Demonstration of lumpy skin disease virus infection in *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* ticks using immunohistochemistry

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Abstract

Lumpy skin disease (LSD) is caused by lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*. Transmission of the virus has been associated with haematophagous insects such as *Stomoxys calcitrans as well as Aedes* and *Culex* species of mosquitoes. Recent studies have reported the transmission of the virus by *Amblyomma hebraeum, Rhipicephalus appendiculatus* and *Rhipicephalus decoloratus* ticks andthe presence of LSDV in saliva of *A. hebraeum* and *R. appendiculatus* ticks.

The aim of this study was to determine which tick organs become infected by LSDV following intrastadial infection and transstadial persistence of the virus in *A. hebraeum* and *R. appendiculatus* ticks. Nymphal and adult ticks were orally infected by feeding them on LSDV infected cattle. Partially fed adult ticks were processed for testing while nymphs were fed to repletion and allowed to moult to adults before being processed for testing. The infection in tick organs was determined by testing for the presence of the viral antigen using monoclonal antibodies with immunohistochemical staining. The viral antigen was detected in salivary glands, haemocytes, synganglia, ovaries, testis, fat bodies and midgut. Since the virus was shown to be able to cross the midgut wall and infect various tick organs, this may indicate potential for biological development and transmission of LSDV in ticks. This study strengthens the previously reported evidence of LSDV in tick saliva.

Introduction

Lumpy skin disease (LSD) is a World Organisation for Animal Health (OIE) listed disease of cattle (World Organisation for Animal Health 2012). It is caused by the lumpy skin disease virus (LSDV), a member of the genus *Capripoxvirus*. Other members of this genus are sheep poxvirus and goat poxvirus (King et al. 2011). Lumpy skin disease was first detected in Northern Rhodesia (Zambia) in 1921 and spread southwards to South Africa (Weiss 1968) and later, northwards up to Egypt (House et al. 1990). The disease has now spread to the Middle East in Israel (1989), Saudi Arabia (1992), Yemen (1995), United Arab Emirates (2000), Oman (2010) and recently also Lebanon (2013) (Tuppurainen & Oura 2012, Woods 1988, Yeruham et al. 1995). Epidemiological evidence implicates biting insects in the transmission of the virus. Most outbreaks have been reported in the wet season and along water courses where there is also an abundance of biting flies (Weiss 1968). The 1957 outbreak in Kenya was associated with high activity of *Culex mirificus* and *Aedes natrionus* mosquitoes (MacOwan 1959). The spread into Israel, in 1986, was associated with the stable fly, *Stomoxys calcitrans* (Yeruham et al. 1995).

Recent studies have reported mechanical transmission of LSDV by *Rhipicephalus appendiculatus* and transovarial *transmission*of the virus by *Rhipicephalus (Boophilus) decoloratus* (Tuppurainen et al. 2013a, Tuppurainen et al. 2013b). Transstadial and intrastadial infection of LSDV in saliva of *R. appendiculatus* and *A. hebraeum* adults previously fed on infected animals as either nymphs or adults, has also been demonstrated (Lubinga et al. 2013a).

Ixodid ticks normally have single prolonged blood meals per development stage. For instance, *R. appendiculatus* nymphs will feed for 5 to 6 days and *A. hebraeum* nymphs for 7 to 8 days (Cupp 1991, Sonenshine 1991, Walker 2003). Pathogens in the blood meal have to survive and cross the midgut barrier before reaching the haemocoele from where other tick organs including the midgut, haemocytes, ovaries, synganglion (tick brain) and the salivary glands become infected (Booth et al. 1989, Booth et al. 1991b). Biological persistence of pathogens allows passage of the pathogens across tick stages (transstadial), vertically in the ovaries (transovarial) and sometimes within the same tick stage following interrupted feeding (intrastadial) (Labuda & Nuttall 2004, Sonenshine 1991). The specific tick organs affected by LSDV have not been previously described.

As part of a broader study to determine the role of ixodid ticks in the transmission of LSDV, this study reports on the specific organ infection of *R. appendiculatus* and *A. hebraeum* adults by LSDV following intrastadial infection and transstadial persistence.

Materials and methods

Cattle

Eleven sero-negative Bonsmara cattle (*Bos indicus* and *Bos taurus* cross-bred cattle), were purchased from a farm in the Pretoria area, South Africa from a herd where vaccination against LSDV is not practised. They were approximately 18 months old and weighed between 190 and 250 kg. They were brought to the Faculty of Veterinary Science (FVS), University of Pretoria (UP) 14 days before onset of the trial for them to acclimatise to the environment and kept in insect-free stables at the UP's Bioscience Research Centre (UPBRC). Five of them were used as donor animals to infect ticks while 6 were used as recipient animals to host potentially infected ticks. The experimental procedures for the animals were approved by the Animal Use and Care Committee (AUCC) of the UP.

Preparation of the virus

A virulent South African LSDV field isolate (248/93), propagated on primary bovine dermis cells was used in the study. It was passaged five times in cell cultures at the Virology Section of the Department of Veterinary Tropical Diseases (DVTD), UP. The final titre of the virus was 5.95 log $TCID_{50}$/ml.

Source of the ticks

Laboratory reared unfed nymph and F1 adult *A. hebraeum* and *R. appendiculatus* ticks were supplied by the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI).

Experimental infection of donor animals

Five donor animals were infected with the prepared LSDV inoculum on Day 0 post infection (pi), by injecting a volume of 2.5 ml of the virus suspension intravenously in the jugular vein and 0.25 ml intradermally at 4 sites, at the back of the animals(Lubinga et al. 2013a, Tuppurainen et al. 2013a). Two donor animals (DA1 and DA2) hosted *A. hebraeum* clean ticks while the other two (DR1 and DR2) hosted *R. appendiculatus* clean ticks. The fifth

donor animal DA3 hosted the second group of unfed F1 *A. hebraeum* and *R. appendiculatus* adults.

