Evidence of vertical transmission of lumpy skin disease virus in *Rhipicephalus decoloratus* ticks

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ABSTRACT

Lumpy skin disease (LSD) is an economically important acute or sub-acute disease of cattle that occurs across Africa and in the Middle East. The aim of this study was to assess whether *Rhipicephalus decoloratus* ticks were able to transmit lumpy skin disease virus (LSDV) transovarially. Uninfected, laboratory-bred *R. decoloratus* larvae were placed to feed on experimentally infected "donor" cattle. After completion of the life cycle on donor animals, fully engorged adult female ticks were harvested and allowed to lay eggs. Larvae that hatched from these eggs were then transferred to feed on uninfected "recipient" cattle. The latter became viraemic and showed mild clinical disease with characteristic skin lesions and markedly enlarged precrural and subscapular lymph nodes. This is the first report of transovarial transmission of poxviruses by *R. decoloratus* ticks, and the importance of this mode of transmission in the spread of LSDV in endemic settings requires further investigation.

Introduction

Lumpy skin disease (LSD) is an economically devastating disease of cattle which is endemic in most African countries and in the Middle East. The recent outbreaks in the Middle East region pose a risk of further spread of the disease into Turkey, Europe, and Asia (Tuppurainen and Oura, 2012). The causative organism is lumpy skin disease virus (LSDV) which belongs to the genus *Capripoxvirus* within the subfamily *Chordopoxvirinae* and the family *Poxviridae* (Buller et al., 2005). Lumpy skin disease inflicts serious constraints on livestock production in its endemic range by causing decreases in milk yield and weight gain and a reduction in the value of hides. Temporary or permanent infertility in both male and female stocks and abortions may occur (Weiss, 1968). In addition to direct losses due to clinical disease, temporary loss of draft power may also occur which remains important in rural regions (Gari et al., 2011). The indirect economic impact resulting from LSD outbreaks is very significant due to long-term restriction of the global trade of live animals and animal products from affected countries, as well as acting as a hindrance to the introduction of new high-producing breeds of cattle to endemic countries (Babiuk et al., 2008a).

It is widely agreed that the most important mode of transmission of LSDV is likely to be through mechanical transmission by a variety of blood-feeding vectors. This hypothesis is supported by the observation that LSD outbreaks are correlated with warm and wet seasons (Weiss, 1968). Previous studies have demonstrated the mechanical transmission of sheep pox and goat pox viruses by *Stomoxys calcitrans* (L.) stable flies (Kitching and Mellor, 1986) and LSDV by *Aedes aegypti* (L.) mosquitoes (Chihota et al., 2001). Mechanical or intrastadial transmission of LSDV by *Rhipicephalus appendiculatus* ticks has also been reported (Tuppurainen et al., 2013). The transmission of LSDV by direct contact is considered to be inefficient, while transmission may also occur, to a lesser extent, via common water troughs or feed contaminated by infected saliva or nasal discharge (Coetzer, 2004). Similarly, LSDV has been isolated from bovine semen (Irons et al., 2005), and seminal transmission has been experimentally demonstrated (Annandale et al., 2013). Some wild ruminant species are known to be susceptible to LSDV infection, but the role of wildlife species as potential reservoirs for LSDV is currently unknown (Barnard, 1997; Greth et al., 1992; Hedger and Hamblin, 1983). *Rhipicephalus decoloratus* ticks are known to transmit anaplasmosis (*Anaplasma marginale*), bovine babesiosis/African redwater (*Babesia bigemina*), and borreliosis (*Borrelia theileri*) (Norval and Horak, 2004).

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The epidemiology of LSDV is not fully understood. In endemic countries, sporadic outbreaks of LSD occur in remote locations without links to animal movements (Weiss, 1968). If LSDV was able to be transmitted transovarially in tick vectors, persistence of the virus in tick eggs laid in soil or vegetation would contaminate the environment, and infected larvae would be a potential source of infection for susceptible domestic and wild ruminants. This could 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. Other transovarially transmitted viruses include Kyasanur Forest disease, Louping ill (Swanepoel, 1968), and tick-borne encephalitis (Lindquist and Vapalahti, 2008) viruses in the *Flaviviridae* family, Crimean-Congo haemorrhagic fever, Nairobi sheep disease, and Uukuniemi viruses in the *Bunyaviridae* family, and African swine fever virus in the *Asfarviridae* family (Nuttall et al., 1994). In this paper, we examine the potential for transovarial transmission of LSDV in the African blue tick (*R. decoloratus*).

