-specific, with only a few known exceptions, such as the KSGP O-240 strain (which has recently been identified as a LSDV) as well as Isiolo and Kedong GTPV strains (Tulman et al., 2002, Capstick, 1959, Coackley and Capstick, 1961). Very few data are available concerning the susceptibility of wild ruminants to LSDV, or on the role of wildlife as potential reservoirs of the virus. Natural infections have been reported in Asian water buffalo (*Bubalus bubalis*), but morbidity is significantly lower in buffalo (1.6%) than in cattle (30.8%) (Ali et al., 1990, El-Nahas et al., 2011). Clinical signs of LSD have been demonstrated after experimental inoculation with LSDV, in impala (*Aepyceros melampus*) and giraffe (*Giraffa camelopardalis*) (Young et al., 1970). Lumpy skin disease was also reported in an Arabian oryx (*Oryx leucoryx*) in Saudi Arabia (Greth et al., 1992). Capripoxvirus was identified using electron microscopy, in skin nodules of the oryx and raised antibody levels against capripoxvirus were detected in paired serum samples using a neutralization test (Greth et al., 1992). However, whether the disease was actually caused by LSDV or SPPV was never confirmed. More recently, live virus was isolated and the presence of LSDV nucleic acid was reported in skin samples collected from springbok (*Antidorcas marsupialis*) in South Africa (Lamien et al., 2011a, Le Goff et al., 2005).

Hardly any data exist on the susceptibility of rodents for capripoxviruses, although some information does exist for other poxviruses, such as zoonotic cowpoxvirus within the genus *Orthopoxvirus* infecting rodents, particularly ground squirrels, gerbils, voles and woodmice and occasionally cattle or cats (Chantrey et al., 1999).

The presence of antibodies in an animal species indicates its susceptibility to the virus and its potential involvement in the epidemiology of the disease (Barnard, 1997). However, antibody-positive animals do not necessarily generate a productive infection and may not be able to transmit virus. Antibodies against capripoxvirus have been detected in blue wildebeest (*Connochaetes taurinus*), black wildebeest (*Connochaetes gnu*), springbok, eland (*Taurotragus oryx*) and impala (Barnard, 1997). Seroprevalence varied from 10 to 27%, averaging 17% in a grassland and 33% in a forest transition environment (Barnard, 1997). Antibodies were also detected in serum samples collected from African buffalo (*Syncerus caffer*) in Kenya (Davies, 1982). In another study low levels of antibodies were detected in kudu (*Tragelaphus strepsiceros*), two waterbuck species (*Kobus ellipsiprymnus* and *Kobus defassa*), reedbuck (*Redunca arundinum*), impala, springbok and giraffe, leading to the conclusion that the samples may have contained non-specific virus inhibitors (Hedger and Hamblin, 1983). However, the antibody titres in the giraffe and reedbuck samples were as high as in convalescent cattle, suggesting potential infection in the past (Hedger and Hamblin, 1983).

Animals with mild or inapparent infections with LSDV do not always show antibody levels that are detectable in a neutralization assay. It is therefore possible that the number of LSDV-infected wild ruminants may be considerably higher than revealed by this test. Wild animals showing clinical signs of LSD are likely to be more susceptible to predators which could explain the lack of reports of clinical disease in wildlife species. Due to the natural selection wild ruminants may be genetically more resistant and rapidly clear the infection. Also the presence of clinical signs of LSD in wildlife is easily missed as the monitoring of skin lesions is difficult or impossible, especially in mild cases (Barnard, 1997).

1.8. DISEASE CONTROL BY VACCINATION

Due to the presence of the virus in the environment and in arthropod vectors, once the disease has arrived into a country it is very difficult if not impossible to eradicate it from domestic cattle without vaccination. For a vector-borne disease like LSD, immunization of cattle using effective vaccines, creating and maintaining sufficient (at least 80%) herd immunity, is considered essential for successful control of the disease. Attenuated LSDV and SPPV vaccines can be and are currently used to protect cattle against LSDV in endemic regions (Coakley and Capstick, 1961, Kitching, 1983). The use of an SPP vaccine against LSDV has been restricted to those countries where SPP and GTP are also endemic, such as the Middle East, Turkey and Iraq. However, vaccination using an homologous vaccine is generally recommended for capripox diseases (Bhanuprakash et al., 2010). This is supported by experience from field outbreaks, including the 2012-2013 LSDV outbreak in Israel, which indicated the superiority of LSDV vaccines (compared to SPPV vaccines) for cattle against LSDV (Dr N. Galon, chief veterinary officer, Israel, personal communication). However, no inactivated LSD vaccines are currently commercially available.

The use of live attenuated LSDV vaccines is not recommended in countries that were previously disease-free. However, during LSDV outbreaks in Egypt and Israel (in 2006-2007 and 2012-2013), cattle were vaccinated with an SPP vaccine, as the same vaccine was already being used in the area to protect sheep against SPP (Brenner et al., 2009). During the 2006 outbreak of LSD in Egypt it was reported that the live attenuated SPPV vaccine (comprising Kenyan sheep and goat pox virus, O-240 strain) did not provide cattle with complete protection against LSD (Tuppurainen, 2006). Incomplete protection was also observed when the Yugoslavian RM65 (Ramyar) SPP vaccine was used to vaccinate cattle against LSD in Israel from 2006 to 2007 (Brenner et al., 2009). A retrospective study carried out by Brenner et al. (2009), involving 4607 vaccinated cattle showed that the number of clinical LSD cases

was 5 times greater in unvaccinated compared to vaccinated herds. This demonstrated that increased levels of protection were indeed achieved in vaccinated animals, as compared to those not vaccinated. However, 11.1% of the vaccinated animals still developed cutaneous lesions after exposure to LSDV in the field. Skin nodules collected from these animals tested LSDV-positive using PCR methods that differentiated between SPPV and LSDV (Brenner et al., 2009, Stram et al., 2008). The authors were therefore able to exclude the possibility that the vaccine virus itself was responsible for the skin lesions.

It is well known that during vaccination campaigns not all animals will necessarily develop absolute protective immunity against LSDV. Several factors have been reported to contribute to real or apparent vaccine breakdown (Carn, 1993, Kitching, 2003). Cattle may be incubating the disease when vaccinated, or some animals may be "missed" during a vaccination campaign. If proper needle hygiene is not practised, needles or diluents contaminated with virulent LSDV during the actual vaccination procedure may also transmit the virus. Inappropriate storage of vaccine or a failure in one or more steps of the cold-chain can occur, or vaccine may be inactivated due to exposure to direct sunlight or high environmental temperatures during the vaccination process. In some cases vaccine may be poorly administrated, or an incorrect dosage used. Also maternally-derived antibodies are known to cause interference in the development of active immunity in calves up to six months of age, so calves vaccinated before 6 months of age that were born to naturally infected or vaccinated dams, may not be protected (Kitching, 2003).

Live attenuated vaccines may cause adverse reactions, such as a drop in milk yield, relatively large local reaction at the vaccination site or even a mild generalized disease in some animals. During quiescent periods between outbreaks and without a threat of a new outbreak, farmers have not been willing to vaccinate their cattle, leading to a low overall herd immunity against LSDV (Hunter and Wallace, 2001).

Although vaccination may not result in a complete protection against the disease in each vaccinated animal, it is currently the only effective way to control the spread of LSDV. In non-endemic regions the use of live attenuated vaccines could, however, compromise the disease-free status of the country, and would be highly questionable on the grounds of safety. In addition, the use of genetically modified recombinant live LSDV vaccines may not be permitted. The use of inactivated vaccines could be considered as a short term solution in an emergency (Tuppurainen and Oura, 2012); however the protection provided by inactivated vaccines is believed to be not solid and is only short-lived (Kitching, 1983). It is not possible to differentiate infected from vaccinated animals using currently available tests. The heightened risk of LSD spreading from the Middle East to the rest of Asia, or to Europe,

underlines the need for the development of a DIVA (Differentiating Infected from Vaccinated Animals) vaccine and associated diagnostic tests for all capripoxviruses (Tuppurainen and Oura, 2012).

Experimentally, LSDV has been successfully used as a backbone for several recombinant subunit vaccines, such as rabies (Aspden et al., 2002), rinderpest (Ngichabe et al., 1999, Ngichabe et al., 1997), Rift Valley fever (Wallace et al., 2006) and HIV (Burgers et al., 2014, Shen et al., 2008, Shen et al., 2011).

1.9.   TRANSMISSION

1.9.1.   DIRECT AND INDIRECT CONTACT

Although transmission of LSDV by direct contact does occur, it is not believed to be the main route of transmission (Haig, 1957, Henning, 1956). Deliberate attempts to transmit LSDV via manual handling of infected animals, immediately prior to contact of the handler with susceptible cattle, or keeping naïve and infected animals in the same pen, both failed to transmit infection (Weiss, 1968). Therefore it was concluded that direct or indirect contact between infected and susceptible animals is an inefficient method of transmission (Carn and Kitching, 1995a, Weiss, 1968). However, successful transmission was achieved when naïve animals were allowed to share a drinking trough with severely infected animals (Haig, 1957). Although transmission via direct contact is not considered to be important, the most recent studies have shown that infected animals start to excrete the virus in saliva, eye and nasal discharges soon after the onset of clinical signs (Babiuk et al., 2008b). Consequently they may become infectious in the early stages of the disease. Highly sensitive molecular diagnostic tools were not available when the earlier transmission experiments were conducted and thus further studies, using the improved diagnostic techniques are needed to re-investigate transmission by direct contact. Sucking calves may become infected via the skin of infected teats and infected milk. Transplacental transmission of LSDV can occur and infected cows are known to give birth to calves with skin lesions (OIE Manual, Lumpy skin disease-Chapter 2.4.14).

1.9.2.   SEMINAL TRANSMISSION

Lumpy skin disease virus has been isolated from the semen of experimentally infected bulls for 22 dpi (Weiss, 1968). A more recent study demonstrated the persistence of live virus in bovine semen for up to 42 dpi and viral DNA was detected until 159 dpi (Irons et al., 2005). In both studies the virus was

isolated from the semen of bulls with no apparent disease. The epididymis and testis were identified as the sites of persistence of LSDV and viral DNA was detected in all fractions of semen (Annandale et al., 2010). Vaccination of bulls with the South African live attenuated Neethling strain prevented shedding of LSDV in the semen of animals subsequently challenged with LSDV, and vaccinated animals did not shed vaccine virus in their semen (Osuagwuh et al., 2007). Transmission of LSDV via artificial insemination has recently been experimentally demonstrated (Annandale et al., 2013).

1.9.3. ARTHROPOD TRANSMISSION

During the first LSD outbreaks in southern Africa it was observed that isolated outbreaks occurred in widely-scattered herds, in the absence of cattle movements. These outbreaks were associated with wet and warm weather conditions with an abundance of a variety of blood-feeding insect populations, and it was not possible to control the spread of the disease effectively by quarantine measures alone (Thomas, 1945, Thomas and Mare, 1945, Weiss, 1968). Transmission of LSDV is believed to occur mainly mechanically by biting arthropods (Chihota et al., 2001, Chihota et al., 2003, Kitching and Mellor, 1986, Weiss, 1968). Female *Aedes aegypti* (L.) mosquitoes were shown to transmit LSDV from infected to susceptible cattle for 2 to 6 days post-feeding on experimentally infected animals (Chihota et al., 2001). An intravenous mode of feeding makes these mosquitos ideal candidates for mechanical transmission. Experimentally, stable flies (*Stomoxys calcitrans*) are able to mechanically transmit SPPV between sheep (Mellor et al., 1987) and live LSDV has been isolated from stable flies after feeding on infected cattle (Weiss, 1968). However, attempts to transmit LSDV between experimentally infected and naïve cattle, by *Stomoxys calcitrans* failed (Chihota et al., 2003), as did attempts to transmit LSDV via adults of two mosquito species (*Anopheles stephensi* and *Culex quinquefasciatus*), or a biting midge (*Culicoides nubeculosus*) (Chihota et al., 2003). Attempts to isolate LSDV from ticks collected from infected animals during the early outbreaks in South Africa were unsuccessful *(*Weiss, 1968*)*.

Camelpox virus belongs to the genus *Orthopoxvirus* within the family *Poxviridae* (Buller et al., 2005). The clinical signs and epidemiology of camelpox are very similar to those caused by capripoxviruses. Wernery et al (1997) were able to isolate camelpox virus from camel ticks (*Hyalomma dromedarii*) on cell culture, the virus was initially detected by electron microscopy in ticks collected from infected camels (Wernery et al., 1997). This study was the first report concerning the vector capacity of three common sub-Saharan hard ticks *Rhipicephalus appediculatus, Amblyomma hebraeum* and *Rhipicephalus decoloratus*.

1.10. IXODID (HARD) TICKS

1.10.1. TICK TAXONOMY

Rhipicephalus appendiculatus, *R. decoloratus* and *A. hebraeum* species are classified in the hard tick family *Ixodidae* within the suborder *Ixodida* and the class *Arachnida*

Table 2. Taxonomy of ticks

Kingdom	*Animalia*		
Phylum	*Arthropoda* (ticks, mites, spiders, insects, crustaceans and others)		
Class	*Arachnida* (ticks, mites, spiders, scorpions and others)		
Subclass	*Acari* (ticks and mites)		
Order	*Parasitiformes Leach, 1815*		
Suborder	*Ixodida Leach, 1815*		
Families	*Ixodidae* (hard ticks)	*Argasidae* (soft ticks)	*Nuttalliellidae*

1.10.2. TICK SPECIES

1.10.2.1. *RHIPICEPHALUS APPENDICULATUS*

Three-host *Rhipicephalus appendiculatus* ticks are widespread and abundant in Africa and have been implicated in the transmission of many livestock pathogens such as *Theileria* spp. (East Coast fever, Corridor disease, and Zimbabwe theileriosis), *Ehrlichia bovis* (bovine ehrlichiosis), *Rickettsia conori* and Nairobi sheep disease virus (Norval and Horak, 2004). Each instar feeds on a different host, then drops off the host and develops to the next life cycle stage, or lays eggs, in a suitable site on the ground. Emerging larvae, nymphs or adults climb vegetation of a suitable height and wait until a new host appears.

In the subtropical, central and southern regions of Africa the occurrence of different *R. appendiculatus* life stages is seasonal: most adult ticks are found from mid to late summer. *R. appendiculatus* species feed on the ears of the host where the skin is thinner than in other parts of the body, which allows them to feed in very close proximity to blood vessels. Adult *R. appendiculatus* ticks prefer to feed on

large and medium-sized ruminants, while in addition to feeding on most domestic and wild ruminants, larvae and nymphs, also feed on small mammals, birds and tortoises (Norval and Horak, 2004).

1.10.2.2. *RHIPICEPHALUS DECOLORATUS*

One-host ticks *R. decoloratus* (known originally as *Boophilus decoloratus)* are common in southern, western, eastern and central Africa, infesting mainly cattle but also small ruminants and some wild ungulates. The life cycle of this tick species is short (21 to 23 days on the host and approximately five weeks off the host) (Arthur and Londt, 1973). Low winter temperatures synchronize egg development and hatching, causing an abundance of larvae when the weather starts to warm up at the beginning of the summer season (Norval and Horak, 2004). *R. decoloratus* ticks are known to transmit anaplasmosis (*Anaplasma marginale*), bovine babesiosis/African redwater (*Babesia bigemina*) and borreliosis (*Borrelia theileri*) (Norval and Horak, 2004). *R. decoloratus* larvae ambush passing cattle from vegetation of appropriate height and are attracted by different stimulants such as odour, CO_2 or vibrations. *R. decoloratus* ticks can only transmit the pathogen via larvae, originating from infected females (Norval and Horak, 2004).