Test for intrastadial infection in *A. hebraeum*

One of the virus inoculation sites on the lateral aspects of the body of donor animal DA3 was covered by a cotton cloth containment bag adhered to the skin with Genkem contact adhesive glue (Qualichem and Genkem, South Africa). On Day 4 pi, 75 unfed F1 *A. hebraeum* males were placed in the lateral bag. Four days later, i.e. Day 8 pi, 25 F1 *A. hebraeum* females were added to the same bag with males since *A. hebraeum* females only attach in response to aggregation and attachment pheromones secreted by males that had fed for approximately four days (Norval, Andrew & Yunker 1989, Norval et al. 1992). All the ticks (males and females) were collected after females fed for 4 days (Day12 pi) to test for LSDV using immunohistochemistry, specifically immunoperoxidase staining (IHC), electron microscopy (EM) and real-time PCR.

Test for transstadial persistence in *A. hebraeum*

To demonstrate transstadial passage of LSDV in *A. hebraeum* ticks, 500 unfed *A. hebraeum* nymphs were placed on the ears in containment bags attached to the base of the ear of each donor animal (DA1 and DA2) on Day 7 pi as described by Lubinga et al (2013a). After feeding to repletion for 7-8 days, the engorged nymphs were collected, washed, dried and incubated for moulting in an acaridarium at 25°C and 85% relative humidity (RH). Two months after emergence, 200 F2 males were placed on each of the recipient animals RA5 and RA6 in containment bags placed on the lateral sides of the body to test for LSDV transmission, on Day 0 post attachment (pa). Four days later (Day 4 pa), 200 females were added to the bags containing males. Four days after adding females (Day 8 pa), 100 ticks (males and females) were collected for the detection of LSDV by IMPS, EM and real-time PCR.

6.1.1. Test for intrastadial infection in *R. appendiculatus*

To investigate mechanical/intrastadial infection of the virus in *R. appendiculatus,* 100 adult ticks were placed in containment bags attached to the base of one ear of donor animal (DA3). After 4 days of feeding they were collected and tested for the presence of LSDV by IMPS EM and real-time PCR.

6.1.2. Test for transstadial persistence in *R. appendiculatus*

To investigate transstadial passage of LSDV in *R. appendiculatus*, 100 *R. appendiculatus* nymphs (total 200) were placed on one ear of each donor animal (DR1 and DR2) in the same way that adult ticks were placed above. After feeding to repletion for approximately 5 to 6 days, the engorged nymphs were collected and incubated to moult to adults as described above for *A. hebraeum* nymphs. As in *A. hebraeum*, 150 emergent emergent *R. appendiculatus*adults were placed on recipient animal RR3 to test for transstadial transmission of LSDV. Fifty partially fed adults were collected after feeding for 4 days for testing by IMPS, EM and real-time PCR.

6.1.3. Animal infection tests

Infection in donor and recipient animals was determined by monitoring clinical signs and testing blood samples for LSDV by real-time PCR, virus isolation (VI) and serum neutralisation test (SNT) (Tuppurainen et al. 2013a).

6.1.4. Virus isolation

Virus isolation was carried out according to a standard operations procedure (SOP) of the Virology Section of the DVTD, UP, based on OIE guidelines (World Organisation for Animal Health 2012).

6.1.5. Real-time PCR

Real-time PCR was done on the tick homogenate samples as well as on cell culture supernatants. Blood samples from donor and recipient animals were also tested by real-time PCR (Lubinga et al. 2013a, Tuppurainen et al. 2013a).

6.1.6. Immunohistochemistry

To determine the presence of LSDV in specific tick organs following interrupted feeding (intrastadial infection of the virus), 9 F1 *A. hebraeum* and 5 *R. appendiculatus* adults were collected from donor animal DA3. They were sectioned in the sagittal plane and fixed overnight in 10% phosphate buffered formalin. Both halves of each tick were then embedded in paraffin and processed according to standard protocols of the Pathology Section in the Department of Paraclinical Sciences, FVS, UP.

To determine the presence of LSDV antigens in specific tick organs following transstadial passage of the virus, 5 unfed F2 and 10 partially fed F2 adults from recipient animals (RA5 and RA6) were processed as above. Similarly, to demonstrate the presence of the virus during transstadial infection in *R. appendiculatus*, 4 unfed F2 adults and 17 partially fed F2 adults on recipient animal RR3 were processed as above. Four un-infected ticks were processed in the same way and used as negative controls.