Materials and methods

Cattle hosts and experimental design

Four seronegative Bonsmara cross heifers from a herd in which vaccination against LSDV was not practised were used as experimental hosts. The cattle were approximately 13 months of age and 250 kg in body weight. Two of them were used as donors for LSDV (DB1 and DB2), and two were used as recipient animals (RB2 and RB3). The experiment was carried out in the insect-proofed, high-containment animal facility of the University of Pretoria's Biological Research Unit (UPBRC), Faculty of Veterinary Science, Onderstepoort, South Africa. Donor cattle were infected in October 2010 and 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. The recipient animals RB2 and RB3 were brought into the facilities in the beginning of February 2011, and during the time that the recipient cattle were housed at the insect-free isolation unit, there were no animals infected with LSDV or other capripoxviruses. All the experimental procedures were approved by the Animal Use and Care Committee of the University of Pretoria.

Both donor animals were infected by intravenous (IV) as well as intradermal (ID) routes with a virulent South African LSDV field isolate (248/93) which had been passaged 5 or 6 times on primary bovine dermis cell cultures and was used at a titre of 5.95 log $TCID_{50}$/ml. A volume of 2.5 ml was inoculated into the jugular vein, and 0.25 ml was injected ID at 4 sites on the back of the donor animals DB1 and DB2. The donor animals were then monitored closely for clinical signs, and body temperatures were recorded daily. Blood samples (EDTA and serum) were collected at 0, 4, 7, 9, 10, 11, 14, 15, 16, 17, 18, 21, 22, 23, and 24 days post infection (dpi).

Uninfected *R. decoloratus* larvae were reared at the Agricultural Research Council's Onderstepoort Veterinary Institute (ARC-OVI), South Africa. Ticks were fed on donor and recipient animals inside cotton cloth bags which were glued using Genkem Contact Adhesive Glue to shaved skin on the back of the animals. After experimental infection with LSDV, the skin bags were placed to cover the intradermal inoculum sites of LSDV. Larvae (approximately 2500) produced by 5 *R. decoloratus* female ticks were placed to feed on each donor animal at 3 dpi. This ensured that the ticks were feeding on viraemic donor animals. Approximately 30 fully fed females were harvested between 23 and 29 dpi by collecting them from the bottom of the skin bags. The females were then washed with phosphate buffered saline, placed into plastic containers (one female per tube) and maintained at 28 °C and 85% relative humidity until oviposition. Larvae hatched from these eggs were kept in the incubator for 2 months for maturation to complete and were then transferred to feed on naïve recipient animals (approximately 2500 larvae per animal). (Note that for recipient animals the day of attachment of potentially infected *R. decoloratus* larvae is referred to as 0 dpi.)

The recipient animals were closely monitored for clinical signs of disease and their body temperatures were recorded daily. Blood samples (EDTA, heparin, and serum) were collected from recipient animals at 0, 4, 5, 6, 7, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 24, 25, 26, 27, 28, and 31 dpi. Skin samples were collected from recipient animals under local anaesthesia from skin lesions and tick feeding sites using appropriate aseptic surgical techniques and a 0.6- or 0.8-cm diameter biopsy punch (Kruuse, Sherburn in Elmet, UK). Skin samples were taken from recipient animal RB2 at 0, 19, and 28 dpi and from RB3 at 0 and 27 dpi.