1.10.2.3. *AMBLYOMMA HEBRAEUM*

Amblyomma hebraeum is a three-host tick, common in south eastern Africa. Adults are large with long mouthparts. Adults feed on larger wild and domestic ruminants, whereas their larvae and nymphs parasitize several species of large and small mammals, birds and tortoises (Norval and Horak, 2004). Adults become active in early summer and autumn, while larvae are most active in late summer, although all life cycle stages may be found on hosts throughout the year. Moulting and questing may take several months (Norval and Horak, 2004). Instead of waiting, *A. hebraeum* nymphs and newly hatched adults actively seek hosts to feed on. Over-wintering occurs as the nymphal stage and is regulated by environmental factors, such as temperature and humidity. *A. hebraeum* is known to transmit the causative organism of heartwater and bovine theileriosis (Norval and Horak, 2004).

1.10.3. TICK MORPHOLOGY

The morphology of ticks has been described by several researchers and is reviewed by Sonenshine (1991). The body of an ixodid tick is divided into two functional units, referred as the idiosoma and gnathosoma. The idiosoma is dorsally covered by scutum in males, although in females and immature ticks the scutum covers only a small anterior part the dorsal side of the tick and the rest of the cuticle is only weakly sclerotized. This allows substantial enlargement of females, nymphs and larvae during the final steps of feeding. The genital openings on the ventral side of the tick are detected only in adults. Larvae have three pairs of legs, while nymphs and adults are eight-legged (Sonenshine, 1991)(Fig. 5).

The gnathosoma (capitulum) includes the mouthparts, mounted on the basis capituli and comprising the paired chelicerae (cutting organ), two segmented palps, and the ventrally situated, toothed hypostome (the attachment organ) (Sonenshine, 1991) (Fig. 5).

Digestive tract, respiratory, reproductive and central nervous organs and fat body are inside the body cavity (haemocoel). The digestive system comprises the preoral canal, which includes the pharynx, oesophagus and salivary glands. The salivary glands are grape-like organs on both lateral sides of the body cavity. The salivary glands play a crucial role on excretion of excess water and salts from the ingested blood meal back to the host. Anticoagulants, vasodilators and immunosuppressants present in tick saliva are essential for successful feeding. Salivary glands also produce cement-like substances that anchor the tick mouthparts to the feeding site on the host (Sonenshine, 1991).

The digestion of the blood meal occurs inside the endothelial cells of the midgut wall. The hindgut includes the rectal sack, to where excess nitrogenous waste is eliminated via Malpighian tubes (Sonenshine, 1991).

The male reproductive system includes paired testes, vasa deferentia and seminal vesicle, ejaculatory duct and genital accessory glands. The female reproductive system comprises two ovaries and oviducts, uterus and connecting tube. The female genital aperture is connected with the uterus by cervical and vestibular vagina. Interestingly, above the cervical vagina lies a seminal receptacle, where the male tick inserts the sperm package (spermatophore) with its mouthparts during copulation (Sonenshine, 1991).

The tick respiratory organs include small tracheal tubes, spiracles that are connected to spiracular plates on the outer surface of the tick. An open circulatory system includes heart, aorta and short arteries leading to main organs, muscles, tissues, mouthparts and legs. Haemolymph enters the heart after filtering through the wall of the pericardial sinus which surrounds the heart (Sonenshine, 1991).

The central nervous system includes synganglion, located to the anterior ventral region of haemocoel. Peripheral nerves lead to mouthparts, legs, muscles and different organs. Peripheral sensory organs provide information from the environment (Sonenshine, 1991).

The fat body is the major source of vitallogenin, which is essential for egg production in females. The organ has been compared to the liver in mammals, due to its function as a storage site for food reserves, waste detoxifier and hormone producer (Sonenshine, 1991).
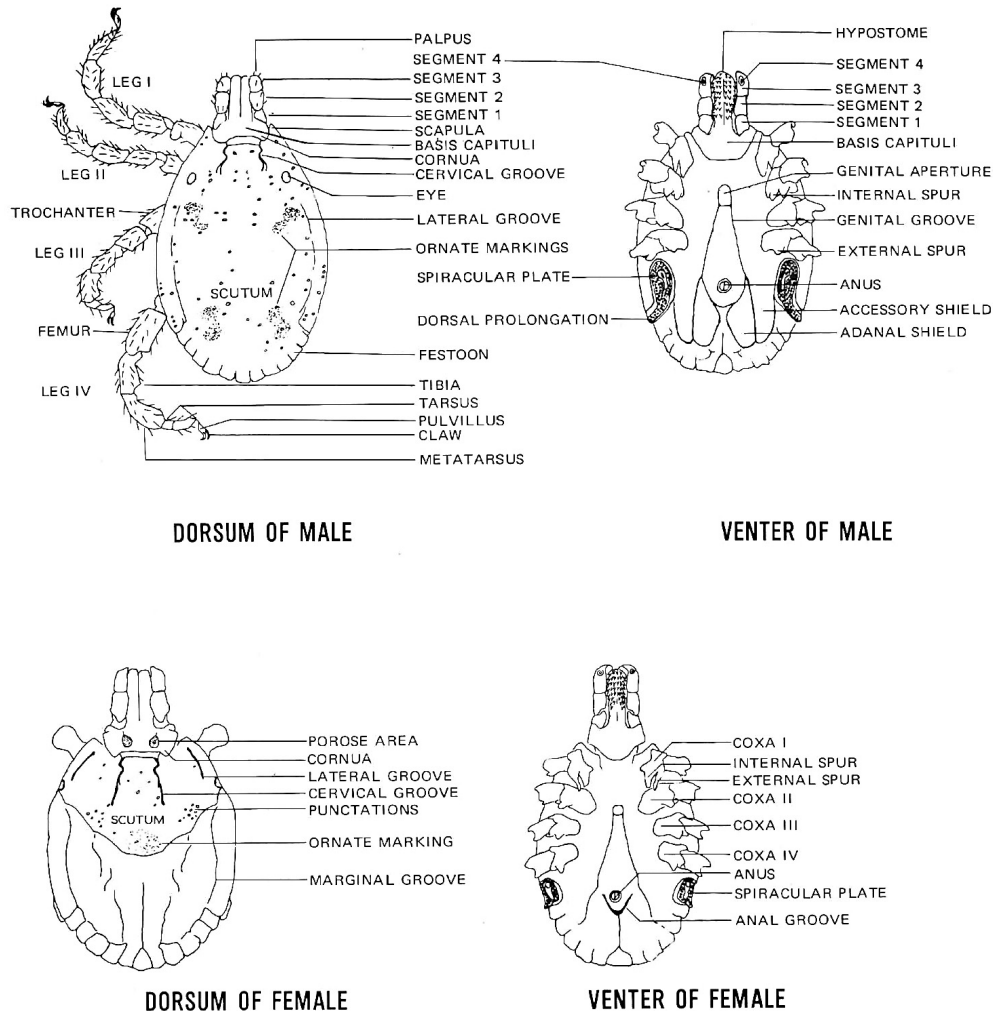
Figure 5. Dorsal and ventral side of hypothetical ixodid male and female ticks (U.S.D.A. Agriculture Handbook No 48, 1973)

One-host species *(R. decoloratus):* After mating and feeding to repletion, gravid females drop off the host, lay their eggs in a suitable sheltered site in the environment and then the female dies. After hatching, larvae climb to the tips of vegetation to quest for a new host to feed on. Moulting occurs on the host and nymphs emerge from larval skin then reattach for feeding. The development of the nymphs to adults occurs similarly on the same host (Fig. 6) (Sonenshine, 1991, Arthur and Londt, 1973).
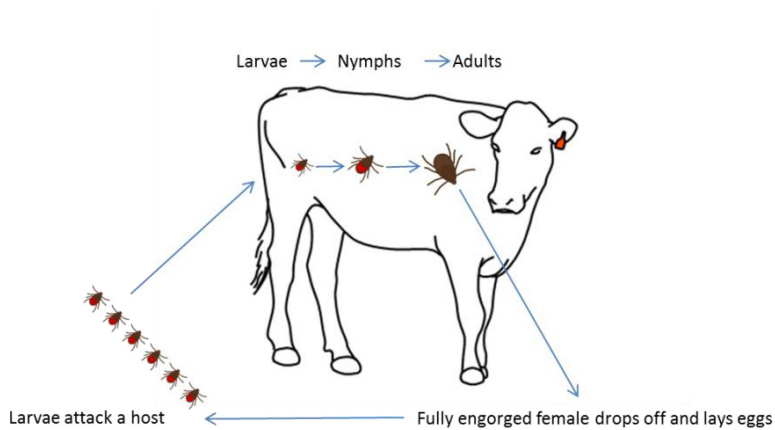
Figure 6. Schematic representation of the life cycle of a one-host tick (E. Tuppurainen)

Three-host species *(R. appendiculatus* and *A. hebraeum):* Larvae, nymphs and adults ticks feed on separate hosts. When fully engorged, each life cycle stage drops off the host and moulting to the next stage, or oviposition occurs on the ground. Newly hatched *R. appendiculatus* larvae, nymphs or adults climb vegetation and passively wait for a host, whereas *A. hebraeum* nymphs and adults actively look for the new host (Sonenshine, 1991) (Fig. 7).
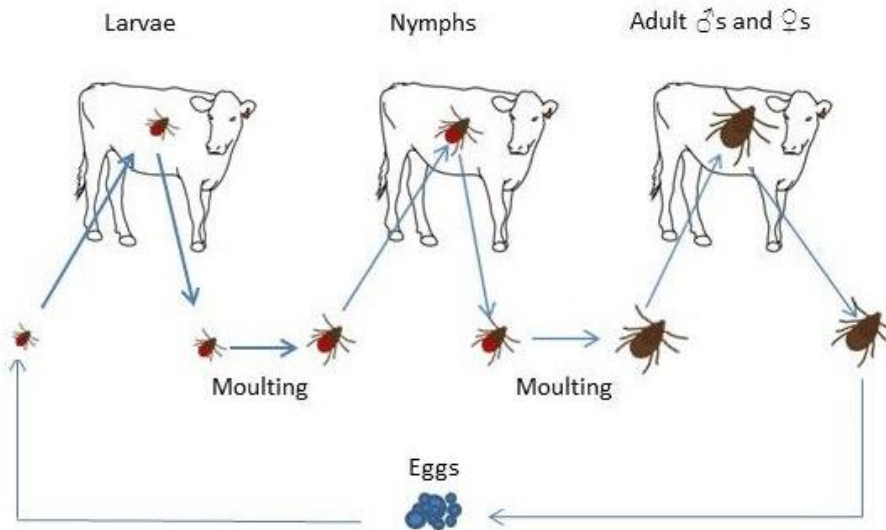
Figure 7. Schematic representation of the life cycle of a three-host tick (E. Tuppurainen)

<div align="center">1.10.5. TICKS AS VECTORS</div>

The vector capacity of ticks for viruses and other pathogens of medical and veterinary importance depends on the ability of the tick to acquire, maintain and transmit a pathogen. If the pathogen is able to survive and/or replicate in tick tissue, many characteristics features of the tick's biology, ecology and physiology make them ideal vectors or reservoirs for tick-borne diseases (Hoogstraal, 1985).

Ticks are known vectors for spirochaetes e.g. *Borrelia* spp., intracellular bacteria such as *Rickettsia* and *Ehrlichia* species, and protozoa like *Babesia* and *Theileria*. Other obligatory intracellular bacterial pathogens transmitted by ticks include genera *Coxiella*, *Francisella*, and some *Anaplasma* species (Norval and Horak, 2004).

Ixodid ticks have been shown act as biological vectors for several viruses, infecting humans and domestic animals such as those belonging to the families *Flaviviridae* causing infections of the central nervous system (tick-borne encephalitis, Kyasanur Forest Disease, and Louping ill) (Gritsun et al., 2003), *Bunyaviridae* causing haemorrhagic fevers (e.g. Nairobi sheep disease and Crimean-Congo haemorrhagic fever) or febrile disease associated with thrombocytopenia and leukopenia (Xu et al., 2011) and *Reoviridae* causing Colorado tick fever (Nuttall et al., 1994). Biological transmission of African swine fever virus, another DNA virus, belonging to the genus *Asfivirus* of the family *Asfarviridae*

(Dixon et al., 2005), has been demonstrated by argasid (soft) *Ornithodoros* ticks, in which the virus replicates, survives the moulting process and can be transmitted via the salivary glands or sexual routes (Plowright et al., 1974, Plowright et al., 1970a, Plowright et al., 1970b).

1.10.5.1.   LONGEVITY OF TICKS

The life span of ixodid ticks ranges from several months to two to three years and unfed instars may survive for more than a year in the environment off the host (Sonenshine, 1991). Histiolytic enzymes and tissue replacement during moulting may create a hostile environment for the virus (Balashov, 1972). After feeding on an infected host, if the virus is able to remain viable in tick tissues and survive the moulting process, with or without replication, this allows ticks to act as a reservoir and vector for the virus.

1.10.5.2.   HOST SPECIFICITY

In order to develop to the next life cycle stage, three-host tick larvae, nymphs and adult females feed on different hosts within one species, or they may use different species of mammals, reptiles or birds. Infected ticks, particularly those feeding on birds or wildlife, could effectively spread the virus over long distances to previously disease-free regions (Hasle et al., 2009). In cases where wild ruminants are susceptible to infection, ticks may be the link between domestic and wild ruminants. In general, little data is available on the susceptibility of wild ruminants and other large or small mammals for capripoxviruses.

1.10.5.3.   SEASONALITY AND DIAPAUSE

Three-host ticks drop off the host after feeding to repletion and spend the majority of their time (95%) off the host (Needham and Teel, 1991). Fully engorged one-host female ticks lay their eggs in a suitable sheltered environment in soil and vegetation. Oviposition and development of one life cycle stage to another is regulated by environmental factors such as temperature, humidity and amount of light. Larvae, nymphs and adults may diapause before (developmental or ovipositional) or after (behavioural) moulting. Desiccation is a major hazard to the developing ticks and one-host ticks are more sensitive for desiccation than three-host ticks (Sonenshine, 1991).

The feeding process of ixodid ticks is slow because the tick body wall needs to grow before the tick is able to ingest a large blood meal. Most of the blood meal is obtained during the last 24 hours before the detachment of the tick (Kaufman, 2010). Compared to insects, the long attachment time to the host has been demonstrated to influence the transmission of a pathogen between the host and the tick vector. The importance of the tick attachment duration has been demonstrated for *Theileria parva* protozoa and *R. appendiculatus* vector, using a mouse model (Konnai et al., 2007).

Digestion of a blood meal occurs inside the cells of the midgut (Sonenshine, 1991) which provides the virus an entrance into the tick cells, then potentially further to the haemocoel and into the salivary glands. The intracellular digestion is also different from insects in which the digestion occurs inside the lumen of the gut. Proteolytic enzymes may not be present inside the midgut of ticks (Nuttall et al., 1994) enhancing the likelihood of pathogen survival and their ability to infect tick tissues.

In general, the main route of virus transmission from infected ticks is via saliva secreted during feeding (Nuttall et al., 1994). Salivary glands excrete the excess water and salts of the ingested blood meal back to the host in saliva (Tatchell, 1967, Gregson, 1967, Kuhnert et al., 1995). The total volume of saliva excreted by a large female tick, such as an *Amblyomma* species, during its attachment may exceed one millilitre (Kaufman, 2010). The volume of the blood meal in males is much smaller and osmoregulation has not been so thoroughly investigated in males. Salivary glands undergo resorption and regeneration during moulting and therefore it can be assumed that cells of the salivary glands get infected later during the life cycle of infected ticks (Nuttall et al., 1994). However, it has been demonstrated that the virus can pass from the haemocoel into the salivary glands and into saliva without infecting the salivary gland cells (Kaufman and Nuttall, 1999).

