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Mechanical transmission of lumpy skin disease virus by *Aedes aegypti* (Diptera: Culicidae)

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SUMMARY

Aedes aegypti female mosquitoes are capable of the mechanical transmission of lumpy skin disease virus (LSDV) from infected to susceptible cattle. Mosquitoes that had fed upon lesions of LSDV-infected cattle were able to transmit virus to susceptible cattle over a period of 2–6 days post-infective feeding. Virus was isolated from the recipient animals in 5 out of 7 cases. The clinical disease recorded in the animals exposed to infected mosquitoes was generally of a mild nature, with only one case being moderate. LSDV has long been suspected to be insect transmitted, but these findings are the first to demonstrate this unequivocally, and they suggest that mosquito species are competent vectors.

INTRODUCTION

Capripoxviruses cause economically significant diseases of sheep (sheep pox), goats (goat pox) and cattle (lumpy skin disease, LSD). The diseases are characterized by pyrexia, generalized or localized pock lesions and lymphadenopathy [1, 2]. LSD, an OIE list A disease, causes major production losses, especially in high-producing exotic breeds, and is a constraint on trade [3]. Sheep and goat pox are endemic throughout Asia and Africa while LSD is restricted to Africa, with only one confirmed report from outside this continent in Israel in 1989 [4, 5]. Epidemiological evidence indicates the involvement of biting insects in the transmission of lumpy skin disease virus (LSDV) [6, 7]. The incidence of disease is highest during wet periods coinciding with periods of biting fly abundance and wanes with the onset of the dry season. In the 1959 Kenyan outbreak of LSD, there were reports of high infestation of *Aedes natronius* and *Culex mirificus* during some outbreaks [7]. Similarly, the 1989 Israeli outbreak of capripox is thought to have been the result of infected *Stomoxys calcitrans* being carried on the wind from Ismailiya in Egypt [4, 5]. *Stomoxys calcitrans*, has also been shown to mechanically transmit capripoxvirus between sheep in the laboratory [8–10]. Mechanical transmission of a number of poxviruses by biting arthropods is well documented, and includes myxoma virus where *Ae. aegypti* has been identified as an important vector [11]. Mosquitoes have also been shown to mechanically transmit Shope fibroma virus and fowl pox virus [12, 13]. The present study was undertaken to determine whether *Ae. aegypti* can act as an efficient mechanical vector of LSDV.

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MATERIALS AND METHODS

Virus isolate

The LSDV isolate used in this study was supplied by Dr J. A. House (Plum Island Animal Disease Centre, USA) and was originally recovered from cattle in an outbreak in Ismailiya, Egypt in 1989. The virus was grown on lamb testis (LT) cell cultures and harvested when 90% of the cells showed cytopathic effect (CPE). The LT cultures were freeze-thawed three

times, clarified by low speed centrifugation (200 g, 15 min) and stored at −20 °C until required.

Cell cultures

Primary LT tissue cultures were prepared from the testes of prepubertal lambs [14]. LT cells were grown in 175 cm^2 plastic tissue culture flasks in 50 ml of Glasgow Modified Eagle's Medium (GMEM) supplemented with 10% foetal calf serum and antibiotics. The flasks were kept at 37 °C and 4% CO_2. Once a confluent monolayer was formed, the cells were split at a ratio of 1:3. The LT cells were used to a maximum of 10 passages, to ensure sensitivity to capripoxvirus.

Virus isolation

Blood samples collected in heparinized vacutainers were placed in plastic universal bottles and centrifuged at 600 g for 20 min. The buffy coats were pippeted off and carefully placed in fresh universal bottles to which 20 ml of cold GMEM had been added. The bottles were then centrifuged at 600 g for 20 min and the supernatants decanted. Red blood cell lysis was achieved by adding 10 ml of cold double-distilled water followed by 10 ml of cold 2 × GMEM and the universal bottles were centrifuged at 600 g for 20 min. This was repeated until clear pellets were obtained. The pellets were then resuspended and overlaid on LT cell cultures, which were observed for signs of CPE at 9 days. If no CPE was observed a second passage was carried out.

Virus neutralization index

Blood samples from experimental animals were collected in non-heparinized vacutainers on day 31 post mosquito feeding. The sera were separated, diluted 1:5 with GMEM and then heat inactivated at 56 °C for 30 min. Aliquots of 100 μl of the diluted sera were placed in each well of 96-well microtitre plates. Serial tenfold dilutions of LSDV (Ismailiya) from neat to 10^{-8} were prepared in GMEM. To each well of the plates, 100 μl aliquots of the virus dilutions were added using four replicates per dilution and, following incubation at 37 °C for 1 h, LT cells (50 μl) were added to each well at a concentration of 4×10^5 cells/ml. The wells were examined for evidence of CPE by light microscopy after 7 days and the end-points determined [15]. The virus neutralization index was taken as the difference between the titre in the presence of test serum and day 0 serum from the same animal. An index greater than or equal to 1·5 was considered positive [16].