Real-time polymerase chain reaction (PCR)

The presence of viral DNA in blood and skin samples was quantified using a real-time PCR. Briefly, DNA was extracted using either QIAamp®All Nucleic Acid Kit MDx Kit (Qiagen, Crawley, UK) and robotic extraction techniques (Qiagen BioRobot Universal System) (Stubbs et al., 2012) or a manual phenol:chloroform:isoamylalcohol extraction method (Tuppurainen et al., 2011). Primers and a probe (Bowden et al., 2008) were used in combination with a QuantiFast™ Probe PCR Kit (Qiagen, Crawley, UK) in a Mx3005p Multiplex Quantitative PCR System (Strategene, Netherlands).

Virus isolation (VI)

VI from skin and blood samples was carried out on bovine dermis primary LB9.D cells (LGC Promochem, Teddington, UK) in 25-cm^2 tissue culture flasks or 12-well tissue culture plates (Cellstar, Greiner Bio-One, Stroudwater, UK) as previously described (Tuppurainen et al., 2005). Cell cultures showing no cytopathic effect (CPE) were blind-passaged once or twice. In addition to the observation of CPE in infected cell culture, the presence of LSDV was confirmed using real-time PCR.

Serum neutralization test (SNT)

Serum samples were tested for neutralizing antibodies using a constant-virus/varying-serum neutralization test (Beard et al., 2010). The positive control serum, collected on 37 dpi from cattle experimentally infected with LSDV that exhibited severe clinical disease, was produced at The Pirbright Institute, UK. Lumpy skin disease is exotic to the United Kingdom, and the negative control serum was collected from healthy cattle in the UK. Titres were determined as the last dilution that gave a 50% end point.

Results

Donor animals

Both donor animals showed mild clinical signs of LSD. Donor animal DB1 did not develop any skin lesions, whereas donor animal DB2 developed nodules on the side of the neck (Fig. 1), which disappeared within a few days. No skin nodules, other than those at the inoculation sites of the LSDV were detected in these animals inside the skin bags. The precrural and subscapular lymph nodes of DB1 started to enlarge at 7 dpi and those of DB2 at 9 dpi. Blood samples collected from donor animal DB1 tested PCR-positive on the days 4–11 pi. Blood cycle threshold values (C_t values) varied between 34 and 37 (Table 1). Donor animal DB2 was PCR-positive from 4 to 14 dpi. Blood C_t values varied between 34 and 39 (Table 1). The high C_t values observed in the donor animals indicated that only

Fig. 1. Skin lesions detected in an experimentally infected donor animal used for the oral infection of female *Rhipicephalus decoloratus* ticks.

Fig. 2. Skin lesions detected in a recipient animal (RB2) 18 days after the attachment of infected *Rhipicephalus decoloratus* larvae.

low levels of viral DNA were present in the blood of both donor animals after the experimental inoculation of a virulent LSDV isolate via the IV and ID routes. The onset of viraemia at 4 dpi (measured by PCR) was also associated with a short peak in body temperature (DB1 39.8 °C and DB2 39.9 °C degrees). Both donor animals seroconverted during 16–27 dpi.

Recipient animals

In both recipient animals (RB2 and RB3), a short peak in body temperatures (39.4 and 39.1 °C degrees, respectively) was observed at 18–20 days post-attachment of *R. decoloratus* larvae. In recipient animal RB2, enlargement of the precrural lymph nodes was suspected from as early as 4 dpi, and by 12 dpi the subscapular lymph nodes were also enlarged. Blood and skin samples collected from RB2 and RB3 prior to the attachment of ticks (0 dpi) tested negative. Blood samples taken from recipient animal RB2 tested real-time PCR-positive from 18 to 24 dpi. C_t values varied between 35 and 39 (Table 1). The blood sample collected from RB2 at 24 dpi was inoculated onto bovine dermis cells. In the first passage, no CPE was detected, but tissue culture supernatant tested positive by real-time PCR (C_t 38). In the second passage, CPE was observed, and the presence of the viral DNA was confirmed using the real-time PCR (C_t 36). Small skin lesions appeared on the side of the neck and around the feeding sites of the ticks of recipient animal RB2 at 18 dpi (Fig. 2). Skin lesions collected from the neck area of RB2 at 19 dpi tested real-time PCR-positive (C_t values 38 and 39). Excessive salivation was observed at 21 dpi in RB2, and small erosions

were detected in the mucous membranes of the mouth and on the edge of the nostrils at 26 dpi.