Pilot studies were conducted to optimise the immunoperoxidase labelling technique. Briefly, three 3 to 4 µm-thick tick sections were mounted on positively charged microscope slides and dried overnight in an oven at 38°C. After de-waxing in xylene for 10 minutes, the specimens were rehydrated through a graded ethanol and distilled water series (3 minutes each in 100%, 96% and 70% ethanol). Endogenous peroxidase activity was quenched by incubating the tick sections in 3% hydrogen peroxide (in methanol) for 15 minutes at room temperature (22-25°C) and rinsed three times in distilled water. For the purpose of antigen retrieval, slides were incubated in citrate buffer (pH of 6) at 96°C for 14 minutes, followed by cooling for 15 minutes at room temperature and then rinsed twice in distilled water and in PBS buffer for 5 minutes. To block non-specific immunoglobulin binding, the slides were incubated with normal horse serum (1:10 dilution) for 20 minutes at room temperature. The blocking serum was decanted and replaced with the primary F80G5 monoclonal antibody (anti–S057) (Babiuk et al. 2008), diluted 1:1000 and incubated overnight with the sections. The slides were then rinsed three times in distilled water and then in PBS buffer for 10 minutes. The secondary antibody, a biotinylated polyclonal rabbit anti-mouse antibody (Catalogue no: EO354, DakoCytomation, Denmark), was incubated with the tick sections for 30 minutes in a humidified chamber at room temperature. The slides were rinsed in distilled water for 3 minutes before rinsing in PBS for 10 minutes. The sections were incubated with the peroxidase conjugated avidin biotinylated complex (Catalogue no: PK6100, Vector laboratories, USA) for 30 minutes at room temperature and rinsed twice as before. A Vector® Nova red substrate (catalogue no: SK-4800, Vector laboratories, USA) was reconstituted according to manufacturer's instructions and incubated with the sections at room temperature. During this time, the positive-tissue control, a section of skin with characteristic lesions from a cow with confirmed LSD (PCR, EM and IHC-positive on a skin sample) was monitored at 100X magnification for positive labelling, using the light microscope in the IHC laboratory. As soon as there was evidence of clear, specific positive labelling in the positive-tissue control section, all of the tick sections were immediately rinsed in a distilled water bath to

halt the substrate reaction. The sections were then counterstained with Mayer's haematoxylin for 20 seconds and rinsed under running tap water for 10 minutes. The sections were routinely dehydrated through 70%, 96% and 100% alcohol, cleared in xylene and mounted in Entellan® (Merck Chemicals, Darmstadt, Germany). Specific positive labelling was confined to cytoplasmic granular labelling in tick cells and tissues, comparable with the labelling in target cells in the positive-tissue control.

To determine the limit of detection for the IMPS, bovine dermal cell monolayers were cultured on 8-well glass-slides (AEC-Amersham, LTD, South Africa). In each well, 400 µl of MEM containing 480,000 bovine dermis cells per ml was aliquoted and incubated for a day to allow the cells to form a confluent monolayer. Ten-fold serial dilutions of the virus (titre 4.5 Log $TCID_{50}$/ml) were performed and 10 µl of the respective virus dilution was inoculated into individual wells. After four days incubation, the wells were examined for cytopathic effects. The supernatant was removed for testing by real-time PCR to confirm presence of LSDV. The monolayers were air-dried and went through the process of immunoperoxidase staining according to protocols of the Section of Pathology. The stained slides were examined for the presence of LSDV. The viral titre for the highest dilution to show LSDV antigen staining was taken as the limit of detection.

Results

Infection of donor and recipient animals

All donor animals (DA1, DA2, DA3, DR1 and DR2) developed clinical signs for LSD and tested positive for LSDV. The detailed results for DA1, DA2, DR1 and DR2 are described in separate reports (Tuppurainen et al, 2013; Lubinga et al 2013a). Donor animal DA3 showed mild LSDV signs with mild prescapular lymph node enlargement from Day 11 pi until Day 27 pi. Lumpy skin disease virus DNA was detected by real-time PCR, from blood samples, between Day 11 and Day 26 pi with C_t values between 34. 4 and 39.8.

To demonstrate transstadial transmission by *R. appendiculatus* the recipient animal was assessed for lumpy skin disease. Recipient animal, RR3, developed small circumscribed skin lesions on the neck on Day 12 post attachment (pa) until Day 22 pa. The highest body temperature was 39.1°C on Days 5 and 13 pa. Lumpy skin disease virus DNA was detected by real-time PCR from blood samples on Days 16, 20, 21, and 23 pa with Ct values between 34 and 39 but no sero-conversion was detected by SNT. Cytopathic effect was observed from

blood samples on cell cultures after the second passage between Days 9 and 27 pa which was confirmed by real time PCR to be due to LSDV on samples for Day 20 pa (C_t value 38.7). There was both intrastadial and transstadial transmission by *A. hebraeum.* These are reported separately (Lubinga et al, 2013b)

Infection in ticks

Of the 50 ticks examined by IHC, 41 (82%) showed positive labelling for LSDV. The *A. hebraeum* ticks which partially fed on the infected animal as adults (F1 adults) showed positive labelling in seven (78%) of the samples. The *A. hebraeum* F2 adults, which fed on a infected blood meal as nymphs, were positive in four (80%) of the unfed adults and eight (80%) of the fed F2 adults. *Rhipicephalus appendiculatus* showed positive labelling in four (80%) of the F1 partially fed adults, two (50%) unfed F2 adults and 16 (94%) fed adults. Non-infected negative-tissue control ticks did not show any specific labelling (Figure 1). The IHC results are summarised in Table 1.

The salivary glands were most affected with the highest infection occurring in F2 fed *R. appendiculatus* adults (82%), followed by F1 *R. appendiculatus* fed adults (80%) and F2 *A. hebraeum* fed adults (60%) (Table 1). In salivary glands, granular labelling of the LSDV antigen was seen in the cytoplasm of secretory cells of both type II and III alveoli (Figures 2, 3 & 4). Specific positive labelling was also seen in the cuticular layer of the salivary lobular ducts and in the bicuspid valves of the salivary gland acini.