Infection of animals and mosquitoes

Two steers (a Holstein–Friesian and an Angus cross Jersey) were inoculated at six sites each on the neck, flank and abdomen with 1 ml of LSDV inoculum per site delivered intradermally/subcutaneously. The titre of the LSDV inoculum was $10^{5·5}TCID_{50}$/ml. All animals were housed in biosecure isolation facilities. Animal attendants wore rubber protective clothing, which was washed with an iodophor disinfectant (FAM, Evans) before moving between animal boxes, and before leaving the isolation unit.

One-week-old adult female *Ae. aegypti* mosquitoes, in cardboard cages with fine mesh screen tops, were fed to engorgement through the netting on LSD lesions of the infected steers 3 days after the appearance of the lesions. Approximately 50 *Ae. aegypti* females were allowed to feed on a lesion. The blood-fed females were then selected in the laboratory, and placed in separate cages and any non blood-fed females were discarded. The mosquitoes were maintained at 25 °C, 60% humidity and were fed on a 10% sucrose solution, given daily, which was placed on a cotton pad on the terylene netting tops of their cages. To encourage oviposition, filter paper was placed on top of moistened cotton wool in a tubular support at the base of the cages.

Transmission of virus was then attempted by allowing the potentially infected mosquitoes to feed on six susceptible cattle at various times post-infective feeding. Transmission was confirmed by recording clinical signs of LSD or recovering live virus from lesion material or the blood of susceptible animals.

DNA extraction from mosquitoes

After an infected blood meal, 10 engorged mosquitoes were removed from the holding cages. Of these, 5 mosquitoes were subjected to the DNA extraction protocol of Cheung et al. [17]. The DNA extracted was assayed by the polymerase chain reaction (PCR) method described by Ireland and Biinepal (1999) [18]. Five mosquitoes that had previously engorged were removed from the holding cages on each of days 2, 3,

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Table 1. *The clinical response of animals exposed to LSDV infected mosquitoes measured using the following scale*

Reaction score	Generalization	Lymph node enlargement	Local lesion	Other comments
Mild	−	+	Transient	Transient reaction at inoculation site Mild regional lymphadenopathy No systemic disturbance 2–3 secondary lesions Heat and pain at inoculation site
Moderate	+/−	+++	< 6 cm diam	Pyrexia and depression Conjunctivitis and rhinitis Severe general lymphadenopathy Anorexia Severe general lymphadenopathy
Severe	++++	++++	> 6 cm diam	Severe conjunctivitis/rhinitis Numerous secondary papules Humane destruction necessary

4, 5 and 6 post-feeding on an infected animal. DNA was similarly extracted from these mosquitoes, and assayed by PCR.

Experimental design

Non-heparinized blood was collected for serology from a healthy Holstein-Friesian and five Angus cross Jersey steers. The animals were then exposed to potentially infected mosquitoes at intervals of 48 h, 3, 4, 5 and 6 days post-infective feeding. Non-heparinized blood from each susceptible steer was collected for serology immediately before mosquito feeding took place. The susceptible animals had four shaved feeding sites on their necks and flanks. A total of approximately 200 mosquitoes were allowed to feed to engorgement on the four shaven sites on the neck and flank of each animal. Feeding was deemed to be complete when probing activity ceased. The susceptible animals were then monitored for clinical signs of LSD. Blood samples for serology and virus isolation were collected daily. The animals were scored on the severity of the clinical reaction to infection with the mosquito-transmitted virus (Table 1). Five mosquitoes from each batch were assayed for LSDV by PCR and virus isolation immediately post re-feeding.

RESULTS

Table 1 shows the criteria used to determine the severity of disease in infected animals. The clinical response to LSD, of cattle exposed to the bites of infected mosquitoes is summarized in Table 2. Five out of six susceptible animals developed clinical LSD after infected mosquitoes were allowed to feed on them. Four out of five susceptible animals (TW6-9) developed mild clinical LSD, mainly localized swelling at feeding sites with regional lymphadenopathy. Steer TW10 did not develop clinical LSD. However, steer TU39 showed a more severe response with the development of conjunctivitis, rhinitis and pyrexia lasting 3 days. Also, a swelling was observed on one feeding site 24 h later and this developed into a nodule about 3 cm in diameter. Primary lesions were seen on two other feeding sites. At 15 days post-feeding, three secondary lesions were observed around the first feeding site. The primary lesion developed into an ulcer, which subsequently formed a scab. Using the reaction score system outlined in Table 1, steer TU39 developed moderate LSD. Table 2 summarizes the results of virus isolation from the buffy coat of the animals in the study. Virus isolation and PCR both detected capripoxvirus confirming transmission of LSDV from 48 h up to 6 days post-feeding by infected mosquitoes. One animal (TW10) did not develop LSD lesions but virus was detected in the buffy coat showing that transmission of LSDV had occurred. The virus neutralization index showed 3 animals with a neutralization index of greater than $\log_{10}1{\cdot}5$, which is considered indicative of exposure and response to LSDV. Table 3 shows the results of PCR and virus isolation from samples of mosquitoes immediately after the infective feed and just before re-feeding on susceptible animals. It is evident that many mosquitoes were able to retain virus for a period up to 6 days post feeding on an infected animal. Table 3 also shows that mosquitoes acquired a mean infection of $10^{3{\cdot}3}$ $TCID_{50}$ of virus/mosquito on day 0 immediately