Interrupted feeding is a natural behavioural pattern for adult *Rhipicephalus* and *Amblyomma* males but may also occur in females if the host dies or if vigorous grooming by the host interrupts the feeding of the tick at an early stage (Wang et al., 1999). The salivary glands are able to switch their function between parasitic and non-parasitic physiological stages, which allow the tick to survive and re-feed (Wang et al., 1999). Males remain on the host for a long time (Norval and Horak, 2004) and feed several times during this period (Wang et al., 1999). After finding and mating with a female, the male attaches adjacent to the female feeding site and at the same time secretes immunosuppressive proteins into its saliva that assist the female to engorge (Wang et al., 1998). During this co-feeding period the male may also detach and reattach. When host animals are in close contact, or the first host dies, males may swap their hosts. Consequently, by interrupted feeding, transmission of the virus may occur mechanically via the tick mouthparts. Alternatively, it may occur intrastadially if the virus

survives in the salivary glands and is flushed out when ticks excretes saliva or cement to the host. Partially fed ticks have been demonstrated to transmit Thogoto (Davies et al., 1987) and Kyasanur Forest Disease viruses (Sreenivasan et al., 1979).

During transstadial transmission, the virus is able to survive and remains virulent or replicates in tick tissues throughout moulting from one life cycle stage to another and is then passed to the host by an unengorged tick via its saliva during feeding.

Tick saliva is known to contain vasodilators, anticoagulants and immunosuppressive substances such as saliva–assisted transmission (SAT)-factor that favour the transmission of the pathogen (Steen et al., 2006). This factor may either attach to the surface of a pathogen inhibiting the host immune mechanism to destroy the pathogen (Ramamoorthi et al., 2005) or it may supress the immunity of the host locally at the feeding site (Jones et al., 1989). Saliva–assisted transmission factor is also likely to assist in transmission of a pathogen through infection of leucocytes between infected and uninfected ticks that are co-feeding on a vertebrae host that is showing either very low levels of viraemia or no viraemia at all, or even in immune animals (Labuda et al., 1993, Nuttall and Jones, 1991). It has been postulated that the transmission of the virus by co-feeding will promote survival of tick-borne diseases in nature, by ensuring that the host will not die of the disease, before ticks have completed their blood meal. In addition, transmission of the virus between ticks is not delayed by the time required for host to become viraemic (Nuttall et al., 1994).

In addition of spreading infections, tick saliva contains harmful substances that may cause allergic reactions or paralysis of the host. In heavily infested hosts a large amount of ticks on a single host may lead even to death by acute anaemia (Howell et al., 1987).

1.10.5.5.   MATING BEHAVIOUR

Mating of *Rhipicephalus* and *Amblyomma* species occurs on the host. Females, attached to the host produce sex pheromones to attract males. In order to find females, males remain on the host for longer periods and feed repeatedly during that time. Copulation is required for females to be able to complete feeding whereas males are able to produce sperm immediately after moulting. After finding a female, the male moves around the posterior end of the female and places the capitulum next to her genital aperture. The male oscillates his mouthparts in the female genital opening several times to distend it prior to sperm transfer. The male ejects a mass of sperm into the centre of a balloon-shaped spermatophore and grasps it with his chelicerae then implants it in the female gonophore. The sack of sperm is then pushed into the female genital tract. For females a blood meal seems to be

necessary to be able to attract males and copulation is required for full engorgement. Males copulate several times with the same or different females between repeated feeding (Varma, 1993, Sonenshine, 1991).

1.11. TICK CELL LINES

The capability of a tick cell line to support replication of a pathogen reflects the natural virus-vector relationship (Mussgay et al., 1975) and the growth of virus in tick cells could be a criteria for arboviruses (Rehacek, 1965). Currently, over 60 continuous cell lines derived from different hard and soft tick species are available at The Pirbright Institute for *in vitro* studies (Bell-Sakyi and Attoui, 2013). These tick cell lines may provide a valuable tool to study tick-borne diseases, to assess the vector potential of different ticks for different arboviruses and to study the interaction between the virus and tick cells, particularly using molecular techniques (Bell-Sakyi et al., 2007). The cells in tick cell lines are heterogeneous, originating from different types of tissues of embryonic or moulting nymphal ticks (Bell-Sakyi et al., 2012). In order to survive within moulting larvae or nymphs, the virus presumably has to establish infection in at least one cell type that does not undergo histolysis during the moulting process (Nuttall et al., 1994). *In vivo* different tick-borne pathogens are known to prefer different tick tissues or cells. All the cells present in the cell culture may, however not support the growth of the virus in live ticks.

Propagation of tick-borne viruses in tick cells have been reported for several viruses such as tick-borne encephalitis virus (Ruzek et al., 2008), West Nile, Powassan, Langat, Louping ill and Venezuelan equine encephalitis viruses (Lawrie et al., 2004), Dugbe virus and Hazara virus (Garcia et al., 2005), Crimean-Congo haemorrhagic fever virus (CCHFV) (Bell-Sakyi et al., 2012), Nairobi sheep disease (Munz et al., 1980) and Thogoto virus (Bell-Sakyi et al., 2007). Continuous tick cell lines are also able to support the growth of rickettsial intracellular pathogens such as *Ehrlichia ruminantium,* the causative agent of heartwater (Bell-Sakyi, 2004).

Previous attempts to propagate poxviruses in tick cells included a study with vaccinia virus in primary *Hyalomma dromedarii* cell cultures (Rehacek, 1965) and another study on vaccinia, ectromelia, fowl pox and orf viruses in the RA243 cell line (Munz et al., 1980). The incubation time in the first study was eight days and in the second study five days. Neither of these studies resulted in poxvirus replication in tick cells detectable by endpoint titration in vertebrate cells.

Arbovirus infection does not cause detectable CPE changes in tick cell cultures (Bell-Sakyi et al., 2012) and potential replication of any virus in tick cells must be investigated using other diagnostic tools such as determination of endpoint titre in vertebrate cells, quantitative PCR or other molecular tools. Compared to mammalian cell cultures, many tick-borne viruses are known to multiply slowly in tick cell cultures, generating only low titres of progeny virus (Rehacek, 1965).

2. AIMS OF THE STUDY

The aim of the pilot study was to investigate *in vivo* if transmission of LSDV from infected to naïve cattle occurs by common sub-Saharan hard (ixodid) *R. appendiculatus*, *A. hebraeum* and *R. decoloratus* ticks by monitoring the clinical signs of LSD, viraemia and seroconversion in recipient cattle and by testing whole ticks and dissected tick organs for LSDV after feeding on infected cattle as well as eggs laid by females previously fed on infected animals.

The CIDLID study included three objectives:

1. to investigate the mechanical mode of transmission by *Rhipicephalus appendiculatus* species by monitoring characteristic clinical signs, viraemia and seroconversion in recipient cattle

2. to investigate the transovarial mode of transmission by *Rhipicephalus decoloratus* species by monitoring characteristic clinical signs, viraemia and seroconversion in recipient cattle

3. to determine whether feeding on infected animals during the viraemic stage is sufficient, or whether feeding on skin lesions is required in order for ticks to be able to transmit the virus

The final objective was to investigate if the virus is able to multiply *in vitro* in tick cell lines derived from *Rhipicephalus* species and to investigate the presence of LSDV in ticks collected from naturally infected cattle.

3. MATERIALS AND METHODS

This chapter presents and discusses the materials and methods used in this study on a general level. Roman numbers I, II, III, IV in brackets refer to the original publication in which the materials and methods are described in detail.

3.1. ANIMAL EXPERIMENTS (I, II, III)

In order to obtain proof-of-concept for the hypothesis that LSDV can be transmitted by ixodid tick vectors, a pilot cattle experiment was carried out in South Africa during October-November 2008 by The Pirbright Institute and the Department of Veterinary Tropical Diseases (DVTD), University of Pretoria (I). Later, based on the tentative evidence obtained from the pilot study and in order to investigate the mode of transmission in greater detail, a research project entitled "Determination of the role of hard (ixodid) ticks in the transmission of lumpy skin disease virus in cattle" (BB/H009361/1) was conducted by the same research group, between October 2010 and March 2011 (II, III). The latter study was funded by 'Combating Infectious Diseases of Livestock for International Development' (CIDLID), UK and included a PhD student at the DVTD. The second experiment is referred to as the 'CIDLID experiment'. In the present dissertation, the findings of the pilot study (I), the mechanical transmission of LSDV by *R. appendiculatus* males (II) and the transovarial mode of transmission by *R. decoloratus* species (III) sections of the CIDLID project are described.

3.1.1. GENERAL DESIGN OF THE ANIMAL EXPERIMENTS

Both animal experiments were conducted in the insect-proof, high-containment animal facility of the University of Pretoria's Biological Research Unit (UPBRC), Faculty of Veterinary Science, Onderstepoort, South Africa. However, the facility did not contain tick gutters. All experimental procedures were approved by the Animal Ethics Committee of the University of Pretoria.

The experimental cattle were purchased from cattle farms near Pretoria. Selected animals were not vaccinated against LSDV and they tested sero-negative prior to purchase and the start of the experiment. On arrival, the animals were allowed to acclimatize for one to two weeks before the onset of the trial. Experimentally infected donors and naïve recipient animals were housed and handled separately.

Laboratory-reared larvae of *R. decoloratus* and both nymphal and adult of *R. appendiculatus* and *A. hebraeum* ticks were provided by ClinVet International (Pty) Ltd laboratories, Bloemfontein (I) and the Agriculture Research Council's Onderstepoort Veterinary Institute (ARC-OVI), South Africa (II, III).

R. decoloratus ticks and *A. hebraeum* adults were fed inside cotton cloth bags which were glued to the shaved skin on the back of donors and recipients. Nymphal and adult *R. appendiculatus* ticks and *A. hebraeum* nymphs were fed inside ear bags glued around the base of the ears of the cattle (Fig 8).

All life cycle stages of *R. appendiculatus, A. hebraeum* and *R. decoloratus* ticks were fed on infected donor cattle during the viraemic stage and on skin lesions.

The general design of the experiments is illustrated in Figure 9. In both experiments the mechanical mode of transmission was investigated by feeding *R. appendiculatus* and *A. hebraeum* males on donors and then transferring semi-engorged males within 24 hours to feed on the skin of naïve recipient cattle.

In the pilot experiment, engorged *R. appendiculatus* and *A. hebraeum* nymphs were tested two to four weeks post-feeding on infected cattle, using PCR and virus isolation (I) but were not placed to feed on naïve animals. In the CIDLID experiment, the intra- and transstadial mode of transmission were further investigated by allowing infected nymphs to moult and the emerging adults were then transferred to feed on fully susceptible recipient animals and this evidence of transmission of LSDV by emerging *R. appendiculatus* and *A. hebraeum* adults is reported by Lubinga et al. (Lubinga et al., 2014b, Lubinga et al., 2013a, Lubinga et al., 2013b).

Figure 8. Feeding of ticks inside the skin bags on the back and around the ears of donor and recipient animals

In the pilot experiment, eggs produced by fully engorged females of all three tick species were tested using PCR and VI using bovine dermis cells (I). In the CIDLID trial, tick eggs were incubated until hatching and emerging larvae were placed to feed on non-infected recipient animals. In this study we

report the results obtained concerning transovarial transmission by *R. decoloratus* species (III). The results of the transovarial mode of transmission of LSDV by *R. appendiculatus* and *A. hebraeum* are reported by (Lubinga et al., 2014c).

Post-attachment of infected ticks, recipient cattle were closely monitored for clinical signs of LSD and both skin and blood samples were collected and analysed using real-time PCR, gel-based PCR and VI. Serum samples were analysed using SNT.
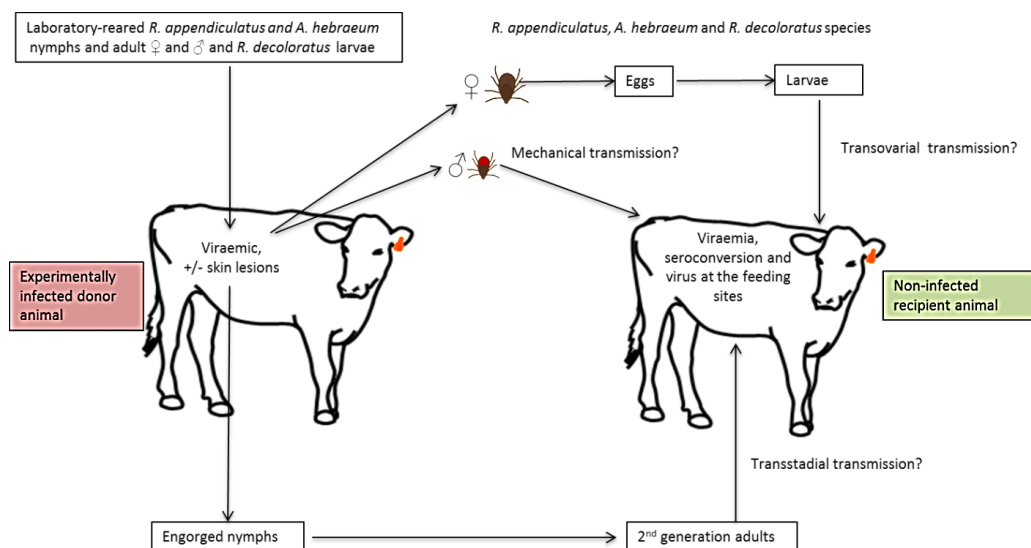
Figure 9. Schematic representation of the general design of the animal experiments

3.1.2. PILOT CATTLE EXPERIMENT IN 2008 (I)

The pilot experiment included eight Pinzgauer and Pinzgauer cross heifers, approximately 13 months of age. The body weight of the animals varied between 195 and 225 kg. Five animals (numbers 604, 605, 609, 610 and 611) were infected with a virulent LSDV field strain, using both the intravenous (IV) via the jugular vein (2.5ml /4.5 log of tissue culture infective dose (TCID$_{50}$)/ml) and intradermal (ID) routes (0.25ml /3.5 log TCID$_{50}$/ml) at four sites on the back of the animals. Laboratory-reared ticks were placed to feed on the donor animals (Table 3). Partially fed male ticks and some female ticks were then transferred to three naïve recipient animals (numbers 613, 701 and 702) on which they were allowed to complete their blood meal (Table 4). Nymphal ticks were allowed to complete their

blood meal on the infected host and drop off into the ear bag, from where they were then harvested. *R. appendiculatus* nymphs were incubated for 17-24 days and *A. hebraeum* nymphs were incubated for 30 days in the acaridarium at 25-28°C and 85% relative humidity (RH) before tested. Fully-engorged females were washed in phosphate buffered saline (PBS), placed into an acaridarium for oviposition and the eggs were then tested using a conventional PCR (section 3.2.2) and real-time PCR (section 3.2.3).

Table 3. Number of ticks and duration of feeding on donor cattle

Donor	*R. decoloratus*	*R. appendiculatus*		*A. hebraeum*		Feeding time/days
	larvae	nymphs	adults	nymphs	adults	
604	500					14
		400				5-7
			100			4-7
					25x♂	7-8
605	1500					18-24
			30			4-7
609		300				6
610			200			6
611				200		4-8
					15x♂, 5x♀	9

Table 4. Origin and number of ticks and duration of feeding on recipient animals

Recipient	*R.decoloratus*	*R. appendiculatus*	*A. hebraeum*	Feeding time/days	Donor
613		<100 ♂		9-12	604, 605, 610
701	>20 ♂			N/S	605
702			<30 ♂	10-14	604, 605, 611

N/S = not successful

Donor and recipient animals were closely monitored for the appearance of characteristic clinical signs of LSD. Body temperatures were recorded daily and blood and serum samples were collected on regular intervals for PCR, VI and SNT. Full-thickness skin biopsies as well as biopsy punches were aseptically collected from the skin at the feeding sites of *R. appendiculatus* and *A. hebraeum* males on the recipient animals after 4 to 14 days of attachment.

