

Potential role of ticks as vectors of bluetongue virus

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Received: 22 February 2010 / Accepted: 8 March 2010 / Published online: 1 April 2010
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Abstract When the first outbreak of bluetongue virus serotype 8 (BTV8) was recorded in North-West Europe in August 2006 and renewed outbreaks occurred in the summer of 2007 and again in 2008, the question was raised how the virus survived the winter. Since most adult *Culicoides* vector midges are assumed not to survive the northern European winter, and transovarial transmission in *Culicoides* is not recorded, we examined the potential vector role of ixodid and argasid ticks for bluetongue virus. Four species of ixodid ticks (*Ixodes ricinus*, *Ixodes hexagonus*, *Dermacentor reticulatus* and *Rhipicephalus bursa*) and one soft tick species, *Ornithodoros savignyi*, ingested BTV8-containing blood either through capillary feeding or by feeding on artificial membranes. The virus was taken up by the ticks and was found to pass through the gut barrier and spread via the haemolymph into the salivary glands, ovaries and testes, as demonstrated by real-time reverse transcriptase PCR (PCR-test). BTV8 was detected in various tissues of ixodid ticks for up to 21 days post feeding and in *Ornithodoros* ticks for up to 26 days. It was found after moulting in adult *Ixodes hexagonus* and was also able to pass through the ovaries into the eggs of an *Ornithodoros savignyi* tick. This study demonstrates that ticks can become infected with bluetongue virus serotype 8. The transstadial passage in hard ticks and transovarial passage in soft ticks suggest that ticks have potential vectorial capacity for bluetongue virus.

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Further studies are required to investigate transmission from infected ticks to domestic livestock. This route of transmission could provide an additional clue in the unresolved mystery of the epidemiology of Bluetongue in Europe by considering ticks as a potential overwintering mechanism for bluetongue virus.

Keywords Ticks · Bluetongue virus · Artificial infection · In vitro feeding

Introduction

Bluetongue is a non-contagious infectious disease transmitted by the bites of *Culicoides* midges and affects domestic livestock with clinical signs ranging from apathy and weight loss to swollen heads, tender feet and death (Verwoerd and Erasmus 2004). Bluetongue virus (BTV) is a double stranded RNA-virus, belonging to the genus *Orbivirus* of the family *Reoviridae* with 24 different known serotypes (Verwoerd and Erasmus 2004; Mellor and Boorman 1995). In August 2006, bluetongue virus serotype 8 (BTV8) emerged and spread rapidly in North-West Europe through local vectors (Enserink 2006), and clinical cases disappeared in early January 2007. The re-emergence of the outbreak many months later affected a large number of holdings and clearly demonstrated that BTV8 had somehow survived the winter. This most economically damaging outbreak of bluetongue ever seen came to a halt towards the end of 2007. In the summer of 2008 new outbreaks of BTV8 occurred again in large parts of North-West Europe (Schwartz-Cornil et al. 2008). The ability of BTV to “overwinter” has been recognised for decades in other parts of the world (Nevill 1971). In South-Africa transmission is interrupted when both *Culicoides* midge activity as well as virus replication in the vector midge ceases at cool temperatures (Nevill 1971). However, if outbreaks resume only after several months, far longer than the lifespan of the local adult *Culicoides* vector or the duration of viraemia in the mammalian host, the question is raised where the virus persists without detection during the winter months (Wilson et al. 2008).

Of the indigenous European *Culicoides* species, principally *Culicoides dewulfi*, species of the *obsoletus* complex and *Culicoides chiopterus*, are considered to be the main vectors of BTV8 (Nevill 1971; Wilson et al. 2008). Although it is assumed that BTV is unable to survive when most adult midges succumb during the North-West European winters, its overwintering ability may be enhanced in the local vectors (Dijkstra et al. 2008; Meiswinkel et al. 2007). Alternatively, because most species of *Culicoides* at northern latitudes survive the winter as larvae, it might appear that the most likely mechanism for overwintering is the vertical transmission of virus from infected vectors to offspring via the developing egg. However, experiments designed to look for vertical transmission of BTV in *Culicoides* have consistently given negative results (Mellor 1990).

Even if BTV may persist in local vectors or in various mammalian hosts, alternative vectors could also play a role. For instance, mechanical transmission of BTV has been reported in *Melophagus ovinus*, a wingless fly that lives in the fleece of sheep (Luedke et al. 1965).