Infection in the reproductive organs was especially prominent in fed F2 *R. appendiculatus* adults (65%), fed F2 *A. hebraeum* (50%) and unfed F2 *A. hebraeum* (60%) adults but was not seen in unfed F2 *R. appendiculatus* adults. In males, the most obvious infection of the reproductive tract was observed in the spermatogonia and spermatozoa of the testes (Figure 3) and in the vas deferens (Figures 4 & 5). labelling was also seen in the cells of the accessory sex glands. Infection in the female reproductive tract was noted in the cytoplasm of oogonia and

Table 1: Summary of results obtained by immunohistochemistry

Tick species	**examined**	**Pos**	**S/ gl**	**epidermis**	**pharynx**	**muscles**	**syng**	**rep system**	**midgut**	**faty body**	**h/cytes**	**MT**	**trachea**
A. hebraeum –IST	***9***	7 (78%)	3 (33%)	1 (11%)	0 (0%)	2 (22%)	0 (0%)	2 (22%)	2 (22%)	0 (0%)	0 (0%)	0 (0%)	5 (56%)
A. hebraeum-TST, unfed	**5**	4 (80%)	1 (20%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)	3 (60%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	3 (60%)
A. hebraeum-TST, fed	**10**	8 (80%)	6 (60%)	3 (30%)	0 (0%)	3 (30%)	1 (10%)	5 (50%)	1 (10%)	0 (0%)	5 (50%)	1 (10%)	2 (20%)
R. appendiculatus-IST	**5**	4 (80%)	4 (80%)	2 (40%)	0 (0%)	2 (40%)	0 (0%)	2 (40%)	1 (20%)	0 (0%)	2 (40%)	0 (0%)	3 (60%)
R. appendiculatus-TST, fed	**17**	16 (94%)	14 (82%)	5 (29%)	2 (12%)	10 (59%)	3 (18%)	11 (65%)	5 (29%)	6 (35%)	5 (29%)	1 (6%)	8 (47%)
R. appendiculatus-TST, unfed	**4**	2 (50%)	2 (50%)	0 (0%)	0 (0%)	2 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (25%)
Overall	***50***	**41 (82%)**	**30 (60%)**	**11 (22%)**	**2 (4%)**	**20 (40%)**	**4 (8%)**	**23 (46%)**	**9 (18%)**	**7 (14%)**	**12 (24%)**	**2 (4%)**	**22 (44%)**

IST= Intrastadial infection, TST= transstadial passage, rep system= reproductive system, S/gl= salivary glands, syng= synganglion, h/cytes= haemocytes, MT= malpighian tubules, Pos= total positive samples

in the lumen of the ovary, and in the walls and lumen of oviducts and connecting tubes (Figures 6, 7 & 8).

Cytoplasmic labelling was also seen in the haemocytes of five (50%) F2-fed adult *A. hebraeum*, five (29%) fed F2 adult *R. appendiculatus* and five (40%) F1 adult *R. appendiculatus* ticks where all types of haemocytes, including the prohemocytes and plasmocytes were labelled (Figures 4 & 9).

Infection of the muscles was seen in all groups of ticks with the highest rates in fed F2 *R. appendiculatus* (59%). labelling of muscles was mostly seen in the cytoplasm of connective sheaths enclosing the muscle bundles (Figure 8). The midgut was labelled in the epithelial cellsof two (22%) F1 *A. hebraeum* adults, one (10%) F2 fed *A. hebraeum* adult, one (20%) F1 *R. appendiculatus* adult and five (29%) F2 fed *R. appendiculatus* adults (Figures 6 & 7). The synganglia showed focal/multifocal granular labelling in one (10%) unfed F2 *A. hebraeum* adult and three (18%) fed F2 *R. appendiculatus* adults. labelling was seen in the cytoplasm of neuro-secretory cells of the cortex (Figure 6). Labelling of the fat body was seen in one (20%) unfed F2 *A. hebraeum* adult and six (35%) fed F2 *R. appendiculatus* adults. Labelling was seen in the cytoplasm of both cells and in the matrix of fat bodies (Figures 6 & 7). Labelling was also seen in the malpighian tubules.

The limit of detection on stained monolayers was at a dilution of 10^{-4}, which corresponded to a viral titre of 10^{-1} log $TCID_{50}$/ml. This is also to the highest dilution where cytopathic effects were seen. Real-time PCR was able to detect the virus in monolayers up to a dilution of 10^{-7}, which corresponded to a concentration of log 10^{-3} log $TCID_{50}$/ml.

Real-time PCR confirmed the presence of LSDV (C_t value 36.69) in a sample of F2 *A. hebraeum* adults and in a sample of F1 *R. appendiculatus* (C_t value 36.05). These ticks were from the same batch as those examined by IHC.

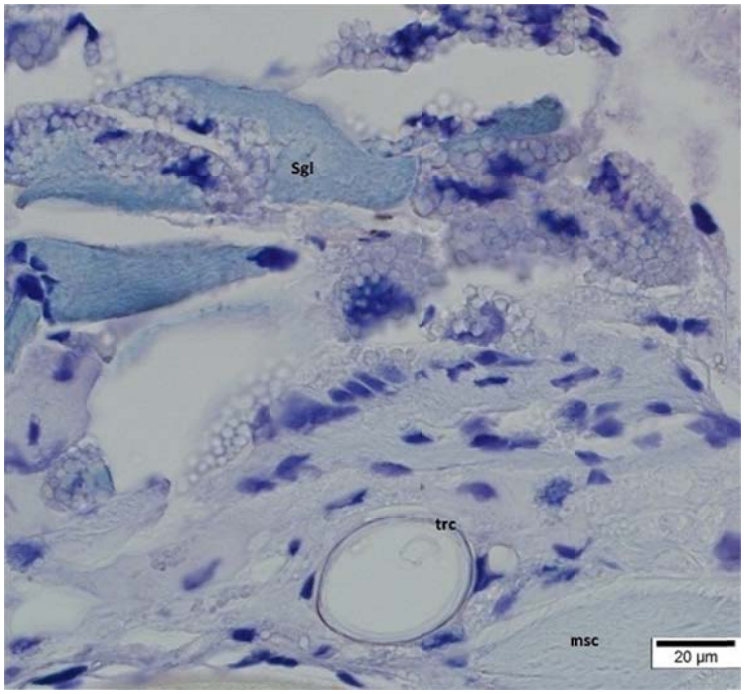

Figure 1: Non-infected negative-tissue control tick showing a lack of LSDV-specific immunoreactivity in salivary glands (sgl), muscle fibres (msc), tracheae (trc)