Animal 604 was euthanized for humane reasons at 17 dpi which was not sufficiently long for one-host *R. decoloratus* ticks to complete the development from larvae to fully engorged adults. Therefore, the feeding of *R. decoloratus* ticks on this animal was not considered as successful. Less than 10 fully engorged females were harvested for oviposition from animal 605.

3.1.3. CIDLID CATTLE EXPERIMENT IN OCTOBER 2010 - MARCH 2011 (II, III)

Bonsmara cross heifers were used as experimental hosts. The heifers were approximately 13 months of age and the body weight of the donors varied between 231– 314 kg and recipient animals between 178 and 188 kg.

All donor animals were infected by both the IV route via the jugular vein (2.5ml) and ID routes (0.25ml) at 4 sites on the back of the donor animals using a virulent South African LSDV field isolate (5.95 log $TCID_{50}$/ml). The donors were then monitored closely for clinical signs, and body temperatures were recorded daily using a rectal thermometer. Blood samples (in EDTA) and serum were collected on regular intervals for testing by real-time PCR (section 3.2.3) and VI.

In order to investigate the mechanical transmission of LSDV by *R. appendiculatus* males, two cattle were used as donors (DR1 and DR2) and one animal (RR1) was used as a recipient. The numbers of ticks placed to feed on the donors and recipient, and the time ticks were allowed to feed on the hosts are indicated in Table 5.

Semi-engorged *R. appendiculatus* males were fed on the earlobes of cattle. Detached semiengorged males were harvested and transferred from donors to recipient animals within 24 hours. The development of clinical signs, viraemia and seroconversion in recipient animal was monitored (Fig 10).

In order to study the vertical transmission of LSDV by *R. decoloratus* ticks, two of heifers were used as donors for LSDV (DB1 and DB2) and two were used as recipient animals (RB2 and RB3). Donor cattle were infected in October 2010 and after ticks were allowed to feed on them, they were removed from the animal facilities by the end of November 2010. The facilities were then cleaned and disinfected according to the standard operating procedures of UPBRC.

Table 5. Number of *Rhipicephalus appendiculatus* ticks and duration of feeding on the host

	Animal ID	*R. appendiculatus* males	Time on the host/days
Donors	DR1	200	7
	DR2	200	7
Recipients	RR1	140	24

After collection of fully engorged *R. decoloratus* females from infected donors, the ticks were rinsed with phosphate buffered saline (PBS) and then incubated in the acaridarium at 25-28°C degrees at 85% RH until oviposition (4-6 days). The dead females were removed from the containers. Larvae emerged by the middle of December 2010 and were kept for maturation for two months before being placed to feed on naïve recipients (Fig 11). The numbers of ticks placed to feed on the donors and recipients, and the time ticks were allowed to feed on the hosts, are indicated in Table 6. Recipient animals RB2 and RB3 were brought into the animal facilities in the beginning of February 2011. During the time that recipient cattle were housed at the insect-free isolation unit, no animals were infected with LSDV or other capripoxviruses.
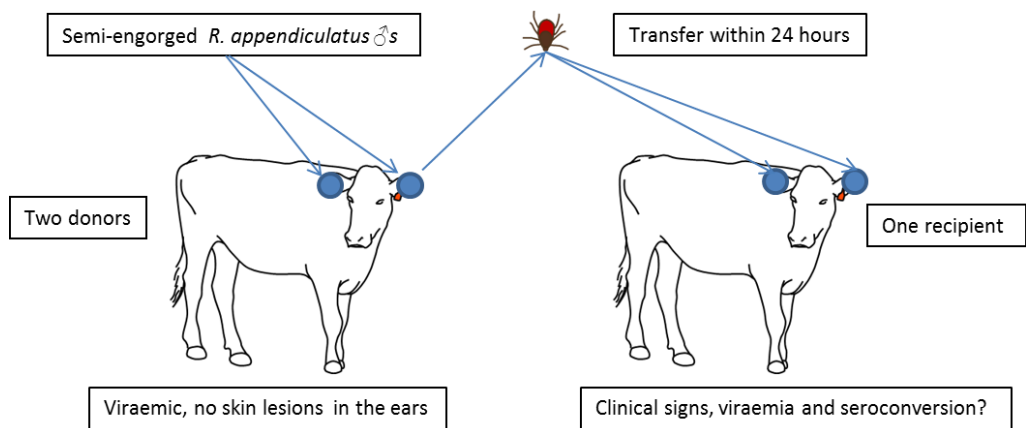
Figure 10. Schematic representation of the study design on mechanical transmission of lumpy skin disease virus by *R. appendiculatus* males

Table 6. Number of *Rhipicephalus decoloratus* ticks and duration of feeding on the host

	Animal ID	*R. decoloratus* larvae	Time on the host/days
Donors	DB1	2500	20-26
	DB2	2500	20-24
Recipients	RB2	2500*	20-30**
	RB3	2500*	20-30**

*Emerging larvae originating from eggs laid by females previously fed on DB1 and DB2

** Until fully engorged females detached
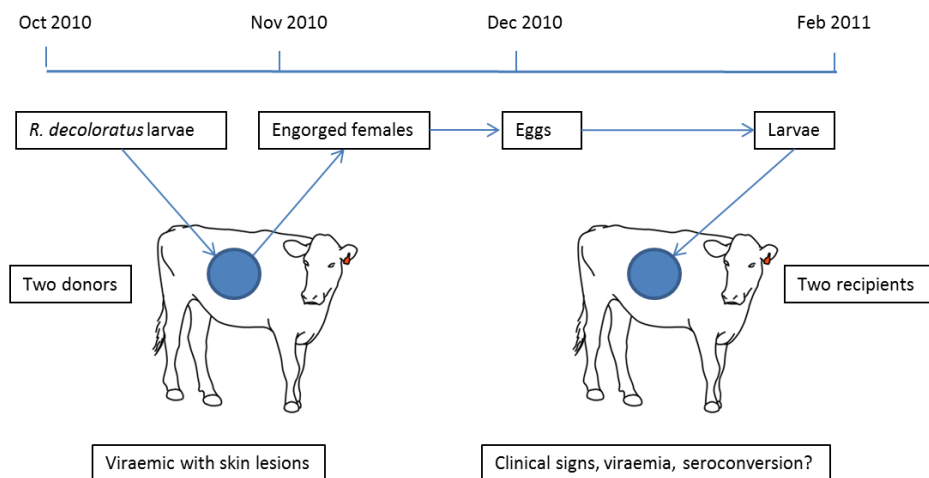
Figure 11. Schematic representation of the study design on the vertical transmission of lumpy skin disease virus by *R. decoloratus* ticks

3.2.   TEST METHODS

3.2.1. DNA EXTRACTION FOR CONVENTIONAL AND REAL-TIME PCR METHODS (I, II, III, IV)

In order to extract the maximum yield of nucleic acid from tick samples, a phenol : chloroform : isoamyl alcohol extraction method (Tuppurainen et al., 2005) with some modifications, was performed on tick samples. Proteins were digested by adding 2-4 IU of Proteinase K (Invitrogen, Life Technologies, Paisley, UK) and the samples were incubated at 56°C overnight. DNA was precipitated in 2 volumes of 100 % ethanol and 1/10 of 3 M sodium acetate (pH 5.3). Tick extraction method including sample volumes is described in detail in the original publication (I).

Viral DNA was extracted from blood samples in EDTA and from tick cell cultures (a volume of 50 µl), using "QIAamp®All Nucleic Acid Kit MDx Kit" (Qiagen, Crawley, UK) and robotic extraction techniques (Qiagen BioRobot Universal System) according to manufactures recommendations.

3.2.2. CONVENTIONAL PCR (I, IV)

Primers designed from the viral attachment gene (PCRA) (Ireland and Binepal, 1998) were used in combination with Platinium Quantitative PCR Supermix-UDGx2 Kit (Invitrogen, Life Technologies,

Paisley, UK). The primers have the following sequences: Forward primer 5'-TCCGAGCTCTTTCCTGATTTTTCTTACTAT-3', reverse primer 5'-TATGGTACCTAAATTATATACGTAAATAAC-3'. The PCR reaction was carried out in Eppendorf Mastercycler PCR system (Eppendorf UK Ltd., Cambridge, UK). The thermal profile was 1 x 42 °C for 2 min and 94 °C for 10 min, 1x 94 °C for 1 min, 50 °C for 30 sec and 72 °C for 1 min, followed by 40 x 94 °C for 1 min, 50 °C for 30 sec, and 72 °C for 1 min and 1 x 72 °C for 1 min (Ireland and Binepal, 1998). Positive samples gave products of the expected size of 192 bp (Ireland and Binepal, 1998). Amplified products were analysed by agarose gel electrophoresis. The gel was viewed using the Bio-Rad Molecular Imager® Gel Doc ™XR System 170 (Bio-Rad Laboratories, Ltd., Hemel Hampstead, UK).

Extracted DNA from non-infected ticks of those species used in this study, or DNA from non-infected lamb testis cells, were used as negative controls. Extracted DNA from scabs or skin lesions collected from experimentally infected donor animals were used as positive controls.

3.2.3. REAL-TIME PCR (II, III, IV)

Primers and a probe, designed by Bowden *et al.* (2008), were used in combination with QuantiFast Probe PCR Kit (Qiagen, Crawley, UK) in Mx3005p Multiplex Quantitative PCR System (Strategene, Netherlands) (Bowden et al., 2008). This real-time PCR assay targets an 89 bp region within the P32 gene and utilises forward primer 5'-AAA ACG GTA TAT GGA ATA GAG TTG GAA-3', reverse primer 5'-AAA TGA AAC CAA TGG ATG GGA TA-3' and TaqMan probe 5'-*6FAM*-TGG CTC ATA GAT TTC CT-*MGB/NFQ-3'*. The thermal profile was 1 x 50°C for 2 min, 95°C for 10 min, and 45 x 95°C for 15 s and 60°C for 1 min. Samples were examined with reference to cycle threshold (C_t) values. Samples tested below C_t 39.5 were considered as positive. C_t values between 39.5 and 40 indicated inconclusive test result and C_t values over 40 were recorded as negative test results. (Bowden et al., 2008, Stubbs et al., 2012). Positive and negative controls, as described in section 3.2.2 and water control were included in each PCR run. The optimized capripox real-time test has an analytical sensitivity of at least 63 target DNA copies per reaction and a greater sensitivity compared to the conventional, gel-based PCR assay (Stubbs et al., 2012). The assay is validated for use as a primary diagnostic method for the detection of LSDV DNA from samples submitted to the OIE Capripoxvirus Reference Laboratory at The Pirbright Institute, and is an ISO 17025 accredited method.

3.2.4. VIRUS ISOLATION FROM TICKS, TICK CELLS AND SKIN SAMPLES (I, II, III, IV)

In the pilot study ticks collected from donor or recipient animals were individually dissected in Dulbecco's Modified Eagle's medium (DMEM), their mouthparts being collected before the idiosoma was cut open. Salivary glands and the gut of each tick were separated. The dissected organs of five ticks of the same species, gender and life cycle stage were pooled in 500 µl of DMEM containing antibiotics and then tested as one sample. The remainder of the viscera of each tick were also collected, pooled and tested using a real-time PCR (section 3.2.3) and VI. Tick dissection and sample preparation is described in detail in the original publication (I).

Virus isolation was carried out in bovine dermis cells primary LB9.D cells (LGC Promochem, Teddington, UK) in 25 cm^2 tissue culture flasks, or microtitre plates of different sizes. Tissue materials were ground with sterile sand in PBS. In order to release intracellular virus from the infected cells the samples were sonicated twice for 15 seconds using Thisle Scientific Branson Sonifier 150. Samples were then centrifuged and the supernatant decanted onto bovine dermis cell monolayers. After incubation for 2 hours at 37°C, cells were washed with PBS and fresh medium containing antibiotics was added to the flasks or tissue culture plates which were incubated at 37 °C for 12 days. Cell cultures showing no CPE were blind passaged once: cells were detached and sonicated, cell debris was spun down and 500 µl of supernatant was decanted onto a fresh cell monolayer.

3.2.5. VIRUS TITRATION (IV)

The virus was titrated in 96-well flat bottom microtiter plates on bovine dermis cells LB9.D (LGC Promochem, Teddington, UK) in 0.5 log dilutions. TCID$_{50}$/ml values were calculated according to Spearman-Kärber method (Kärber, 1931, Spearman, 1908).

3.2.6. SERUM NEUTRALISATION ASSAY (I, II, III)

Neutralizing antibodies were measured using a constant-virus/varying-serum neutralization test (Beard et al., 2010). The positive control serum was collected from cattle experimentally infected with LSDV and collected 37 dpi. Serum collected from cattle in the United Kingdom was used as the negative control. Titres were determined as the last dilution in the serum virus mixtures that gave a 50 % end point.

3.3. PROPAGATION OF LUMPY SKIN DISEASE VIRUS ON TICK CELL LINES (IV)

3.3.1. LUMPY SKIN DISEASE VIRUS ISOLATE

The aim of this experiment was to investigate the replication of LSDV *in vitro* on tick cell lines. The LSDV isolate used in this study was isolated from a skin nodule collected from a bull experimentally infected with a South African LSDV isolate (SA 248/93). The virus was passaged twice on bovine dermis primary LB9.D cells (LGC Promochem, Teddington, UK). The titre of the virus was 6.5 log $TCID_{50}$/ml. The volume of virus inoculum for each tick cell culture tube was 200 µl and the day of inoculation is referred to as Day 0 post-infection (pi).

3.3.2. TICK CELL LINES AND GROWTH MEDIUM

The tick cell lines used in this study, the origin of the cells, growth medium used for each cell line and references are listed in Table 7. All cell lines were propagated in 2 ml growth medium in ambient air in sealed flat-sided culture tubes (Nunc) at 28°C with weekly medium changes (removal and replacement of 1.5 ml medium).

Table 7. *Rhipicephalus* spp. tick cell lines tested for ability to support lumpy skin disease virus replication

Tick cell line	Species and instar of origin	Medium	Reference
RAE/CTVM1	*R. appendiculatus* embryos	L-15	(Bell-Sakyi, 2004)
RA243	*R. appendiculatus* developing adults	L-15	(Varma et al., 1975)
RAN/CTVM3	*R. appendiculatus* developing adults	H-Lac	(Bekker et al., 2002)
REE/CTVM29	*R. evertsi* embryos	L-15	(Alberdi et al., 2012b)
BDE/CTVM16	*R. decoloratus* embryos	L-15	(Bell-Sakyi, 2004)
BDE/CTVM14	*R. decoloratus* embryos	H-Lac	(Lallinger et al., 2010)

Rhipicephalus appendiculatus cell lines (RAE/CTVM1 and RA243), *R. evertsi* (REE/CTVM29) and *R. decoloratus* cells (BDE/CTVM16) were grown in Leibovitz L-15 medium (PAA Laboratories, Yeovil, UK) supplemented with 10% tryptose phosphate broth, 20% foetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

Rhipicephalus appendiculatus (RAN/CTVM3) and *R. decoloratus* (BDE/CTVM14) cells were grown in Hanks' Balanced Salt Solution (H-Lac) (Sigma-Aldrich, Gillingham, UK) supplemented with 0.5% lactalbumin hydrolysate (Sigma-Aldrich, Gillingham, UK), 20% FBS, 2 mM L-glutamine, and antibiotics as above. Subcultures were carried out when required by adding fresh growth medium, re-suspending the cells and dispensing 2 ml volumes into fresh culture tubes while leaving 2 ml cell suspension in the parent tube.

Inoculation of LSDV onto tick cell lines was carried out in triplicate. Four tubes for each cell line were seeded on Day -4. On Day 0, 200 μl of virus suspension was added directly to the growth medium of three of the tubes containing tick cells and to cell-free control tubes containing only either growth medium (L-15 or H-Lac) or PBS. The fourth tube of each cell line was used as an uninfected negative control.