The precrural and subscabular lymph nodes of recipient animal RB3 started to enlarge at 14 dpi. Blood samples from this animal tested PCR-positive at 20 dpi and remained PCR-positive until 28 dpi. Blood C_t values varied between 33 and 39 (Table 1). Small skin lesions appeared on the neck of recipient animal RB3 and adjacent to the feeding sites of the larvae at 18 dpi. Skin biopsies, collected adjacent to feeding sites of the larvae at 27 dpi, tested positive by real-time PCR (C_t values varied between 32 and 35). Neither recipient animal RB2 nor RB3 produced antibody levels detectable using SNT. No live virus was isolated from skin samples collected from recipient animals RB2 and RB3.

Discussion

It has previously been reported that after feeding on experimentally infected cattle, *R. decoloratus* females and subsequently their eggs tested positive by a conventional PCR method (Tuppurainen et al., 2011). In the current study, it was shown that infected *R. decoloratus* females were able to pass the infection vertically, via their eggs, to the next generation of larvae, which in turn were able to transmit the virus to their bovine hosts, causing mild clinical LSD in naïve recipient animals. Despite washing the fully fed females prior to oviposition, the possibility of surface contamination of the newly hatched larvae, originating from the surface of adult female ticks or from their faeces, cannot be totally excluded. Currently very little, if any, data are available about the survival of poxviruses in tick vectors, and it is not known if infected ticks secrete the virus into their faeces. Although poxviruses are known to be stable and to survive for long periods inside desiccated crusts shed from healing skin lesions into the environment, survival of infectious LSDV for 3 months in the tick containers is unlikely to occur, and therefore surface contamination of the larvae is highly unlikely.

R. decoloratus are common in southern Africa, infesting mainly cattle, but also small ruminants and some wild ungulates. The life cycle of this tick species is short (approximately 3 weeks on and 5 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), which coincides with the peak of LSD cases.

The development of *R. decoloratus* larvae to nymphs and then to adults occurs on the skin of the same host (Norval and Horak, 2004). In order to enhance the possibility of oral infection of the *R. decoloratus* females by LSDV, the ticks were allowed to feed

Table 1
Real-time polymerase chain reaction (PCR) results of blood samples collected from experimentally infected donor animals (DB1 and DB2) and from recipient animals (RB2 and RB3) infected by *Rhipicephalus decoloratus* larvae.

dpi	DB1	DB2	dpi	RB2	RB3
0–3	n/c	n/c	0–17	No C_t	No C_t
4	34.21	36.41	18	38.48	No C_t
5	n/c	n/c	19	35.39	No C_t
6	n/c	n/c	20	No C_t	39.46
7	35.37	34.9	21	38.52	38.65
8	n/c	n/c	22	n/c	n/c
9	36.0	34.32	23	n/c	n/c
10	37.31	36.38	24	38.55	No C_t
11	36.29	37.53	25	No C_t	No C_t
12	n/c	n/c	26	No C_t	32.89
13	n/c	n/c	27	No C_t	No C_t
14	No C_t	38.62	28	No C_t	38.07

on the intradermal inoculation sites of the virus on donor animals. *R. decoloratus* larvae were placed on experimentally infected donor animals at 3 dpi. One donor animal (DB1) was real-time PCR-positive on the days 4–11 pi, and the second donor animal (DB2) was PCR-positive from 4 to 14 dpi. As the feeding pattern of the larvae usually takes 5–9 days, it can be assumed that these larvae fed on the hosts when they were viraemic. Moulting from larvae to nymphs takes for 1–2 two days, and nymphs usually feed for 5–9 days prior to moulting to adults (Arthur and Londt, 1973). Consequently, the nymphs may or may not have been feeding on the donor animals during the viraemic period. However, when the adult female ticks emerged and commenced feeding, it is highly likely that both donor animals were no longer in the viraemic phase.