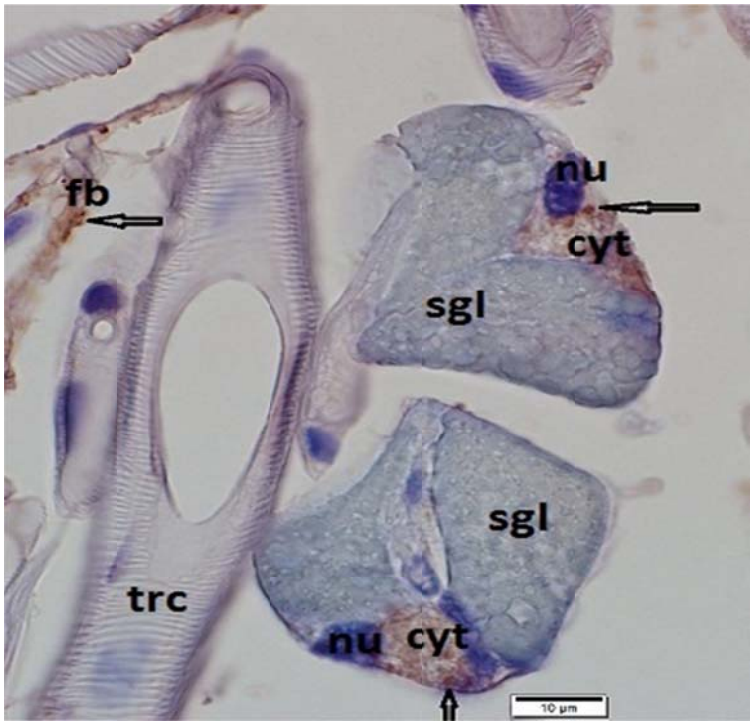

Figure 2: Salivary glands. Granular red-brown (LSDV-positive) labelling (arrows) in the cytoplasm (cyt) of alveolar cells of salivary glands (sgl) and fatbody (fb). Nu=nucleus.

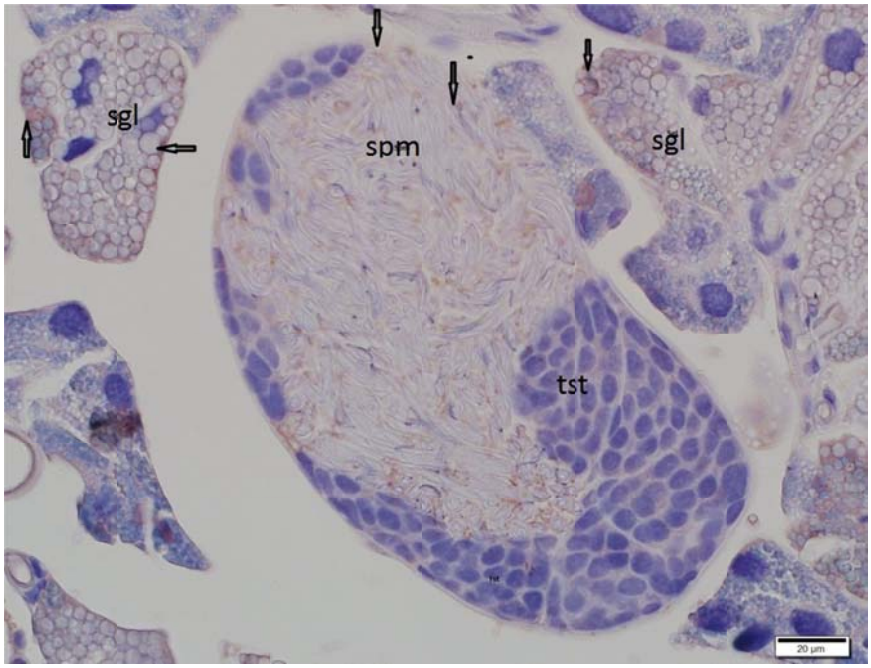

Figure 3: Red-brown granular labelling (arrows) in spermatozoa (spm) of testes (tst) and secretory granules of salivary glands (sgl)