The optimal growth temperature for tick cell lines used in this study is 28°C which is likely to be lower than optimum temperature for the virus to replicate (37°C). In the first experiment, in an attempt to increase the adaptation of the virus to replicate in tick cells, infected and uninfected tick cells, as well as LSDV-infected L-15 control tubes were incubated first for seven days at 37°C followed by four weeks at 28°C. Because it was known that tick cells would not survive at 37°C for long (author's unpublished observation), another group was incubated at 28°C for 35 days.

In the second experiment, infected and uninfected tick cells and infected L-15 (C1), H-Lac (C2) and PBS (C3) control tubes were incubated at 28°C for 35 days. Additional control tubes C1D and C2D were identical to C1 and C2 except medium changes were carried out at the same time and in the same manner as those of the tubes, containing tick cells.

Medium was changed weekly on Days 6, 13, 20, 27, 34 pi in all tick cell culture tubes, maintaining a constant volume of 2 ml throughout the experiment; the tubes were held upright for a few minutes to allow floating cells to sink to the bottom, 1.5 ml spent medium was removed without disturbing the cell pellet, 1.5 ml fresh growth medium was added and the tubes were incubated horizontally.

All tick cell cultures were monitored by weekly inverted microscope examination; general appearance and density of LSDV-infected cultures was compared with that of uninfected control cultures.

3.3.3. SAMPLE COLLECTION

In order to determine the baseline C_t values and virus titres, samples were collected from all inoculated tubes immediately after adding the virus inoculum on Day 0 pi and then on Day 35 pi. Prior to sample collection, cells attached to the cell culture tube were gently detached by flushing with the growth medium, the cell suspension was mixed well and a 200 µl sample was collected. In order to disrupt the cells, samples were then sonicated twice for 15 seconds using a Thistle Scientific Branson Sonifier 150 at a power setting of 2. Cell debris was spun down by centrifugation at 600 x g for 5 min at room temperature and the supernatants were collected and stored at −80°C until tested.

3.3.4. DNA EXTRACTION AND REAL-TIME PCR METHOD FOR TICK CELL CULTURE SAMPLES

In order to standardise the DNA extraction method, 50 µl of each cell culture sample was extracted using a robotic extraction technique (BioRobot Universal System, Qiagen). The presence of viral DNA in the samples was quantified using a real-time PCR as described in paragraph 3.2.3.

3.3.5. VIRUS TITRATION

Tick cell culture samples and cell-free control samples were titrated on bovine dermis cells (section 3.2.5) at 0.5 log dilutions from 10^{-1} to 10^{-6}. Infected wells were identified by microscopic detection of CPE in cell monolayers. $TCID_{50}$/ml values were calculated according to the Spearman-Kärber method (Kärber, 1931; Spearman, 1908).

3.3.6. COLLECTION OF TICKS FROM NATURALLY INFECTED CATTLE DURING LSDV OUTBREAKS

In September 2006 four semi-engorged *Rhipicephalus (Boophilus)* sp. female ticks were collected from three Holstein-Friesian cattle, recovering from LSD but still showing some skin lesions with scabs, at two privately owned dairy farms in Menofilia Governorate in Egypt. The collected ticks were transported to The Pirbright Institute in PBS with 10% glycerol at room temperature and then stored at -20°C until tested in November 2006.

In March 2007, adult *Rhipicephalus*, *Amblyomma* and *Hyalomma* spp. ticks were collected from infected *Bos indicus* cross cattle from three smallholdings near Pretoria, Gauteng Province, South Africa and from cattle brought to a dip tank station in the same area.

Between March and May 2013, *Amblyomma* and *Rhipicephalus (Boophilus)* ticks were collected from Sanga cattle at several dip tank stations in the Mnisi community area which lies in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province, in close proximity to the Kruger National Park border in South Africa.

All ticks collected from South African cattle were placed in cryo tubes without any medium and transported in dry ice. The tick samples were stored at -80°C without medium. Samples collected in 2007 were tested in early 2012. The tick samples originating from the LSDV outbreak in 2013 were tested nine months later. Ticks were identified where possible to species level. For ticks collected from South Africa in 2007, the gender and degree of engorgement of the ticks were recorded.

3.3.7. PREPARATION OF THE WILD TICKS FOR REAL-TIME PCR AND VIRUS ISOLATION

Prior to testing, ticks were washed with 70% ethanol and then rinsed with Schneider's Drosophila Medium (Lonza, Walkersville, MD, USA) containing 200 IU/ml streptomycin and 0.5 μg/ml amphotericin B. Ticks were then cut into small pieces and snap-frozen in liquid nitrogen for 5 min before the samples were mixed with 500 μl of Dulbecco's Modified Eagle's medium. The samples were then lysed twice using a Qiagen Tissue Lyser with 3 mm Tungsten beads at 25 Hz for 2 x 30 s. A further 500 μl of DMEM was added to the samples, followed by centrifugation at 600 x *g* for 5 min at room temperature and then supernatant was collected. One half of the supernatant was used for PCR. DNA was extracted, as described in section 3.2.1, followed by real-time PCR (section 3.2.3). The other half of each sample was used for virus isolation (section 3.2.4). Tick samples collected from Egypt in 2006 were tested using conventional PCR method (section 3.2.2) and did not contain sufficiently sample material for VI.

4. RESULTS

Samples collected from the pilot experiment were originally tested using conventional PCR method (section 3.2.2). After publication of these results (I), a more sensitive real-time PCR assay (section

3.2.3) became available and the same samples were re-tested. Therefore, all the real-time PCR results of the pilot experiment that are reported here, represent unpublished data.

4.1. CLINICAL SIGNS, VIRAEMIA AND SEROCONVERSION IN DONOR ANIMALS

Development of the characteristic clinical signs of LSD, such as high fever, skin lesions, enlargement of subscapular and precrural lymph nodes were closely monitored in donor animals to evaluate the probability of the ticks getting infected, either by ingesting viraemic blood, or by obtaining the virus from skin lesions which are known to contain high virus titres (Babiuk et al., 2008b).

Only two of the five donor cattle (604 and 605) of the pilot experiment developed generalized LSD with multiple skin lesions. Donors 609, 610 and 611 showed transient fever but only a few, if any, skin lesions, apart from the local reaction at the intradermal injection sites. The subscapular and precrural lymph nodes were visibly enlarged in all donors. In addition to multiple skin lesions, animals 604 and 605 showed high fever, excessive salivation and nasal and ocular discharges. In animal 604 pox lesions were also detected in the muzzle and in the mucous membranes of the mouth and conjunctivae. Animal 604 was euthanized for humane reasons at 17 dpi. Post-mortem examination revealed several pox lesions in the mucous membranes of the respiratory and alimentary tracts.

All donor animals became viraemic, as measured by conventional PCR. The same samples were later tested using a real-time PCR method. In animal 604 viraemia started at 4 dpi and lasted until this animal was euthanized at 17 dpi (13 days). In 605 viraemia lasted for 7 days from 7 to 14 dpi. Animal 609 was viraemic for 11 days from 7 to 18 dpi, 610 for 14 days from 4 to 18 dpi and 611 for 17 days from 4 to 21 dpi. The C_t values of the blood samples collected from severely infected animal 604, varied between C_t values of 23.4-29.4 but in all other donor animals blood samples collected during the viraemic stage tested between C_t values of 33 and 39 (Table 8).

All donor animals (except animal 604) had seroconverted by 35 dpi, when the last serum samples were collected. Only one animal (605) already showed low antibody levels at 14 dpi. In samples collected for the rest of the animals a rise in antibody titres started approximately 21 dpi. No increased serum antibody levels were detected in animal 604 before it was euthanized at 17 dpi.

During the CIDLID experiment *R. appendiculatus* donor cattle (DR1 and DR2) did not exhibit severe clinical signs of disease at any time-point pi, but both animals became viraemic. DR1 had a mild form of LSD and did not develop any skin lesions, other than local lesions, approximately 5 cm in diameter at the intradermal inoculation sites. DR2 showed multiple small skin lesions on the side of the neck and in the muzzle and some ulcerative lesions in the mucous membranes of the mouth. No skin lesions were detected in the skin of the ear lobes, or at the base of the ears in either donor animal. However the precrural and subscapular lymph nodes were noticeably enlarged in both donor animals.

Viraemia started in the *R. appendiculatus* donor animals DR1 and DR2, at 7 and 4 dpi, respectively and EDTA blood samples from both animals tested positive for LSDV by real-time PCR (section 3.2.3), for up to 24 dpi. For DR1, the C_t values varied between 32 and 36, and for DR2 between 32 and 38. This indicated that the *R. appendiculatus* male ticks had fed on the donor animals during the viraemic period but (since there were no skin lesions on the ears) not on skin lesions. For both DR1 and DR2 the onset of viraemia was associated with a short peak in body temperatures. The highest body temperature measured in DR1 was 39.4°C degrees for one day only (8 dpi), after which the temperature remained within normal limits. DR2 showed rectal temperature of 40.4°C at 4 dpi and then the body temperature remained at 39.5°C for the three following days. The donor cattle had seroconverted by the end of the experiment (24 dpi).

Both *R. decoloratus* donor animals (DB1 and DB2) showed mild clinical signs of LSD. Donor animal DB1 did not develop any skin lesions, while donor animal DB2 developed small skin nodules on the side of the neck at 7 dpi. No skin nodules, other than those at the inoculation sites of the LSDV were detected on these animals inside the skin bags. The precrural and subscapular lymph nodes of DB1 started to enlarge at 7 dpi, while those of DB2 enlarged at 9 dpi. Blood samples collected from donor animal DB1 tested PCR positive between 4 and 11 dpi, with C_t values between 34 and 37. Donor animal DB2 tested positive between 4 and 14 dpi (C_t values between 34 and 39). The high C_t values observed in the donor animals indicated that only low levels of viral DNA were present in blood of both cases after the experimental inoculation of a virulent LSDV isolate via the IV and ID routes. The onset of viraemia at 4 dpi was also associated with a short peak in body temperature (DB1 39.8°C and DB2 39.9°C). Both donor animals seroconverted between 16 and 27 dpi.

Table 8. Duration of the viraemic stage and real-time PRC (C_t) results in experimentally infected donor animals

Animal	DPI	Total	PCR results (C_t)	Severity of LSD
604	4-17	13	23-29	Severe
605	7-14	7	33-38	Severe
609	7-18	11	34-39	Mild
610	4-18	14	34-38	Mild
611	4-21	21	33-38	Mild
DR1	7-24	17	32-36	Mild
DR2	4-24	20	32-38	Moderate
DB1	4-11	7	34-37	Mild
DB2	4-14	10	34-39	Mild

4.2. *RHIPICEPHALUS APPENDICULATUS*

4.2.1. MECHANICAL TRANSMISSION BY *R. APPENDICULATUS* MALES

In the pilot study, *R. appendiculatus* recipient animal 613 showed a slight transient elevation in body temperature approximately 7 days post-attachment (dpa) of infected ticks and a second peak between 14 and 16 dpa. Superficial lymph nodes, regional to the tick attachment sites were slightly enlarged. No skin lesions or any other clinical signs characteristic of LSD were observed. Blood samples collected from animal 613 tested negative using conventional PCR. However, when the same samples were tested with a more sensitive real-time PCR method (section 3.2.3), one blood sample collected at 13 dpa tested positive (C_t 37). The positive blood sample preceded a rise in body temperature at 14 to 16 dpa. Recipient animal 613 did not seroconvert.

In the CIDLID trial, *R. appendiculatus* recipient animal RR1 did not develop visible skin lesions. However, swelling of the subscapular and precrural lymph nodes were observed at 6 dpa and the animal became viraemic (as measured by PCR) at 10 dpa of *R. appendiculatus* males. The transfer time between collection of the ticks from the donors and their placement on the recipient was less than 24

hours. As in animal 613, the onset of the viraemic stage in RR1 correlated with a transient peak in body temperature (39.2°C on Day 10 pa) and the animal remained viraemic for 13 days. Blood test gave C_t values between 34 and 38. The recipient animal started to seroconvert at 20 dpa and remained seropositive until the last serum sample was collected at 26 dpa. Antibody titres were low in the recipient animal, varying from 1:5 to 1:10. The skin samples collected from the feeding sites of the ticks 19 dpa, tested PCR negative.

Full-thickness skin biopsies were collected from recipient animal 613 from sites where several *R. appendiculatus* males were co-feeding in clusters. Skin samples collected from the base of the left ear of recipient animal 613 all tested negative by conventional PCR (known to be less sensitive than the real-time method) and VI, while samples collected from the right ear tested positive using conventional PCR. However, when real-time PCR methods became available, both left and right ear samples tested positive at 12 to 14 dpa, with C_t values varying from 37 to 38. These results are in line with the C_t value (37.1) of the blood sample collected from the same animal at 13 dpa. No live virus was isolated from these skin samples.

Semi-engorged *R. appendiculatus* males were transferred to the left ear of animal 613 from donor animal 610, which was viraemic but did not have skin lesions. A smaller group of *R. appendiculatus* males previously fed on the skin lesions of donor 605, were added to the same skin bag (left ear). These males were left to feed on the recipient 613 for 4 to 14 days before sample collection.

Semi-engorged *R. appendiculatus* males which had previously fed on the skin lesions of severely infected, viraemic donors 604 and 605, were also allowed to feed on the skin of the right ear of animal 613 for 9 to 12 days prior to taking of the skin biopsies.

When the skin samples were tested by conventional PCR, only the samples collected from the right ear tested positive. However, real-time PCR results for these skin samples confirmed that samples collected from both ears were equally positive (C_t values of 37.4-37.8) and based on these results it was not possible to draw any conclusion whether the severity of the clinical disease in the donors had any effect on the transmission efficiency of the virus.

The mouthparts of semi-engorged *R. appendiculatus* males, which had initially fed on donors and then on recipient animal 613, tested positive by conventional PCR. These results were later confirmed by

real-time PCR (Ct 35.7-38.1). After feeding on the skin lesions of viraemic donor animals (604 and 605), the mouthparts tested positive with slightly lower C_t values 34.3-37.1 (Table 9).

After feeding only on infected donors, or initially on a donor and then on recipient animals, the viscera of *R. appendiculatus* males tested positive with C_t values of 35.5-37.8 (Table 9).

4.2.2.   FEMALES AND EGGS

In the pilot study, fully fed females were allowed to oviposit after feeding on donor animals. Then dead females were removed from the containers and the egg samples were tested for LSDV DNA using conventional PCR assays. Isolation of the virus from the eggs was also attempted but without success, due to the toxicity of tick eggs for primary bovine dermis cell cultures.

Fully engorged female *R. appendiculatus* ticks tested strongly positive by conventional PCR. The females showed much lower C_t values (23.7-28.8) than males after feeding on donor animals which was probably due to the greater volume of ingested blood in females compared to males (Table 9).

The egg samples originated from *R. appendiculatus* females previously fed on viraemic animal 610 (without skin lesions), tested negative in conventional PCR. Later, the same egg samples were tested by real-time PCR. The positive C_t values of egg samples originating from females fed on donors with mild clinical disease showed more variation (C_t values of 35.4-39.4) than samples collected from severely infected donors with multiple skin lesion (C_t values of 36.4 -36.9) (Table 9).

4.2.3.   NYMPHS

In a pilot study, after feeding on the skin lesions of the viraemic donor animal 604, nymphs were incubated in the acaridarium at 85% RH and 28°C for 5 days, then tested by conventional PCR with negative results. Nymphs were incubated in the acaridarium for 2 to 3 weeks prior to testing by real-time PCR. The C_t values obtained for their mouthparts varied between 37 and 38, while fully-fed whole nymphs gave C_t of 32.0-32.9 (Table 9).