Ticks have a much longer lifespan than *Culicoides* midges, and may also act as biological vectors of BTV. Interestingly, the vectorial capacity of the American soft tick *Ornithodoros coriaceus* for BTV has already been reported in 1985 (Stott et al. 1985). *O. coriaceus* ticks could become infected by feeding through an artificial membrane on BTV-containing cell culture material or on BTV-containing sheep blood. The ticks were also infected by feeding on viraemic sheep or cattle. It was also possible to transmit BTV

to cattle by soft ticks after feeding on BTV-containing cell culture material (Stott et al. 1985).

Ticks have a relatively long lifespan. *Ixodes ricinus*, the predominant ixodid tick in North-West Europe, feeds on a broad range of hosts including domestic ruminants and takes two to three years to complete its lifecycle. As a typical three-host tick, *I. ricinus* feeds in total approximately 3 weeks divided over three different hosts. The remaining time is spent in the vegetation. Other ticks, such as *Dermacentor* species complete one generation per year. The feeding behaviour of ticks on viraemic hosts on the one hand and the long-term survival of ticks off-host on the other hand, makes ticks excellent candidates to serve as viral reservoirs. In fact, there are many other viruses belonging to the genus *Orbivirus*, which are transmitted by ticks (Labuda and Nuttall 2004).

The objective of this work was to explore the potential role of European ticks as vectors of BTV8. To this end, four species of ixodid (hard) ticks, *I. ricinus*, *Ixodes hexagonus*, *Dermacentor reticulatus*, and *Rhipicephalus bursa*, were artificially infected with BTV8 containing blood through capillary feeding, in order to determine whether the virus could be maintained in these tick species. Moreover, *I. ricinus* ticks and the soft tick *Ornithodoros savignyi* were infected with BTV8 containing blood by feeding on artificial membranes.

Materials and methods

Ticks

Both hard (Ixodidae) and soft (Argasidae) ticks were used in this study. Three species of ixodid ticks were selected on the basis of their feeding preferences for livestock prevalent in North-West Europe (Estrada-Peña et al. 2004). The most commonly found tick *I. ricinus* feeds on a broad range of hosts including cattle, sheep and goats (Estrada-Peña et al. 2004). Adult ticks used in this study originated from the vegetation at different locations in the province of Utrecht in the Netherlands. *Ixodes hexagonus*, a tick commonly found on hedgehogs but also on domestic animals, was obtained from a laboratory colony maintained at the Utrecht Centre for Tick-borne Diseases (UCTD) and originated from an engorged female tick collected from a dog. *Dermacentor reticulatus* adults and nymphs were also available from the UCTD colonies. These ticks originated from the vegetation in the South-West of the Netherlands, where this tick had recently been discovered (Nijhof et al. 2007). *Dermacentor reticulatus* feeds on a range of different hosts, including cattle, small ruminants and companion animals and occurs in typical habitats in much of Europe (Estrada-Peña et al. 2007). Furthermore, *R. bursa*, a two host tick widely distributed on cattle, sheep and goats in southern Europe, was also used in the experiments and was obtained from a laboratory colony of UCTD. In addition, the soft tick *O. savignyi* was used as a control in the sense that previous studies had already shown that *Ornithodoros* ticks are able to transmit BTV to cattle (Stott et al. 1985). *Ornithodoros savignyi* originated from a laboratory colony maintained at the University of Pretoria in South Africa. This tick does not occur in Europe, but is found on domestic animals in semi-arid regions of Southern and Eastern Africa (Hoogstraal 1956).

Laboratory-reared negative control ticks, never exposed to BTV8 and artificial feeding, were also included in each of the experiments described below.

BTV

Bluetongue virus serotype 8 was used to spike blood collected in heparin from a calf negative for BTV and BTV-antibodies. The blood was divided into 1 ml aliquots and stored at -80°C until used in the experiments.