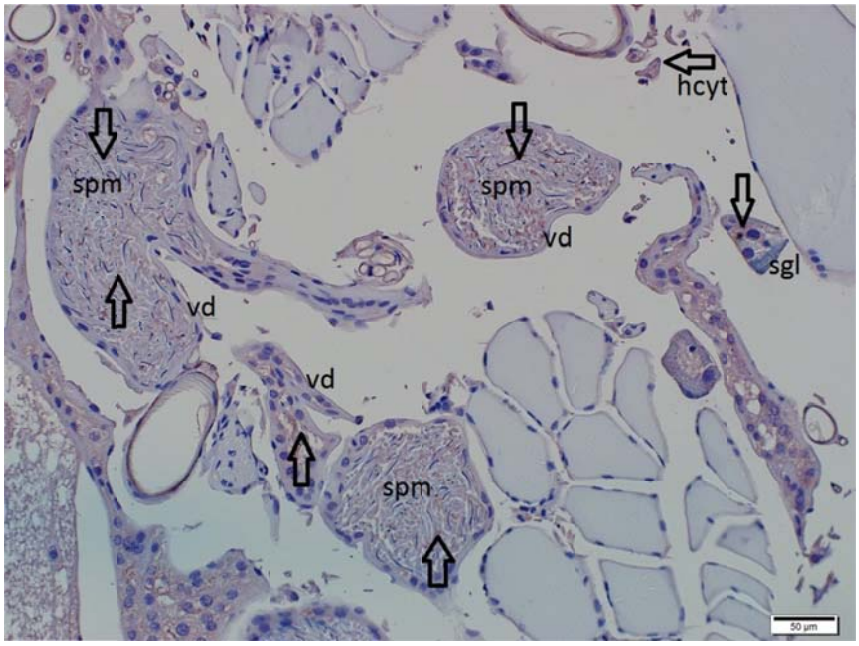

Figure 4: Red-brown granular labelling (arrows) in spermatozoa (spm) of the vas deferens (vd), the cytoplasm of haemocytes (hcyt) and secretory cells of the salivary glands (sgl)

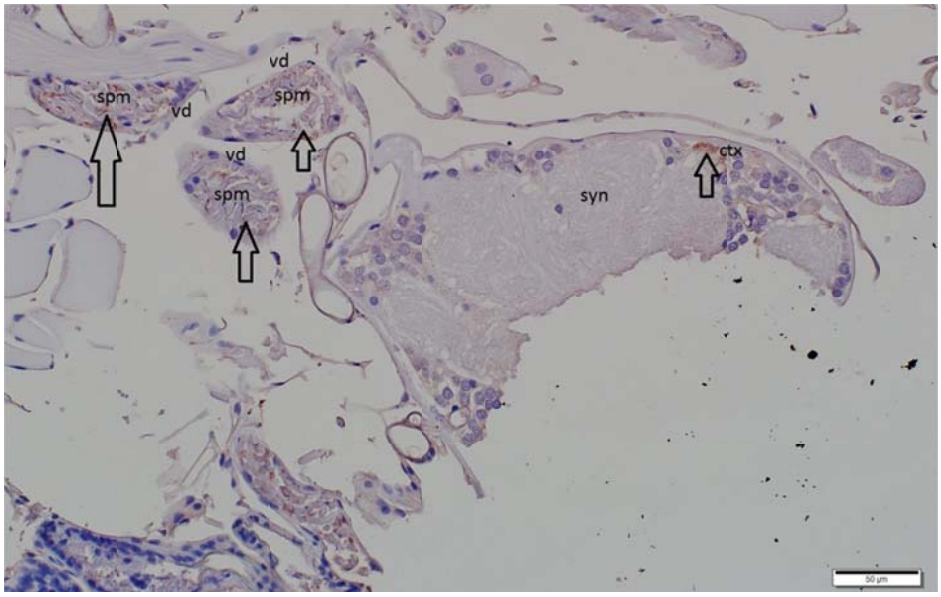

Figure 5: Red-brown granular labelling (arrows) in cortex (ctx) of synganglion (syng) and spermatozoa (spm) in vas deferens (Vd)

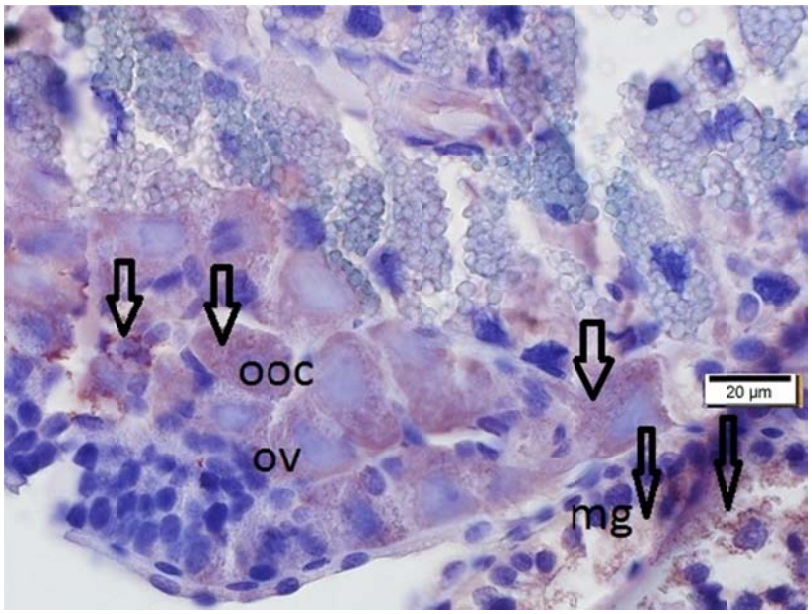

Figure 6: Intracytoplasmic Red-brown granular labelling (arrows) in the oocytes (ooc) of the ovary (ov) and in the midgut (mg)