Table 9. Summary of *Rhipicephalus appendiculatus* results

	Eggs	Larvae	Nymphs	Females	Males
Conventional PCR	-	N/D	-	+	+
Real-time PCR	+	N/D,*	+	+	+
Virus isolation	N/S	N/D,*	N/D,*	N/D	-
Recipient animal	N/A	N/D,*	N/D,*	N/A	+

* Results reported by Lubinga et al. (Lubinga et al., 2014c, Lubinga et al., 2014b, Lubinga et al., 2013b), N/D= Not done by the author, N/S= Not successful, N/A= Not applicable

4.3.   RHIPICEPHALUS DECOLORATUS

4.3.1.   FEMALES AND EGGS

In the pilot experiment animal 604, used as a 'donor' for *R. decoloratus* ticks, was euthanized for humane reasons due to the severity of the disease at 17 dpi. After killing, the area of skin to which the ticks were attached, was excised and ticks of different developmental stages were detached by hand. On the following day, the transfer of these ticks to a new host (animal 701) was attempted. However, because ticks were still so firmly attached to the skin of animal 604, it was difficult to remove them without damaging their mouthparts and consequently the re-attachment of these ticks to recipient animal 701 was not successful.

In the CIDLID trial, evidence on transovarial transmission of the virus after attachment of *R. decoloratus* larvae, originating from females previously fed on infected donor animals, was obtained by demonstration of characteristic clinical signs of LSD in the recipient animals RB2 and RB3. The donor cattle used for feeding of *R. decoloratus* developed only mild clinical disease with few skin nodules.

Both RB2 and RB3 showed enlarged lymph nodes and a short peak in body temperature between 18 to 20 dpa which correlated with the onset of viraemia. In animal RB2, viraemia started at 18 dpa and lasted until 24 dpa. In RB3 viraemia lasted between 20 and 28 dpa. The C_t values of blood samples collected from RB2 and RB3 varied between 35.5 to 38.5 and 32.9 to 39.4, respectively. These values were in agreement with those of the donor animals. Live virus was isolated from the blood sample

collected from RB2 at 24 dpa. As soon as the first CPE was detected in the infected cell monolayer the presence of LSDV was confirmed by real-time PCR and the second passage was done (C_t 38 for the first passage and C_t 36 for the second passage).

RB2 and RB3 developed small skin lesions at 18 dpa at the feeding site and on the side of the neck. These skin lesions were identical to those that developed on the experimentally infected donor animals. Skin samples collected from the nodules of RB2 tested positive at 19 dpa (C_t values of 38-39) and RB3 at 27dpa (C_t value of 32-35).
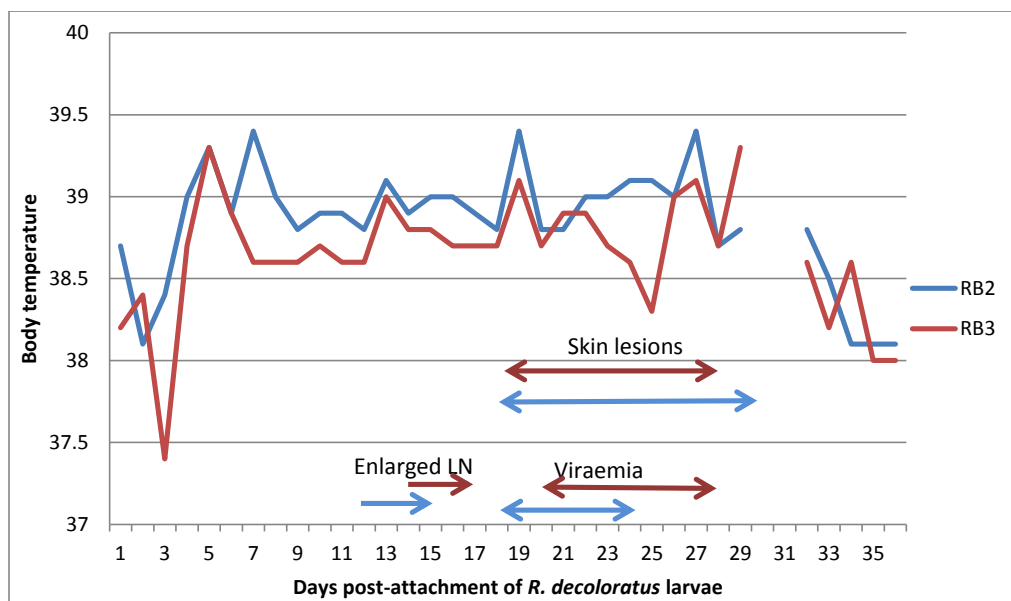
Figure 12. Body temperature and clinical signs of lumpy skin disease observed in recipient cattle RB2 and RB3 post-attachment of *R. decoloratus* larvae, originating from the females, previously fed on infected donor animal

In the pilot experiment flat (unfed), semi- and fully-engorged females were collected from donor animal 605. The fully engorged females tested positive both in conventional and real-time PCRs, which is not surprising because they were feeding on the skin of a viraemic animal. However, only 2 out of 11 flat females tested positive, with very high C_t values (39.8 and 43.1), suggesting that these '+ve' results may have been either very low level of viral DNA or due to cross contamination as normally such a high values (C_t >40) would not have been considered as positive samples. Therefore, this finding remains inconclusive and requires further confirmation by future studies. When the mouthparts of

the fully-fed *R. decoloratus* females were aseptically separated and tested, a pooled sample collected from 4 ticks tested positive (C_t 30.7).

In the pilot experiment the egg samples tested strongly positive using conventional PCR and later also tested positive using a real-time method (C_t values of 34.6 to 36.8).

4.4.   *AMBLYOMMA HEBRAEUM*

4.4.1.   MECHANICAL TRANSMISSION BY *A. HEBRAEUM* MALES

During the pilot experiment *A. hebraeum* recipient animal 702 (which received *A. hebraeum* ticks previously fed on infected donor animals) did not show any clinical signs other than enlarged lymph nodes. A slight raise in body temperature (39.4°C) was detected 10 dpa. The final serum sample from animal 702 was collected 21 dpa. No viraemia or seroconversion was detected.

Amblyomma hebraeum semi-engorged males, harvested from different donor animals, were allowed to feed on recipient animal 702 for 10 to 14 days before collection of a full thickness skin biopsy for testing by a conventional PCR. In addition, some "wild" *A. hebraeum* males that were attached on donor animals 609, 610 and 611 during the viraemic stage (but not on the skin lesions) were transferred to the skin of recipient animal 702. The harvested skin section contained 12 *A. hebraeum* males, feeding in clusters. Viral DNA was detected by conventional PCR but no live virus was isolated from the skin biopsy. When these skin specimens were later tested using real-time PCR, the results varied between C_t values of 28.8 and 37.3. The viscera of semi-engorged males harvested from recipient animal 702 gave C_t values between 33.2 and 37.7.

In some of the mouthpart specimens (collected from bull 702), a flake of skin or the cement-like substance securing the attachment of the tick to the host during the feeding, were still attached. When the mouthparts with skin flake or cement from single ticks were tested, viral DNA was detected on several occasions by conventional PCR and by real-time C_t 32.5- 34.5.

In general, after feeding on severely infected donors 604 and 605, the mouthparts of the semi-engorged *Amblyomma* males tested positive, with C_t values between 34.7 and 35.7. The mouthparts

of semi-engorged males that had fed on donors then on a recipient animal, tested positive with C_t values of 30.3-38.5.

4.4.2. FEMALES AND EGGS

In the pilot experiment *Amblyomma hebraeum* donor animal 611 showed very mild clinical LSD. Fully fed females and their eggs tested negative for LSDV DNA by conventional PCR but with the more sensitive real-time PCR, C_t values for females varied between 36.1 and 37.8. Also the *A. hebraeum* eggs gave C_t values between 33.9 and 38.9. No virus was isolated from the eggs, due to their toxicity for mammalian cell cultures.

4.4.3. NYMPHS

In the pilot experiment, after feeding on infected donor animals (Table 3) nymphs were incubated in the acaridarium at 85% RH and 25-28°C for approximately 8 days before being stored at -20°C or -80°C until tested. The nymphs tested positive for LSDV DNA by conventional PCR. *A. hebraeum* nymphs tested individually by real-time PCR gave C_t values of 32.4-37.1.

Table 10. Summary of *Amblyomma hebraeum* results

	Eggs	Larvae	Nymphs	Females	Males
Conventional PCR	-	N/D	+	-	+
Real-time PCR	+	N/D, *	+	+	+
Virus isolation	N/S	N/D, *	N/S, *	-	-
Recipient animal	N/A	N/D, *	N/D, *	N/A	+

* Results reported by Lubinga et al. (Lubinga et al., 2014c, Lubinga et al., 2013a, Lubinga et al., 2013b) , N/D= Not done by the author, N/S= Not successful, N/A= Not applicable

4.5.   PROPAGATION OF THE VIRUS ON TICK CELLS

A volume of 200 µl of a South African LSDV field isolate was used to infect the tick cell lines. In the first experiment, incubation of infected tick cells at the higher temperature of 37°C for the first seven days did not have any effect on the amount of virus detected in the cultures four weeks later (Table 11). For *R. appendiculatus* (RAE/CTVM1) and *R. evertsi* (REE/CTVM29) cells, whether grown at 37°C/28°C, or at constant 28°C, the final C_t values were practically identical (average C_t values of 27.5) and in all cases, were higher than the Day 0 baseline values, indicating loss of virus from the cultures during the five-week period. However, in both *R. decoloratus* groups the virus survived poorly in comparison with survival in the *R. appendiculatus* and *R. evertsi* cells (Table 11).

The titre of the original virus inoculum (SA 248/93) was 6.5 log $TCID_{50}$/ml (2nd passage on bovine dermis cells). The average Day 0 baseline titre in tick cell culture tubes was 5.6 log $TCID_{50}$/ml, consistent with the 1 in 10 dilution resulting from inoculation into the cultures. After 35 days the titre of the virus in RAE/CTVM1 cells kept at 28°C for 35 days was 3.34 log $TCID_{50}$/ml, while in REE/CTVM29 and BDE/CTVM16 cells the titre was below detection limits of the test (-0.17 log $TCID_{50}$/ml).

There was no change in the C_t values between Days 0 and 35 pi of virus incubated without tick cells in L-15 growth medium. Incubation at 37°C/28°C resulted in a small increase in C_t (from 22.6 to 24.1) in 35 days but still this increase was considerably lower than those seen in the infected tick cell cultures (Table 11).

As incubation at the higher temperature at the beginning of the first experiment did not have any detectable effect on the virus PCR signal, the second experiment was carried out with incubation at 28°C throughout. Three *R. appendiculatus* and two *R. decoloratus* cell lines, with cell-free medium and PBS controls, were infected with LSDV as described above and tested on Days 0 and 35 pi.

The baseline and final C_t values and virus titres of the infected cell lines and for the controls are presented in Table 12. The baseline C_t values (Day 0 pi) for all five cell lines were between 23.4 and 25.5. After 35 days, the C_t values for the three *R. appendiculatus* cell lines and *R. decoloratus* cell line (BDE/CTVM14) had risen to between 30.1 and 30.8, while the mean C_t value obtained for *R. decoloratus* line BDE/CTVM16 was considerably higher at 34.09.

Table 11. Survival of lumpy skin disease virus in three *Rhipicephalus* spp. cell lines incubated at different temperatures in the first experiment. Cycle threshold (C_t) values determined for samples collected on days 0 and 35 post-infection are presented for tick cells incubated with LSDV for 7 days at 37° and thereafter at 28°C, or at 28°C throughout

Tick cell line	Incubation temperature	Day 0 (C_t)	Day 35 (C_t)
RAE/CTVM1	37°/28°C	22.23	27.42
	28°C throughout	21.59	27.02
REE/CTVM29	37°/28°C	21.97	27.66
	28°C throughout	25.94	27.84
BDE/CTVM16	37°/28°C	21.03	35.14
	28°C throughout	23.10	34.61
L-15 control	37°/28°C	22.37	24.10
	28°C throughout	22.78	24.11

At the end of the experiment for both *R. decoloratus* cell lines the virus titres were below the detection limits of the assay, although in BDE/CTVM16 cells, live virus was growing in one out of the six wells of the 10^{-1} dilution. Interestingly, the *R. appendiculatus* cell lines grown in L-15 medium (RAE/CTVM1 and RA243) seemed to support the viability of the virus better than the cell line grown in H-Lac medium (RAN/CTVM3). This was reflected in the approximately 1 $\log^{10}$ lower virus titres in the H-Lac medium controls compared to the L-15 medium controls (Table 12). The C_t values of all the undiluted controls – C1 (L-15), C2 (H-Lac) and C3 (PBS) – showed little or no increase between Days 0 and 35 pi, indicating that viral DNA remained intact. However virus viability decreased in all three undiluted controls by between 0.76 and 2.33 log $TCID_{50}$/ml and by an additional 0.83–1.34 log $TCID_{50}$/ml in the two diluted medium controls C1D (L-15) and C2D (H-Lac), demonstrating that detection of DNA by PCR does not necessarily reflect the viability of the virus.

Table 12. Virus titration and real-time polymerase chain reaction (PCR) results for five lumpy skin disease virus-infected *Rhipicephalus* spp. cell lines and cell-free controls incubated at 28°C for 35 days in the second experiment.

Tick cell line	Medium	Cycle threshold (C_t) values		Virus titre ($TCID_{50}$)	
		0 dpi	35 dpi	0 dpi	35 dpi
RAE/CTVM1	L-15	23.69	30.59	5.63	3.0
RA243	L-15	23.43	30.80	5.63	3.5
RAN/CTVM3	H-Lac	24.31	30.13	5.63	2.5
BDE/CTVM16	L-15	25.46	34.09	5.63	*
BDE/CTVM14	H-Lac	25.52	30.43	5.63	**
Cell-free control					
C1 (undiluted)	L-15	23.34	24.65	5.63	4.84
C1D (diluted)	L-15	23.34	29.80	5.63	3.50
C2 (undiluted)	H-Lac	25.68	25.08	5.63	3.17
C2D (diluted)	H-Lac	24.27	34.04	5.63	2.34
C3 (undiluted)	PBS	24.34	24.03	5.63	3.34

* Below detectable levels, live virus isolated, ** Below detectable levels, no live virus isolated

4.6. PRESENCE OF LUMPY SKIN DISEASE VIRUS IN TICKS COLLECTED FROM NATURALLY INFECTED CATTLE

4.6.1. MENOFILIA GOVERNORATE, EGYPT IN SEPTEMBER 2006

The four semi- or fully engorged female ticks were identified to genus level as *Rhipicephalus (Boophilus)*. All four tested positive for LSDV using the conventional PCR, but there was not sufficient sample material for virus isolation (Table 13).

4.6.2. PRETORIA, GAUTENG, SOUTH AFRICA IN MARCH 2007

Out of nine *R. appendiculatus* females collected from cattle with severe manifestations of LSD, six were flat and three semi-engorged. All of them tested positive in real-time PCR (average C_t value of 35.8). Two partially-fed *R. appendiculatus* males collected from the same animals gave a slightly lower average C_t value of 33.3. Attempts to isolate live virus from these samples were unsuccessful.

A total of 11 *Rhipicephalus (Boophilus) microplus* females were collected for testing, two of which were feeding on animals severely infected with LSDV and nine of which were collected from cattle at the dip tank station. Some of the latter cattle were reported to be vaccinated against LSDV although no vaccination records were presented. None of the animals at the dip station had multiple skin lesions. However, mild cases may have been easily missed as the animals were examined only at herd level. One of the two *R. (B.) microplus* females from severely infected animals and three of the nine other ticks tested positive, with an average C_t value of 35.5.

Six male and three female *A. hebraeum* ticks were collected from one calf with scabs left from previous skin lesions. All the males tested positive (mean C_t value of 35.3). Only one out of three females, tested positive (C_t value of 37.3). Two semi-engorged *Hyalomma truncatum* females were also collected from this animal. Another two were collected from cattle at the dipping station. The mean C_t value of 33.0 was obtained from *Hyalomma* females (Table 13).