Capillary feeding of ixodid ticks

Capillary feeding of different ixodid tick species has been reported and has also been applied to in vitro acquisition of pathogens by ticks (Burgdorfer 1957; Matsumoto et al. 2005; Rechav et al. 1999; Booth et al. 1991; Inokuma and Kemp 1998). In brief, four species of ixodid ticks were fed on a capillary, after feeding for 3 days on a rabbit. They were removed with forceps from the rabbit and placed on sticky tape with their ventral side facing upwards. BTV8-containing blood was thawed and heated to 36°C . A capillary was placed over the hypostome of adult ticks and, in the case of nymphs, the hypostome as well as one palp was inserted into the capillary. Subsequently, each capillary was filled with viraemic blood and attached to the tick with adhesive tape. Ticks and capillaries were incubated overnight at 37°C and 85% relative humidity. Thereafter, capillaries were carefully removed and the ticks were placed in an incubator at 27°C and 85% relative humidity. Several female ticks were allowed to lay eggs. All ticks were weighed before and after the capillary feeding in order to estimate the amount of ingested blood.

Membrane feeding of ixodid ticks

Voluntary feeding of ticks on artificial membranes mimics the natural feeding behaviour better than forced capillary feeding. Therefore, adult *I. ricinus* female ticks, partially fed for 4 days together with male ticks on a rabbit, were placed into a feeding unit consisting of a silicone-impregnated membrane as described by Kröber and Guerin (Kröber and Guerin 2007). Each feeding unit was placed into a well of a six-well plate containing BTV8-containing blood kept at 36°C during the feeding process. The blood was replenished every 12 h and *I. ricinus* females fed for 44 h after attachment until repletion.

Membrane feeding of argasid ticks

Adult *O. savignyi* ticks were allowed to engorge on an artificial membrane consisting of Parafilm[®] according to methods described by Schwan et al. (1991). The viraemic blood under the membrane was kept at a constant temperature of 36°C during the feeding process. Thereafter, ticks were weighed and placed in an incubator at 28°C and 80% relative humidity. Several female ticks were allowed to lay eggs.

Tick dissection

Ixodid ticks were dissected on various days post-feeding under ice-cold phosphate buffered saline (PBS), ventral side upwards and fixed with forceps in a small Petri dish. A transverse incision was made between coxa 2 and 3 and the salivary glands, gut, ovaries or testes, Malpighian tubules and remaining tissue were removed, washed in ice-cold PBS and stored in different vials, which were frozen in liquid nitrogen and stored at -80°C until processed by real time RT-PCR.

Soft ticks were fixed in a paraffin-containing Petri dish with their dorsal side upwards and dissected under ice-cold PBS. A circular incision was made to remove the dorsal part of the integument. Salivary glands, guts, ovaries, Malpighian tubules and remaining tissue were removed, washed in ice-cold PBS, frozen in liquid nitrogen and stored at -80°C in separate vials.

Furthermore, eggs, ixodid larvae and argasid N_1 nymphs were also frozen into liquid nitrogen and stored at -80°C until processed by real time RT-PCR.

Real-time RT-PCR

Various tissues dissected from the ticks as well as tick eggs, larvae and nymphs were tested for the presence of BTV8 by the real-time reverse transcriptase PCR assay (PCR-test). Briefly, viral dsRNA from 200 μl diluted (1:1 with PBS) tick sample was isolated using the Total Nucleic Acid Isolation Kit (Roche Diagnostics Nederland, Almere, Netherlands) using an isolation robot MagNa Pure (Roche Diagnostics Nederland) according to the instructions of the manufacturer. Isolated dsRNA samples were tested using primers (Eurogentec Nederland, Maastricht, Netherlands) and a Taqman probe (Tib MolBiol, Berlin, Germany) targeting segment 10 of the BTV genome. Real-time reverse transcriptase PCR was performed with a LightCycler RNA Master Hybridization Probes kit in a LightCycler 2.0 (Roche Diagnostics Nederland). Template RNA (5 μl) was added to a reaction mixture containing 0.25 μM of the forward and reverse primer, 0.25 μM probe, 2.75 mM MnCl_2 , 7.5 μl Kit and 0.2 μl RNAsin (RNAsin, 40 U/ μl ; Promega Benelux, Leiden, Netherlands) in a final volume of 20 μl .