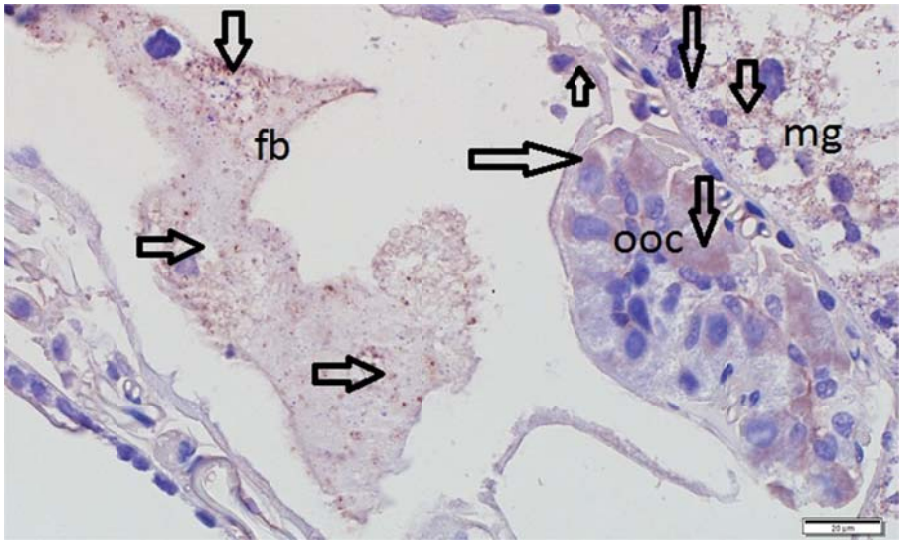

Figure 7: Intracytoplasmic red-brown granular labelling (arrows) in the oocytes (ooc) of the ovary and in the midgut (mg) and fatbody (Fb)

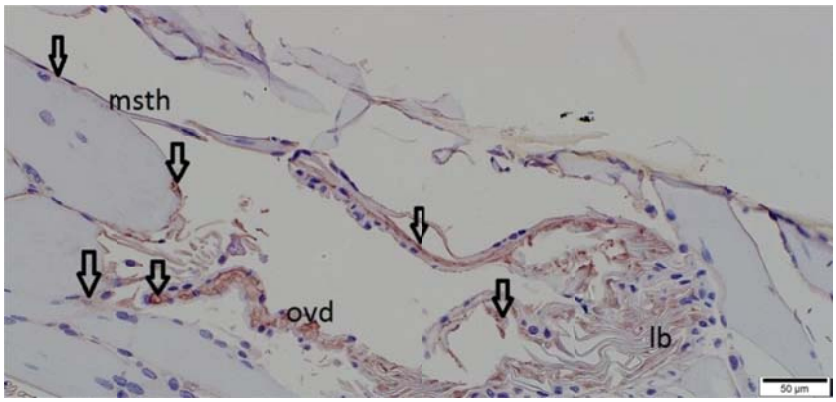

Figure 8: Red-brown granular labelling (arrows) in muscle sheaths (msth) around muscle bundles (ms) and in the oviducts (ovd) and labyrinth (lb) of common uterus.

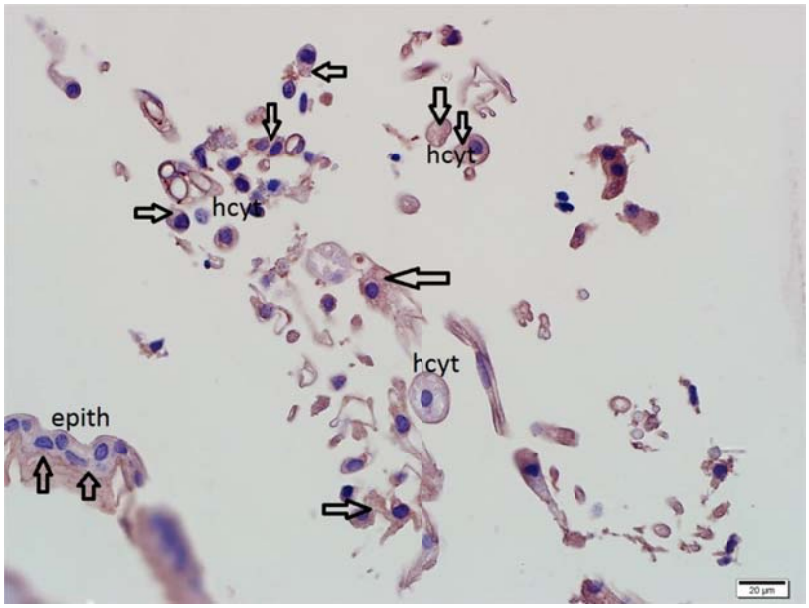

Figure 9: Red-brown granular labelling (arrows) in cytoplasm of haemocytes (hcyt) and epidermis (Epid)

Discussion

The competence of a tick as a vector of a pathogen is determined by the ability of the pathogen to survive the hostile tick midgut environment (Jones et al., 1989) and then enter the salivary gland and saliva. In this study, novel evidence on the vector competence of *A. hebraeum* and *R. appendiculatus* for LSDV was demonstrated by the detection of LSDV antigens, beyond the midgut in the organs of the haemocoele and the epidermis using IHC (Table 1). Similar organ involvement was demonstrated for Thogoto virus in *R. appendiculatus* (Booth et al., 1989; Kaufman and Nuttall, 2003) and Dugbe virus in *A. hebraeum* (Booth et al., 1991a; Booth et al., 1991b). Similarly, *Ixodes hexagonus* ticks were reported to be competent vectors for bluetongue virus when the virus was demonstrated to cross the gut wall and spread to the testes, ovaries and salivary glands (Bouwknegt et al., 2010). Both transstadial and transovarial persistence of Nairobi sheep disease virus in *R. appendiculatus* was reported although the organs affected were not determined (Lewis, 1946; Davies, 1982).