4.6.3. MNISI, MPUMALANGA, SOUTH AFRICA, BETWEEN MARCH AND MAY 2013

A total of six *Amblyomma* ticks were collected from cattle at several dip tank stations and two of them tested positive (mean C_t value of 33.6). From a total of 11 *R. (Boophilus)* females, six ticks tested

positive (mean C_t value of 32.0) (Table 13). No live virus was isolated from any of the field derived tick samples.

Table 13. Detection of lumpy skin disease viral DNA in field ticks collected from cattle during lumpy skin disease outbreaks in Egypt and South Africa.

Sample location	Tick species	Number of ticks (LSDV positive/total)	Mean C_t value of positive ticks
Menofilia, Egypt	*Rhipicephalus* (*Boophilus*) sp. female	4/4*	Not done
Pretoria, Gauteng, South Africa	*R. appendiculatus* female	9/9	35.8
	R. appendiculatus male	2/2	33.3
	R. microplus female	4/11	35.5
	A. hebraeum female	1/3	37.3
	A. hebraeum male	6/6	35.3
	H. truncatum female	4/4	33.0
Mnisi, Mpumalanga, South Africa	*Amblyomma* sp.**	2/6	33.6
	Rhipicephalus (Boophilus) sp. female	6/11	32.0

*Tested by gel-based PCR   ** Gender not recorded

5. DISCUSSION

5.1. GENERAL OBSERVATIONS

Despite the substantial economic impact caused by an outbreak for the cattle farming industry and rural communities in the developing world, LSD has raised very little general interest within the scientific community outside the endemic regions. In order to effectively control the spread of the disease, it is necessary to fully understand the role of different arthropod species in transmission of LSDV. Based on circumstantial evidence obtained during the first LSDV outbreaks in southern Africa in 1940s, transmission of the virus between cattle was suspected to occur by a variety of blood-feeding vectors (Haig, 1957, MacOwan, 1959, Weiss, 1968). However, the attempts to isolate a live virus from ticks failed (Haig, 1957, Weiss, 1968) and since then, no other trials on tick vectors for LSDV were conducted. In addition, only a few transmission studies on the potential vector capacity of different blood-feeding insects for LSDV have been published (Chihota et al., 2001, Chihota et al., 2003, Kitching and Mellor, 1986). Camelpox virus was isolated from camel ticks (*Hyalomma dromedarii*), the virus initially being detected by electron microscopy in ticks collected from naturally infected camels (Wernery et al., 1997).

The overall objective of this study was for the first time to investigate if transmission of the virus between infected and naïve cattle occurs by hard tick vectors. Three common South African tick species were selected for the trial. The same or closely related species are common and abundant across the African continent and in the Middle East (De Clercq et al., 2012, Shoukry et al., 1993), although, none of the tick species included into this study is common in Europe. However, if global warming occurs, it may have an impact on the temperature, humidity, vegetation and other factors, allowing the extension of geographical distribution of these ticks further to the north (Gray et al., 2009). In principal, as mechanical mode of transmission of LSDV was demonstrated by *R. appendiculatus* tick, any tick or insect species capable of interrupted feeding are likely to be able to transmit the virus with their mouthparts.

The role of birds in introducing novel tick species over long distances into new areas, has not been demonstrated but the evidence strongly suggest that it can happen (Hasle, 2013). Birds alone cannot however, spread ticks if environmental temperature, humidity and other factors do not meet the requirements specific for the tick species. Under these circumstances, they would not be able to establish permanent populations, even though they could be introduced to new areas by birds (Hasle, 2013).

Despite the capability of LSDV to multiply in embryonated hen's eggs, no data exists on the susceptibility of wild or domestic birds to LSDV. In infected eggs the highest concentration of the virus was detected in CAMs and the second highest in embryos (Van Rooyen et al., 1969). This finding indicates that it is possible that LSDV is also able to replicate in tissues of other bird species. The potential role of wild birds as intermediate hosts or viral reservoirs for LSDV, transmitting the virus via insect or tick vectors, should be investigated. Co-feeding of infected and uninfected ticks on infected or uninfected birds provides yet another interesting possibility for further investigations.

Clinical signs and viraemia were compared in recipient animals and in experimentally infected donor animals. Cycle threshold values of the blood samples collected from donor and recipient animals of the pilot and CIDLID trials varied between 30 and 39. The high C_t values detected from blood samples collected during the viraemic stage are typical for LSD and the finding is in an agreement with the experience obtained from testing of field samples sent to the Pirbright Institute's Capripoxvirus Reference Laboratory (author's unpublished observations). Particularly in animals manifesting mild clinical disease, the level of viraemia is low, while in severely infected animals C_t values vary between 20 and 30. Hardly any virus titration data from blood samples of viraemic animals have been published. In a previously reported quantitative analysis, the presence of DNA in blood samples, were reported as "intermittently positive" and the DNA copies per ml of blood was reported but not virus titres (Babiuk et al., 2008b).

Another epidemiologically important finding of this study was that the duration of the viraemic stage, does not necessarily correlate with the severity of the clinical signs which in agreement with previously reported data (Carn and Kitching, 1995a). In some cattle with mild or inapparent clinical disease, the viraemia lasted significantly longer than in animals with generalized disease.

It is also noteworthy that a short peak in body temperature was associated with the onset of the viraemic stage in both donor and recipient animals, although the animals did not show high fever at any stage during the course of the disease. Previously, a two phased temperature reaction has been described in LSDV infected animals (Coetzer, 2004). In the present study three recipient animals showed a biphasic fever reaction.

5.2.   MECHANICAL TRANSMISSION BY *R. APPENDICULATUS* TICKS

The first objective of the CIDLID study was to investigate the mechanical mode of transmission by *R. appendiculatus* males. Interrupted feeding is a natural behavioural pattern of *Rhipicephalus* and *Amblyomma* males which detach and reattach several times throughout the period they spend on the host (Wang et al., 1998). When the host animals are in close contact, semi-engorged males, in search for females may easily swap their hosts and consequently, mechanically transmit the virus via their mouthparts from infected to susceptible cattle. Transmission may also occur intrastadially via saliva, if the virus survives in cells of the salivary glands (Nuttall et al., 1994).

The first evidence on mechanical transmission of the virus was obtained already from the pilot study when viral DNA was detected on the mouthparts of *R. appendiculatus* and *A. hebraeum* males after feeding on the donor animals. In addition, the recipient host's skin at the tick feeding site tested positive. After these ticks were collected from recipient animals, they still had PCR positive mouthparts, salivary glands and gut tissue.

In the pilot study the mouthparts and gut of the fully engorged *R. appendiculatus* females which had fed on the skin of the severely affected viraemic donor animal, unsurprisingly, tested PCR positive for LSD viral DNA shortly after their detachment from the host. In any event, these findings have no bearing on transmission between the vertebrate hosts as fully engorged *R. appendiculatus* females do not re-attach to the hosts. However, partially fed *R. appendiculatus* females are still able to detach and re-attach to a new host in the event of interrupted feeding although the frequency depends on the length of time the ticks have been attached to the first host (Wang et al., 1999). Therefore theoretically, such semi-engorged females may be able to transmit LSDV mechanically or intrastadially between cattle, in case of death of the host or as a result of vigorous grooming by the host.

In the pilot trial, only one blood sample collected from *R. appendiculatus* recipient animal tested positive by qPCR whereas in the CIDLID experiment the recipient animal for *R. appendiculatus* males remained viraemic for 13 days. Although the level of the viraemia measured by PCR was low it was at the same level as the viraemia detected in donor animals.

Due to a predominantly cell-mediated immunity against LSDV, seroconversion in vaccinated cattle or in individuals, showing a mild clinical disease is usually low or below the detection limit of SNT although the animals would be fully protected against the virus (Kitching and Hammond, 1992). During the CIDLID trials the only recipient animal for *R. appendiculatus* males became seropositive,

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confirming that the mechanical transmission of the virus via interrupted feeding of these male ticks occurred. However, the absence of seroconversion in the other recipient animals used for the other tick species does not prove with certainty that these animals were not infected.

Rhipicephalus appendiculatus recipient animals did not show any other clinical signs, except enlarged lymph nodes. Previous studies have shown that in order to produce clinical signs in experimentally infected animals, a minimum of six experimental recipient animals are required (Tuppurainen et al., 2014, Annandale et al., 2013, Osuagwuh et al., 2007, Weiss, 1968). Unfortunately, in the pilot and CIDLID trials it was not possible to use sufficient numbers of cattle to demonstrate clinical signs in recipient animals. The presence of viraemia and seroconversion were considered as sufficient proof of transmission and as the aim of this study was only to demonstrate transmission of the virus, the use of several animals was not justified on the grounds of animal welfare.

Further evidence concerning the mechanical or possibly intrastadial mode of transmission after interrupted feeding by *R. appendiculatus* and *Amblyomma hebraeum* males was demonstrated by the South African study group (Lubinga et al., 2013a). Blood samples collected from two recipient animals for *Amblyomma* males both tested positive by real-time PCR although only for one day each (6 dpa and 8 dpa) (Lubinga et al., 2013a). In addition, the presence of the virus was detected in artificially induced saliva samples and using immunohistochemical staining of the salivary glands in both *Amblyomma* and *Rhipicephalus* males after feeding on infected cattle (Lubinga et al., 2014a, Lubinga et al., 2013b). Due to their large mouthparts and interrupted feeding pattern, it is most likely that *Amblyomma* males are equally important as mechanical vectors of LSDV as *Rhipicephalus* males. During this study, attempts to transfer *R. decoloratus* males from infected to a naïve host was not successful due to the small size of these males.

The presence of LSDV has been demonstrated in the endothelial cells of blood vessels (Embury-Hyatt et al., 2012). Thus, these cells may serve as an alternative source of the live virus and viral DNA when ticks are feeding on infected hosts after the viraemic stage. The appearance of neutralizing antibodies 2 to 3 weeks pi is likely to inactivate extracellular virus in the blood and tissues, while the intracellular virus remains virulent despite the presence of antibodies (Kitching, 1986a).

The weakness of the present study is that the findings are mainly based on the detection of viral DNA using molecular assays, which do not necessarily indicate the presence of infectious viruses. Attempts

to isolate LSDV from the tick and blood were not successful as it is difficult to isolate live virus from samples with very low number of virus particles and the reluctance of the virus to grow in cell cultures.

5.3.   VERTICAL TRANSMISSION BY *R. DECOLORATUS* TICKS

The second aim of the CIDLID study was to assess whether after feeding on infected hosts, *R. decoloratus* females were able to pass LSDV to the subsequent larvae and if these larvae were able to transmit the virus to a naïve host. The first evidence of a vertical mode of transmission was obtained already during the pilot study, when *R. decoloratus* eggs, laid by females previously fed on experimentally infected cattle, tested positive in conventional PCR assays for LSDV. Later these positive results were confirmed using real-time PCR. In the pilot and CIDLID study, fully-engorged females were rinsed with PBS before they were placed into the acaridarium to lay eggs and only after the oviposition was completed, dead females were removed from the containers. The potential surface contamination of the eggs and subsequent larvae obtained from the surface of the females cannot be totally excluded, although it is likely that if the virus was attached to the surface of the eggs infectivity would have greatly decreased during the incubation period.

In the CIDLID study the Pirbright scientists were responsible of demonstrating clinical disease, viraemia and seroconversion in two *R. decoloratus* recipient hosts, whereas the presence of the virus in eggs and larvae was investigated by the South African research group (Lubinga et al., 2014c). Both recipient animals developed small lesions in the skin around the tick feeding sites and also outside the skin bags, on the side of the neck. Skin lesions detected in recipient animals were not as big as often seen in naturally infected cattle but the appearance of the nodules were identical to those lesions detected in donor animals and were confirmed to be caused by LSDV using real-time PCR.

The recipient animals for *R. decoloratus* larvae were tested free of disease on arrival and were housed in insect–free bio-containment facilities, in which no other capripoxviruses infected ruminants were kept, that could have served as a source of infection through feeding or handling of animals. The animal facilities were thoroughly disinfected prior to the arrival of recipient cattle, removing the possibility that these animals were infected by virus from the facilities. Detection of viral DNA in blood samples and in skin nodules, particularly from those nodules located outside the skin bags was considered as confirmative proof of transmission of the virus by the *R. decoloratus* larvae. The location of the skin nodules outside the skin bags indicated that the virus must have been transferred from the tick feeding sites to the skin of the neck via blood circulation.

Supportive evidence for vertical transmission was obtained when *R. decoloratus* larvae, originating from females previously fed on LSDV infected cattle were tested positive using real-time PCR. Suspected CPE was detected in attempted virus isolations from larvae samples but the C_t values of these samples were very high and may indicate that the PCR was detecting only residual viral DNA instead of DNA originating from replicating virus (Lubinga et al., 2014c). Using immunohistochemistry, viral antigen was demonstrated in the reproductive organs of *A. hebraeum* and *R. appendiculatus* males and females after feeding on LSDV infected hosts or samples collected from ticks fed on infected cattle as nymphs (Lubinga et al., 2014a).

The incubation time in cattle infected via tick vectors was considerably longer (18 to 20 days) than in donor cattle experimentally infected via IV and ID routes (four days). This is likely to be due to the larger volume of the virus inoculated into donor animals compared to the volumes transferred to recipient animals by *R. decoloratus* larvae. The onset of viraemia was also preceded by a short peak in body temperature on the same day when the viraemic period started in donor and recipient animals. Although the level of viraemia was low measured by real-time PCR, it was on the same level as viraemia detected for donor animals. As in the *R. appendiculatus* trial, C_t values of all skin and blood samples collected from donor and recipient animals were high throughout the experiment. However, live virus was successfully isolated from one blood sample, collected from recipient animal RB2 at 24 dpi. Neither of the recipient animals developed antibody levels detectable using SNT. It is, however, known that none of the serological assays that are currently available, are sufficiently sensitive to detect antibodies consistently during and after mild infections of LSD (Kitching et al., 1987).

Although the number of viral copies transmitted by larvae is likely to be very low, it is known that tick saliva contains immunosuppressive substances that may enhance the replication of pathogens inoculated by ticks into the skin of the host (Jones et al., 1989). The number of recipient animals was, however insufficient for any statistical evaluation of the obtained data.

Previous studies have shown that the levels of vertical transmission of viruses from female ticks to their eggs seem to be generally low (Nuttall et al., 1994). The finding that LSDV can be transmitted transovarially by *R. decoloratus* ticks has a wider epidemiological impact. An engorged *R. decoloratus* female lays approximately 2500 eggs (Howell et al., 1987) and one infected host animal may be infested by up to 70 females (Norval and Horak, 2004). The persistence of the virus in large number of tick eggs laid in the soil or vegetation could effectively contaminate the environment and infected larvae would be a potential source of infection for susceptible domestic and wild ruminants. As the

oviposition and development of *R. decoloratus* eggs are regulated by some environmental factors, including temperature and humidity, the presence of the virus in gravid females, eggs and larvae may explain how the virus is able to survive in the environment for the long periods of time that are often observed in the field between outbreaks.

The mild clinical disease that developed in the donor cattle is possibly an indication that either the field isolate used in this study was not highly virulent, or that the cattle were partially resistant. Previously it has been stated that African or African cross cattle breeds are more resistant to LSD than European thin-skinned breeds of cattle (Coetzer, 2004; Davies, 1982, 1991). Bonsmara breed cattle used in CIDLID study are taurine/zebu hybrids that have been bred to thrive under African field conditions and are therefore likely have some innate immunity against LSD. Due to a restricted budget and lack of availability, it was not possible to use highly susceptible thin-skinned European *Bos taurus* dairy cattle in this experiment.