Thermocycling conditions of the RRT-PCR were: 20 s 98°C , 20 min 61°C , 30 s 95°C , (1 s 95°C , 10 s 61°C , 15 s 72°C) \times 45 followed by 30 s 40°C and storage at 4°C . Amplification was monitored real time by OD530/OD640 ratio using software version 4.05 (Roche Diagnostics Nederland) and expressed as Cp. Cp is defined by the software version 4.05 (Roche Diagnostics Nederland). Briefly, Cp is the cycler number by which the line of the maximal increase of OD530/OD640 ratio is crossing the X-axis. Positive controls were included containing different virus dilutions of BTV1 grown on BHK21 cells in DMEM with 5% fetal bovine serum and diluted in the same growth medium. DNase-free phosphate-buffered saline (PBS) solution was used as negative control.

Results

BTV8 in ixodid ticks

Variable amounts of blood were ingested by each of the 24 female ticks during capillary feeding as demonstrated by an increase in weight which ranged between 1.8 and 74.2 mg. BTV8 was detected by PCR in 20 out of 24 (83.3%) female ticks infected by capillary feeding (Table 1). There was no obvious correlation between the amount of blood ingested and the PCR-results. The virus was found in the gut, ovaries, salivary glands, Malpighian tubules as well as in the remaining tissues derived from *I. ricinus*, *I. hexagonus*, *D. reticulatus* and *R. bursa* ticks, between 3 and 15 days after feeding on BTV8-containing blood. Virus was also detected in the ovaries of 15 out of 24 (62.5%) of female ticks of all four species tested (Table 1). Several engorged females were allowed to lay eggs. Larvae which hatched from eggs derived from six *D. reticulatus* females and from six *R. bursa*

Table 1 Infection of female ixodid ticks with BTV8 by capillary feeding

Tick ID number	Days post-feeding	Species of tick	Bloodmeal (mg) ^a	Gut (Cp) ^b	Ovaries (Cp)	Salivary glands (Cp)	Malpighian tubules (Cp)	Remaining tissue (Cp)
BT 20	3	<i>D. reticulatus</i>	4.3	27.27	– ^c	–	–	–
BT 23	3		19.0	27.17	–	–	–	26.87
BT 24	3		74.2	25.87	29.50	30.44	32.70	–
BT 187	11		33.2	28.03	30.98	30.22	32.70	–
BT 228	2	<i>Ixodes ricinus</i>	44.8	30.47	30.70	–	31.88	28.75
BT 1	5		20.1	29.81	–	–	35.72	28.90
BT 77	6		49.3	28.32	30.84	31.06	–	27.85
BT 79	6		38.0	28.73	30.3	–	–	27.62
BT 107	7		75.1	29.03	29.66	33.00	36.25	27.64
BT 108	7		74.2	28.27	30.53	30.54	–	26.80
BT 109	7		5.5	30.75	31.56	–	33.22	27.67
BT 175	12		68.1	32.60	34.19	33.88	34.46	28.87
BT 95	13		25.6	28.71	30.95	33.06	31.26	29.03
BT 97	13		19.7	29.86	31.86	31.30	37.00	27.21
BT 143	15		35.8	28.96	29.65	–	–	29.42
BT 166	14	<i>I. hexagonus</i>	36.9	30.18	–	34.77	33.99	–
BT 167	14		42.4	33.60	31.53	32.68	34.02	29.77
BT 147	15		7.8	32.44	–	31.73	–	–
BT 117	7	<i>R. bursa</i>	16.7	–	–	–	–	–
BT 119	7		2.5	–	–	–	–	–
BT 120	7		1.80	–	30.65	35.24	–	26.74
BT 160	14		18.6	29.19	31.58	32.61	31.04	28.93
BT 161	14		9.9	30.12	–	–	33.45	–
BT 172	19		3.1	–	–	–	–	–

^a The amount of ingested blood was determined by weighting each tick before and after capillary feeding

^b Cp, Crossing point, see “Materials and methods”

^c Negative

ticks were negative for BTV8. Eggs produced by one of the *I. hexagonus* female ticks were also negative.

Male ticks ingested only very small amounts of blood through the capillaries, with a maximum of 3 mg. Despite these small amounts of ingested blood, 6 out of 12 (50%) *D. reticulatus* males contained BTV8 in their gut, testis, salivary glands, Malpighian tubules and in their remaining tissues (Table 2). Also, two *I. ricinus* males contained virus in their gut, testis, salivary glands and also in the remaining tissues. One *D. reticulatus* tick contained virus in its salivary glands on day 5 after capillary feeding, whereas the salivary glands of one *I. ricinus* tick was positive on day 8 after feeding (Table 2).