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Table 2. *Summary of the responses of animals in experiments 1 and 2 following exposure to LSDV infected mosquitoes*

Steer ID	PCR*	Virus isolation	Clinical score	VNI†	Duration over which transmission was recorded
TU39	+(day 6)	+	Moderate	1·7	48 h
TW6	+(day 8)	+	Mild	1·5	48 h
TW7	+(day 7)	+	Mild	1·5	3 days
TW8	+(day 9)	+	Mild	1·4	4 days
TW9	+(day 10)	+	Mild	1·0	5 days
TW10	+(day 10)	+	No reaction	0·5	6 days

* Figures in parentheses refer to days post feeding that viral DNA was detected by PCR.
† VNI, virus neutralization index.

Table 3. *Results of polymerase chain reaction and virus isolation on mosquitoes fed on an LSDV infected steer**

		Aedes aegypti		
		PCR	Virus isolation Virus titre per mosquito (log_{10})	
Day	Transmission of LSDV to susceptible animal	No infected/ no tested	Range	Mean
0†	Not tested	5/5	2·6–4·0	3·3
2	Yes	3/5	2·9–3·9	3·3
3	Yes	4/5	2·0–2·6	2·4
4	Yes	4/5	2·0–2·7	2·4
5	Yes	2/5	2·3–2·5	2·4
6	Yes	3/5	2·5–2·8	2·5

* Virus content of primary lesion: $10^{6·3}TCID_{50}$/gm tissue.
† Mosquitoes tested immediately after their infective feed on day 0.

after feeding on an infected animal. By day 6, mosquitoes retained an average of $10^{2·5}$ $TCID_{50}$/mosquito. All the groups of mosquitoes sampled from 48 h to 6 days post re-feeding on infected steers were able to transmit LSDV to susceptible cattle.

DISCUSSION

Biting insects have long been implicated in the transmission of LSDV on epidemiological grounds, and several studies have confirmed that the transmission of the virus in an arthropod-free environment does not occur [19, 20; Alexander, personal communication]. In this context, mosquito species are ideal vector candidates since they tend to be intravenous feeders, and previous studies have demonstrated that the intravenous inoculation of LSDV into cattle predisposes to generalization and severe disease [20]. However, until now no data has been published that proves, definitely, that any species of arthropod can transmit LSDV from infected to susceptible vertebrate hosts.

The results of the present investigation show that LSDV can be transmitted by *Ae. aegypti* from infected to susceptible cattle, for at least 6 days after the mosquitoes' infection. Since other work (Chihota, unpublished observations) indicates no evidence of virus replication in the insect vector, the mode of transmission must be mechanical. These findings provide a significant addition to our understanding of the epidemiology of LSD, as this is the first time that transmission of LSDV, by an insect vector, has been confirmed. Although virus has been isolated from biting insects before, attempts at transmission to susceptible animals have been unsuccessful [19]. Previously, speculation on the transmission of LSDV by biting insects had envisaged the mechanism as being merely a short-term 'dirty needle' type of transference, but the results presented in this study suggest otherwise. Virus is able to survive in infected

mosquitoes for periods of at least 6 days without an appreciable loss in titre and is then able to be transmitted. This implies that virus could be localized at a site within the mosquito vector where it is protected from inactivation and suggests a far more complex mode of transmission than a mere 'dirty pin'. Indeed, earlier work has shown that *Ae. aegypti* can harbour infectious LSDV for periods up to 9 days post-infection (Chihota, unpublished observations). These aspects of the relationship between LSDV and its insect vector(s) clearly require further investigation.

This study strongly suggests that *Ae. aegypti* and possibly other biting insects are likely to be involved in the inter-herd spread of LSDV. The study has also shown that it is possible to transmit LSDV by *Ae. aegypti* to susceptible animals without the subsequent development of clinical disease in those animals and this may represent an important aspect of the epidemiology of LSD. Diagnosis of LSD in the field is usually based on observing clinical signs of disease in cattle, but sub-clinically infected animals may also provide additional, "covert" foci of infection for mosquitoes or other biting insects.

To date, *Ae. aegypti* has been shown only to transmit LSDV under experimental conditions. Whether it is also involved in the transmission of this virus in the field remains to be investigated, but should this prove to be the case, the risk of LSD spreading to areas beyond Africa, where *Ae. aegypti* also occurs, may need to be reassessed. In the light of the present work, it would be essential to consider the control of biting flies in the face of LSD outbreaks.

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