As mechanical transmission was demonstrated by male *R. appendiculatus* ticks, it is most likely that male *R. decoloratus* are also able to carry the virus is their mouthparts during interrupted feeding. Thus in addition to the oral route, female *R. decoloratus* ticks may obtain the virus via the venereal route. During interrupted feeding on the skin of a viraemic host, the mouthparts of male ticks are likely to become contaminated with the virus. The male tick uses its mouthparts to enlarge the female genital opening and to insert the sack of sperm inside the female gonophore. In addition, males copulate several times either with the same or different females between repeated feeds (Varma, 1993). The female may be feeding either on viraemic or non-viraemic hosts, as the infected male, delivering the virus may have swapped the host after feeding on a viraemic animal. It is also possible that actual replication of the virus is not required as the virus enters the female sex organs via male mouthparts and sperm. Attached to sperm the virus has an easy entry inside the eggs and the emerging larvae. Venereal transmission of CCHFV has previously been reported in *Hyalomma truncatum* ticks (Gonzalez et al., 1992).

5.4. TRANSMISSION OF THE VIRUS AFTER FEEDING OF TICK VECTORS ON VIRAEMIC HOST WITHOUT SKIN LESIONS

The higher the titre of virus is in a blood meal, the higher number of ticks that become infected (Singh and Anderson, 1968). Because the level of viraemia in LSDV infected animals is low it has been assumed that potential vectors need to feed on skin lesions in order to obtain sufficient amount of

virus (Carn and Kitching, 1995b) as skin lesions are known to contain the highest titres of LSDV (Babiuk et al., 2008b). In addition, LSDV is known to survive for a long period in the skin of cattle: live LSDV has been isolated up to 39 dpi from the skin lesions of the experimentally infected animals, whereas PCR could demonstrate viral DNA until 92 dpi (Tuppurainen et al., 2005).

Mechanical transmission of LSDV occurred by *R. appendiculatus* males through feeding on the healthy-looking skin of viraemic donor animals, manifesting mild or inapparent infection. Consequently, subclinical viraemic animals may serve as an important source of infection via blood-feeding vectors if and when these animals are introduced into a susceptible herd in abundance of biting arthropod vectors. In this study we were able to demonstrate that duration of the viraemic stage in animals is not necessarily associated with the severity of the clinical disease. Introduction of animals with silent infection of LSDV, originating from endemic regions has been associated with the incursion of the disease to previously disease-free areas (Davies, 1991a).

5.5. PROPAGATION OF LUMPY SKIN DISEASE VIRUS ON TICK CELL LINES AND THE PRESENCE OF THE VIRUS IN TICKS COLLECTED FROM NATURALLY-INFECTED CATTLE

The final specific objective of this study was to investigate if LSDV is able to grow *in vitro* in tick cell lines derived from *R. appendiculatus*, *R. evertsi* and *R. decoloratus* species and to investigate the presence of the virus or viral DNA in ticks collected from naturally infected cattle.

In pilot and CIDLID studies on potential biological transmission of LSDV by hard ticks, the results were based mainly on PCR findings and to a lesser extent on virus isolation. When isolation of virus was carried out from tick samples, the presence of the virus indicated by suspected CPE was confirmed by testing infected cell cultures by real-time PCR. The resultant C_t values varied between 35 and 39, indicating that viral DNA was present but not necessarily active replicating virus.

Attempts to grow LSDV on tick cells with increasing titres were not successful. However, infective LSDV survived for 35 days, albeit with some loss of titre, in three *R. appendiculatus* cell lines, tick cell growth medium and in plain PBS. Viral DNA survived at similar levels in a *R. evertsi* cell line. In contrast, much lower levels of viral DNA was obtained in *R. decoloratus* cell lines but the presence of extremely low-titre, infective LSDV was still detected by virus titration after 35 days *in vitro*.

All capripoxviruses are known to grow slowly in mammalian cell culture and sometimes two to three passages are required to successfully propagate these viruses (Weiss, 1968), particularly when they are isolated from samples containing a low number of viral particles. Usually CPE caused by LSDV infection cannot be detected visually before Day 4 pi and in some cases the appearance of CPE may take up to 14 days (Tuppurainen et al., 2005). Due to the physiological differences between tick and mammalian cells *in vivo*, LSDV replicating in the skin or blood cells of cattle is likely to require some adaptation to grow in tick tissues.

During the previous attempts to propagate poxviruses in tick cells, the incubation time in the first study was eight days and in the second study five days (Munz et al., 1980, Rehacek, 1965). In the present study, based on experience obtained from propagation of the virus in mammalian cells, LSDV was incubated for a longer time with the tick cells, 35 days, which involved weekly changes of growth medium. This procedure resulted unavoidably in a loss of a few tick cells, possibly containing intracellular virus and a loss of extracellular virus in the growth medium. Therefore, in order to evaluate the effect of the medium changes on C_t values and virus titres, three controls were included, indicating the survival of the virus in two different growth mediums (L-15 and H-Lac medium) used for tick cells, as well as in plain PBS for 35 days. In addition, dilution controls C1D and C2D demonstrated how much virus was lost due to the weekly changes of the growth medium in case no viral replication would occur.

According to these results it was evident that the virus survived in embryo-derived and nymph-derived *R. appendiculatus* cells, as well as it would have survived in growth medium without any tick cells and no replication of the virus was observed in *R. appendiculatus* cells.

Interestingly, survival of the virus in *R. decoloratus* cells propagated in L-15 (BDE/CTVM16 cells) and H-Lac (BDE/CTVM14 cells) medium decreased to below the detection limit of the virus titration assay, although CPE was still detected in one out of six wells in the 10-1 dilution row on the titration plate after 35 days in BDE/CTVM16 cells. The poor survival of the virus in *R. decoloratus* cells cannot be explained by the difference in the incubation temperature and the same virus isolate was used for all cell lines. Throughout the study, attempts to grow the virus in *R. decoloratus* cells resulted in higher final C_t values than for LSDV in *R. appendiculatus* cells. The only possible factor is the tick cells themselves and in this study we were not able to demonstrate with certainty what caused the poor survival of the virus in *R. decoloratus* cell cultures.

L-15 medium supported the survival of the virus better than H-Lac medium. After incubation of the virus for 35 days the titre of the virus in L-15 had decreased by less than 1 log TCID50, whereas in H-Lac the titre had fallen by nearly 2.5 log TCID50. This may also have affected the survival of the virus in tick cell lines grown in the two different media. Moreover, if the virus was only attached to the surface of the tick cells instead of being inside of the cells, some virus may have been lost when the cell debris was separated by centrifugation from the supernatant during the sample preparation for virus isolation and the proportion of virus retained in the debris might differ between cell lines.

The low cultivation temperature required by tick cells is likely to have a suppressing effect on the replication rate of a vertebrate virus (Rehacek, 1965). The tick cell lines used in this study were maintained at 28°C, which is considerably lower than the average body temperature of cattle (38.5°C), ideal for the replication of LSDV. During the first experiment in which the infected cells were incubated for the first seven days at 37°C, *R. decoloratus* cells in particular started to deteriorate and it was not possible to incubate the infected tick cells longer at the higher temperature. When LSDV was kept in PBS (C3) at a similar temperature as the infected cells (28°C), hardly any difference was detected in the C_t values determined on Days 0 and 35 pi, whereas the virus titre decreased 2.29 log $TCID_{50}$ over the 35 days of incubation; this illustrates, in addition to the exceptional stability of LSDV, how unreliable C_t values are as a measurement of virus viability and infectivity.

Tick cell lines are heterogeneous, comprising cells originating from different tissues of embryonic or developing adult ticks (Bell-Sakyi et al., 2012). In order to survive within moulting larvae or nymphs, the virus presumably has to establish infection in at least one cell type that does not undergo histolysis during the moulting process (Nuttall et al., 1994). It is likely that not all the cells present in the tick cell culture will support survival or growth of the virus which may have affected the results of the present study. In the cell lines, originating from developing adults (RA243 and RAN/CTVM3), the digestive and excretory tissues were removed prior to initiation of the cell lines (Varma et al., 1975) which may have precluded growth of the virus as midgut cells are presumably the site where virus multiplication commences. On the other hand, the embryo-derived cell lines RAE/CTVM1, REE/CTVM28 and the two *R. decoloratus* cell lines may contain cells of midgut origin but equally did not support LSDV growth in the present study. The viral antigen was detected in haemocytes of intrastadially- and transstadially-infected R. *appendiculatus* ticks (Lubinga et al., 2014a), indicating that the virus can infect these cell types which are present in all of the tick cell lines tested (author's unpublished observation).

Most tick cell lines are known to permanently harbour endogenous tick viruses (Alberdi et al., 2012a). Using transmission electron microscopy, endogenous reovirus-like particles were demonstrated in all the tick cell lines included in the present study, except BDE/CTVM16, while putative nairovirus nucleic acid was detected by PCR in all the cell lines except REE/CTVM29. The *R. appendiculatus* cell line RA243 was PCR-positive for the endogenous orbivirus St Croix River virus, while all six cell lines were PCR-negative for flaviviruses (Alberdi et al., 2012a). Very little is known about endogenous tick viruses which may affect the survival and replication of other viruses such as LSDV propagated in tick cell lines (Bell-Sakyi and Attoui, 2013).

In order to survive inside a tick, adaptation of the virus may not necessarily be required, provided the tick cells do not contain substances that are toxic to the virus. LSDV was able to survive for 35 days at 28°C in PBS with a 48% decrease in the titre of the virus. However, similar C_t values detected on Days 0 and 35 pi clearly demonstrate that the real-time PCR method detects viral DNA from both virulent and dead viruses and C_t values cannot replace virus titration as a quantitative assay for viability of the virus.

In this study we were able to demonstrate vertical transmission of LSDV by *R. decoloratus* ticks. Transstadial transmission by *A. hebraeum* adults, moulted from nymphs previously fed on experimentally-infected cattle, has been reported by Lubinga and co-workers (Lubinga et al., 2013a). Survival of infectious LSDV in ticks was demonstrated when live virus was recovered after 90 days from moulting *A. hebraeum* nymphs, following intracoelomic inoculation of virus, and after 96 days from *R. decoloratus* larvae, originating from similarly infected fully engorged females (Lubinga et al., 2014b).

Based on the evidence on vertical transmission obtained from the present study, in addition to the previous investigations, we suggest that viable and infective LSDV may only survive in tick cells and tissues without actual replication; nevertheless this survival may be sufficient to result in transmission by the next life cycle stage following attachment and feeding on a susceptible host. Further immunohistochemical and ultrastructural studies are needed to investigate whether the virus is able to enter tick cells *in vitro,* or it survives attached to the cell surface. The real-time PCR assay used in the present study targets the LSDV structural gene P32, thus quantifying virus DNA but not detecting nucleic acid associated with viral replication. At the time, a validated PCR quantifying a non-structural gene expressed during viral replication was not available. However, in future studies, improved molecular methods measuring viral mRNA associated with viral replication should be developed and

utilised to determine if LSDV actually replicates in tick cells. In addition, more experiments are required in order to obtain statistical confirmation for the results of the present study.

Both the pilot and CIDLID studies utilised laboratory-reared ticks fed on experimentally-infected cattle, while no data have been presented on the persistence of LSDV in ticks collected from naturally infected animals. Here we report for the first time the presence of LSDV nucleic acid detected in naturally-infected ticks, collected from field cases of the disease in Egypt and South Africa. The high infection rates (33-100%) and, in some cases, high levels of viral DNA detected by qPCR in field ticks, combined with the experimental transmission studies, strongly suggest that ticks of several genera may be involved in the epidemiology of LSD. The results obtained from the field ticks indicate that the potential vector capacity of *R. microplus* and *H. truncatum* in the transmission of LSDV should be investigated.

In summary, we were able to demonstrate in *in vitro* studies that the virus survived in tick cell cultures for 35 days, without losing its infectivity. No evidence was obtained for replication of LSDV in *R. appendiculatus*, *R. evertsi* or *R. decoloratus* cell lines. Presence of LSD viral DNA from *Rhipicephalus* and *Amblyomma* ticks, collected from naturally-infected animals, provides supporting evidence for the role of these tick genera in the transmission of LSDV. The results of this study indicate that intra- or extracellular survival of the virus in tick tissues is likely to be more important than actual replication of the virus in tick cells. However, the virus may be able to replicate under certain conditions that were not reproduced in vitro in this study, such as during hot and humid seasons creating optimal conditions for both ticks and the virus, or in the presence of optimal growth or supplementary factors.

6. CONCLUSIONS

The observations made during the pilot study provide, for the first time, evidence that ixodid tick species are associated with the transmission of LSDV. Tentative molecular findings of the pilot study indicated that mechanical or intrastadial transmission occurred by *R. appenduculatus* and *A. hebraeum* ticks, as well as transovarial transmission by *R. decoloratus* species, leading to further investigations and commencement of the CIDLID study.

In the CIDLID trial, mechanical transmission of LSDV via interrupted feeding by *R. appendiculatus* male ticks was confirmed. Consequently any situation where high densities of cattle come into close contact, such as around watering holes, in bomas, animal markets or quarantine stations, enhances the possibility of the virus being mechanically transferred by *R. appendiculatus* males between cattle.

Vertical transmission of the virus was demonstrated by *R. decoloratus* ticks. These females may obtain the virus either via blood meal from the viraemic host, or during copulation by the contaminated mouthparts of male ticks. After repletion *R. decoloratus* female drops off the host and produces 1000 to 2000 eggs which are laid in the soil and vegetation. By contaminating the environment with infected eggs and instars, ticks are likely to assist in the survival of the virus for long periods of time in the environment and serve as a link, transferring the virus between susceptible and infected domestic and wild ruminants, in case common grazing land and pastures are used.

Feeding of *R. appendiculatus* males on a viraemic host without skin lesions was shown to be sufficient for successful mechanical transmission of the virus to naive hosts. This finding underlines the hazard of spreading the disease by introducing viraemic animals with silent infection into susceptible herd in previously diseases-free regions in the presence of arthropod vectors.

No evidence was obtained for replication of LSDV in *R. appendiculatus*, *R. evertsi* or *R. decoloratus* cell lines. We were able to demonstrate in *in vitro* studies that the virus can survive in tick cell cultures for 35 days without losing its infectivity.

The results of *in vivo* and *in vitro* studies indicate that intra- or extracellular survival of the virus in tick tissues is likely to be more important than actual replication of the virus in tick vectors.

Presence of LSD viral DNA from *Rhipicephalus* and *Amblyomma* ticks, the high infection rates (33-100%) and, in some cases, high levels of viral DNA detected by qPCR in field ticks, collected from naturally-infected animals, provides supporting evidence on the role of these tick species in the transmission of LSDV.

The findings of this study will have an impact on the control and eradication measures against LSDV by underlining the importance of tick preventative treatment for cattle and regular immunization using effective vaccines and sufficient vaccination coverage, as well as contributing to the recommendations set for the importation and exportation of live ruminants between endemic and disease-free countries

In order to further enhance our knowledge concerning transmission of LSDV by tick vectors, the following future studies should be conducted:

- The importance of tick vectors for LSDV in the field settings should be investigated in detail
- Infection kinetics, rate and duration of infectivity in ticks should be determined
- The replication of the virus in tick cells requires further molecular investigation
- Potential vector capacity of *R. microplus* and *H. truncatum* for LSDV should be investigated
- Further immunohistochemical and ultrastructural studies are needed to investigate whether the virus is able to enter inside tick cells in vitro or survives attached to the cell surface
- Susceptibility of migrating birds for LSDV should be investigated *in vitro* or *in vivo*

Lumpy skin disease is a major impediment for the development of intensive cattle production in Africa. For small-holders and poor rural communities healthy livestock is the way out of poverty. Lumpy skin disease is on the move in the Middle and Near East, a region which is already burden by political unrest, armed conflicts and movement of hundreds of thousands refugees. Global eradication of smallpox was possible with strong political commitment, global cooperation and sufficient funding. It should be the ultimate goal for LSD as well. As total eradication, however may currently not be realistic, better understanding of various insect and tick vectors and development of effective prophylactic tools is essential for successful control of the disease.

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