Nymphs of *D. reticulatus*, *R. bursa* and *I. hexagonus* were also capillary fed on BTV8-containing blood. After the nymphs had moulted into the adult stage, they were also tested for BTV8. The gut of one out of two *I. hexagonus* adults contained BTV8 on 21 days after capillary feeding.

Table 2 Infection of male ixodid ticks by BTV8 by capillary feeding

Number	Days post-feeding	Species	Gut (Cp) ^a	Testes (Cp)	Salivary glands (Cp)	Malpighian tubules (Cp)	Remaining tissue (Cp)
BT 41	5	<i>D. reticulatus</i>	– ^b	–	–	–	–
BT 42	5		32.85	–	–	35.67	28.86
BT 43	5		–	–	–	–	26.55
BT 44	5		–	–	33.92	34.12	–
BT 46	5		–	–	–	–	–
BT 35	12		32.55	33.56	–	–	29.65
BT 36	12		–	–	–	–	–
BT 40	12		–	–	–	35.70	–
BT 45	12		–	–	–	ND	–
BT 37	16		–	–	–	–	–
BT 39	16		–	–	–	–	26.95
BT 47	16		–	–	–	–	–
BT 86	8	<i>I. ricinus</i>	40.00	36.90	35.96	–	32.19
BT 87	8		36.60	–	–	–	30.58

^a Cp, Crossing point, see “Materials and methods”

^b Negative

ND, not done

Table 3 Transstadial passage of BTV8 from nymphs to adult ixodid ticks after capillary feeding

Tick ID number	Days post-feeding	Tick species	Male/female	Blood-meal (mg) ^a	Gut (Cp) ^b	Ovaries/testes (Cp)	Salivary glands (Cp)	Malpighian tubules (Cp)	Remaining tissue (Cp)
BT 72	68	<i>D. reticulatus</i>	F	4.0	– ^c	–	–	–	–
BT 140	21	<i>I. hexagonus</i>	F	1.0	–	–	–	–	–
BT 141	21		M	0.2	34.04	–	–	–	–
BT 61	39	<i>R. bursa</i>	F	4.1	–	–	–	–	–
BT 62	39		F	1.4	–	–	–	–	–
BT 66	39		F	0.6	–	–	–	–	–
BT 56	40		F	4.2	–	–	–	–	–

^a The amount of ingested blood was determined by weighting each tick before and after capillary feeding

^b Cp, Crossing point, see “Materials and methods”

^c Negative

The other ticks, one *D. reticulatus* female tick and 4 female *R. bursa*, were negative (Table 3).

Furthermore, when *I. ricinus* female ticks fed on artificial membranes, two ticks acquired BTV8, which could be detected 15 days later in their gut, ovary, salivary glands and Malpighian tubules (Table 5).

Finally, laboratory-reared negative control ticks that were not exposed to BTV8 and artificial feeding were also included in the above described experiments as negative controls. All such control ticks were PCR-negative for BTV8.

Table 4 Transovarial passage of BTV8 in *Ornithodoros* ticks after feeding on membranes

Tick ID Number	Days post-feeding	Salivary glands (Cp) ^a	Gut (Cp)	Ovaries/Testes (Cp)	Malpighian tubules (Cp)	Remaining tissue (Cp)	Eggs (Cp)
BT 210	26	–	30.80	32.29	– ^b	31.42	–
BT 211	26	32.89	29.68	30.54	–	31.52	No eggs
BT 212	26	–	26.95	32.67	34.69	32.18	31.72
BT 213	26	33.52	30.35	32.94	–	30.21	No eggs

^a Cp, Crossing point, see “Materials and methods”

^b Negative

BTV8 in soft ticks

Four *Ornithodoros savignyi* females fed on artificial membranes were dissected 26 days after feeding. It was found that the ovaries, gut and remaining tissues of all 4 ticks contained BTV8. In addition, two out of four ticks were positive in the salivary glands. Interestingly, eggs produced by one female *Ornithodoros* tick were also infected by BTV8 (Table 4), but BTV8 could not be detected in the offspring (N_1 nymphs), which emerged from these eggs.