Since tick saliva is the major medium of transmission of tick pathogens (Labuda and Nuttall, 2004; Randolph, 2009), the salivary glands function as the main outlet of pathogens from ticks. The high infection rate in the salivary glands reported in this study suggests the high affinity of

LSDV for the salivary glands and are consistent with the detection of LSDV in the saliva of these ticks reported by Lubinga *et al.* (2013a). . These results, therefore, provide further evidence of vector competence of both *A. hebraeum* and *R. appendiculatus* ticks for LSDV

Both F1 adult *A. hebraeum* and *R. appendiculatus* subjected to interrupted feeding showed infection in organs other than salivary glands (Table 1), thereby demonstrating biological (intrastadial) development of LSDV. In nature, interrupted feeding in ticks has been reported. Males have been shown to detach after their mated female partner has engorged and dropped, in response to attraction pheromones secreted by unmated feeding females (Leahy et al., 1981). *Rhipicephalus sanguineus* males were seen to move between co-housed dogs (Little et al., 2007) while host to host movement of *A. hebraeum* males has also been described (Andrew and Norval, 1989). Therefore, *A. hebraeum* and *R. appendiculatus* males are capable of intrastadial transmission of LSDV as they parasitize other hosts in search of unmated female partners. Intrastadial transmission has also been reported for *Erhlichia canis* by *R. sanguinius* males (Bremer et al., 2005) and *Erhlichia ruminantium* by *A. hebraeum* males (Andrew and Norval, 1989).

All tick stages have been shown to resume feeding when they are interrupted before they have taken enough blood to develop to the next stage, for example following grooming or death of the host (Andrew and Norval, 1989, Wang and Nuttall, 2001). This phenomenon has been attributed to the reprogramming of salivary gland protein expression (Wang et al., 1999). Interrupted feeding, therefore, is not restricted to male ticks and leads to increased disease transmission within an epidemic and may have important implications on the dispersal of ticks from dead animals (Andrew and Norval, 1989, Wang and Nuttall, 2001).

During moulting, some tick organs such as salivary glands undergo histolysis, a process of tissue dissolution (Balashov, 1972, Sonenshine, 1991). The vector competence for transstadial transmission of a pathogen, therefore, depends on the persistence of the pathogen in tissues that are not affected by histolysis during moulting (Nuttall et al., 1994, Kaufman and Nuttall, 2003, Labuda and Nuttall, 2004). The presence of LSDV antigen in the synganglia, epidermis, haemocytes and reproductive organs, which do not undergo histolysis (Balashov et al., 1983, Sonenshine, 1991) reported in this study, suggests these tissues/organs may be sites for LSDV transstadial survival in *A. hebraeum* and *R. appendiculatus*. The involvement of haemocytes

suggests systemic distribution to organs and since haemocytes are responsible for synthesis of non-cellular connective tissue sheaths, the infection of tissue sheaths covering the muscle bundles may be a direct consequence of haemocyte infection. During moulting, the epithelial cells of the epidermis secrete cuticle of the exoskeleton just as the epithelial cells of the tracheae secrete the cuticular intima in the tracheae (Till, 1961; Sonenshine, 1991). Epithelial cells are therefore ideal for persistence of LSDV during transstadial development. Similarly, the fat body plays a storage role during moulting (Balashov et al., 1983, Sonenshine, 1991) and the apparent susceptibility of the fat body to LSDV infection seen in this study, may enable the virus to persist in the fat bodies between stages.

The demonstration of LSDV in female reproductive organs such as the ovaries shows the potential for subsequent transovarial passage of LSDV by both *A. hebraeum* and *R. appendiculatus* females. This confirms the observations of transovarial passage and transmission of LSDV by *A. hebraeum* and *R. appendiculatus* (Lubinga et al, *in press*). The virus, in this study, was also detected in the male reproductive organs especially the sperm cells in the testes and vas deferens. Therefore, apart from the possibility that female ticks become infected from either an infected blood meal or from co-feeding with infected ticks (Labuda et al., 1993, Labuda et al., 1993), uninfected females may also be infected by sexual transmission from infected males, which may then be followed by transovarial passage of the virus. Sexual transmission and transovarial passage has been shown for Crimean Congo Haemorrhagic Fever in *Hyalomma trancatum* (Gonzalez et al., 1992).

Transstadial passage of LSDV in *R. appendiculatus* and *A. hebraeum* adults may play a role in the persistence of the virus during inter-epidemic periods. Both tick species over-winter on the ground after the parasitic nymphal stage as they moult to adults (Short and Norval, 1981, Bryson et al., 2002, Schroder et al., 2006, Horak et al., 2011) and both (*A. hebraeum* and *R. appendiculatus*) adults become active in summer (Short et al., 1989, Horak et al., 2011), notably when the presence of LSD also increases (Weiss, 1968, Coetzer, 2004). This suggests a possible role for ticks in the transmission of the disease. Ticks, especially *A. hebraeum*, are known to have long life spans, i.e. they can survive for several years without a blood meal (Sonenshine, 1991, Walker, 2003) and viruses have the potential to survive in the ticks during this time (Sonenshine, 1991).

The antibody and immunohistochemical technique employed in the present study could detect LSDV in tissues at a viral titre of 10^{-1} log $TCID_{50}$/ml, which is less sensitive than that of real-time PCR which was determined at 10^{-3} log $TCID_{50}$/ml. Immunohistochemistry has the advantage of not being compromised by cross-contamination when compared to real-time PCR. However, it is clear from the study, that only the tissues/organs that were present in the sections were available for immune-detection. The tissues/organs remote from the cut sections were therefore excluded from the IHC test. This limitation could feasibly be reduced by examining serial sections.

This study was able to show for the first time the tick organs and tissues infected by LSDV during both intrastadial infection and transstadial persistence of the virus in ticks. The study also revealed the potential vector competency of *A. hebraeum* and *R. appendiculatus* for LSDV, which is also supported by the reports of mechanical transmission of LSDV by *R. appendiculatus* (Tuppurainen et al., 2013a), mechanical/intrastadial and transstadial transmission by *A. hebraeum* (Lubinga et al, 2013b) and the transstadial transmission by *R. appendiculatus.* Studies to detect LSDV in ticks in natural outbreaks will further validate these results. Further studies are required to investigate if the tick organs affected are influenced by the species and life cycle stage of tick vectors.

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