In experimentally infected animals, live LSDV has also been isolated from healthy looking skin (Weiss, 1968) and for up to 39 dpi in skin lesions (Tuppurainen et al., 2005) that are known to contain higher titres of virus than the blood (Babiuk et al., 2008b). In this study, we were not able to determine if each life cycle stage of the ticks became infected separately or whether the virus survived the moulting process from one stage to the next. Further work is required to investigate the kinetics of infection and transmission dynamics of LSDV in ticks.

In experimentally infected animals, the incubation period for LSD varies from 4–7 days (Coetzer, 2004), as was observed in the donor cattle in this study. Recipient animals RB2 and RB3, infested with *R. decoloratus* larvae, however started to show evidence for viraemia considerably later, at 18 and 20 dpi, respectively. This longer incubation period may be a consequence of the smaller volume of live virus inoculated into the host by infected larvae, compared to high viral loads inoculated experimentally. However, no significant difference in blood C_t values were detected between the blood samples collected from the donor and the recipient animals, indicating that the levels of virus in the blood were similar in the donor and recipient animals. The high C_t values detected in the blood samples of both donor and recipient animals during the viraemic period (Table 1) corroborate also with earlier studies in which experimentally infected animals showed mild clinical disease and low titres of virus in the blood (Tuppurainen et al., 2013).

Both recipient animals developed characteristic clinical signs of LSD, namely small lesions in the skin around the tick feeding sites and also outside the skin bags on the side of the neck (Fig. 2). Markedly enlarged subscapular and precrural lymph nodes were detected in both recipient animals. Clinical disease in these 2 recipient animals was mild, and the skin lesions did not develop into deep ulcers. Donor animal DB2 developed identical small skin lesions as those of the recipient animals during the course of the disease (Fig. 1). In addition, recipient animal RB2 showed excessive salivation and small erosions on the muzzle, both typical signs of LSD (Coetzer, 2004). The recipient animals were free of disease on arrival and were housed in insect-free bio containment facilities in which no other ruminants infected with LSDV or other capripox viruses could serve as a source of infection through the feeding or handling of animals. Therefore, the detection of viral DNA in blood samples and skin nodules, both around the feeding sites of the larvae and outside the skin bags, confirmed the transmission of the virus by the *R. decoloratus* larvae and indicated that the virus had entered the skin located outside the skin bags, via the blood circulation.

The C_t values of all the skin and blood samples collected from donor and recipient animals were high throughout the experiment. It is generally difficult to isolate live LSDV from samples with very low viral copy numbers. Live virus was successfully isolated from only one of the blood samples, collected from recipient animal RB2 at 24 dpi. Neither of the recipient animals developed antibody levels detectable using SNT. It is, however, known that the immunity

against LSDV is predominantly cell-mediated, and 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).

The mild clinical disease that developed in the donor cattle is possibly an indication that either the field isolate (248/93) 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). The Bonsmara breed of cattle are taurine/zebu hybrid that has 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.

During this study, attempts to transfer *R. decoloratus* males from infected to a naïve host were not successful. Depending on farming practises, the potential for LSDV-infected animals suffering from severe tick infestations, to shed fully engorged, infected *R. decoloratus* females over large areas is high. Each female tick can produce 1000–2000 eggs resulting in a relatively small number of infected animals being able to contaminate communal pastures, grazing lands or areas around watering holes with thousands of infected larvae. These larvae could then serve as a continuous source of infection to susceptible domestic and wild ruminants, potentially resulting in the circulation of the virus without evidence of clinical disease, particularly in wild ruminants. In addition, this would make the eradication of the disease from the environment difficult, or even impossible. The survival of LSDV in tick populations may at least partly provide an answer to the question of how the virus survives in the environment for the extended periods of time observed between outbreaks of LSD. The importance of *R. decoloratus* vectors in the transmission of LSDV in a field setting remains to be investigated in detail.

The number of recipient animals used in this experiment was insufficient to provide valid statistical data on this mode of transmission of LSDV by tick vectors. The findings of this study strongly emphasize the importance of carrying out regular vaccination campaigns against LSDV and using tick prevention treatments in cattle in LSD-endemic areas. These results also contribute to the better understanding of the epidemiology of LSDV as a tick-borne poxvirus.

Acknowledgements

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