Discussion

Bluetongue virus serotype 8 was taken up by 4 different species of ixodid ticks, wherein the virus was able to maintain itself, pass through the gut barrier and spread via the haemolymph into the salivary glands, ovaries and testes. Colonisation of the salivary glands is significant, since it would imply that the virus could be transmitted via the saliva when male ticks are feeding on the next host. The successful passage of the virus between stages of the same tick was demonstrated in the case of *I. hexagonus*, during moulting of nymphs to the adult stage. The next step would be to feed such ticks wherein the infection would move to the salivary gland and subsequently transmitted through the saliva. In this study, the infection had reached the gut but not (yet?) the salivary gland of *I. hexagonus* (Table 3). However, in two other male ticks, (*I. ricinus* and *D. reticulatus*) BTV8 was detected in the salivary glands after capillary feeding (Table 2).

It is likely that these ticks are also able to transmit the infection fed on a susceptible host or on an artificial membrane in vitro. A further step in this direction was taken by conducting acquisition experiments with *I. ricinus* ticks which were allowed to feed voluntary on artificial membranes rather than being force-fed through capillaries. The predominant tick on livestock through much of North-West Europe *I. ricinus* is able to take up BTV8 and pass on onto several internal organs including the salivary glands (Table 5).

Further experiments are required to demonstrate whether (artificially) infected ticks are able to transmit BTV either onto a susceptible host or into non-infected blood during artificial membrane feeding.

Transovarial passage and infection of the next generation of ticks could not be demonstrated in *D. reticulatus*, *R. bursa* and *I. hexagonus*: larvae derived from the adult ticks were all PCR-negative. Unfortunately, fungal infections which developed in most *I. ricinus* prohibited further testing of the progeny of this tick.

Table 5 Infection of *Ixodes ricinus* females with BTV8 by membrane feeding

Tick ID number	Days post-feeding	Tick species	Gut (Cp) ^a	Ovaries (Cp)	Salivary glands (Cp)	Malpighian tubules (Cp)	Remaining tissue (Cp)
IR 1	15	<i>I. ricinus</i>	26.32	– ^b	34.78	32.74	26.3
IR 2	15	<i>I. ricinus</i>	28.19	28.79	33.51	29.59	24.75

^a Cp, Crossing point, see “Materials and methods”

^b Negative

The soft tick *O. savignyi* was successfully infected with BTV8-containing blood by feeding on an artificial membrane. BTV8 was detected in various tissues including salivary glands for up to 26 days (Table 4). Since soft ticks do feed multiple times during the same life stage, virus contained in the salivary glands could be transmitted during the same life cycle stage. The infection was also found to pass through the ovaries into the eggs. This finding confirms the possible vectorial capacity of soft ticks for BTV, since transmission of BTV was already reported for *O. coriaceus* (Stott et al., 1985). Soft ticks do not play a role in the epidemiology of the disease in Europe, since these do not occur on livestock in this area, but could play a role in the introduction of bluetongue virus in North-West Europe. In this study soft ticks served as a positive control species. In contrast to soft ticks, 3 of the species of hard ticks used in this study are prevalent on the ruminant population throughout large areas of North-West Europe where the current bluetongue outbreaks occur.

On the other hand, small numbers of adult *Culicoides* vectors were caught throughout the mild winter of 2006–2007 (Losson et al. 2007). A small fraction of the infected adult *Culicoides* population may survive long enough during these winters to bridge the gap between transmission seasons. BTV could also persist in the ruminant population during the winter, whether by chronic or latent infection of some individuals whereas transmission across the placenta from mother to foetus is another possibility.

Finally, the long survival period of ticks off hosts makes them interesting candidates to serve as (additional) reservoir for BTV. The observations presented in this paper would justify further investigations for this hypothesis.

Conclusion

Bluetongue virus serotype 8 can survive for at least 21 days in hard ticks and up to 26 days in the soft tick *O. savignyi*. BTV can pass the gut barrier to other organs of the tick. In this way BTV can be passed trans-stadially and intra-stadially in male *Ixodes* ticks. It can be passaged transovarially in *O. savignyi*. Further research is necessary to determine whether these ticks are able to transmit BTV to susceptible hosts.

Acknowledgments This collaborative project was financially supported by Central Veterinary Institute of Wageningen UR (CVI), Lelystad, The Netherlands. Dr. Armin Elbers (CVI, Lelystad) is thanked for his encouragement to undertake this study.

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