Effect of Culicoides sonorensis *salivary proteins on clinical disease outcome in experimental Bluetongue virus serotype 8 infection of Dorset sheep*

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Biting midges,
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Clinical disease,
Culicoides sonorensis,
Saliva,
Sheep,
Vector-enhanced transmission.

Summary
The severity of Bluetongue clinical disease in ruminants varies greatly depending on the outbreak serotype/strain, animal species/breed, and immune status of the herd. To predict disease risk from any of the 26 Bluetongue virus (BTV) serotypes identified to date, experimental animal susceptibility studies are often conducted. Although sheep are the most susceptible livestock species in the US, infection of domestic breeds by injection of field isolates rarely produces the level of clinical disease observed in natural *Culicoides* midge-transmitted outbreaks. Thus, outbreak risk assessments based on experimental animal infections can underestimate the severity posed by a potential outbreak with a given virus serotype or strain. The aim of this study was to determine whether secreted *Culicoides* salivary proteins injected simultaneously with virus, to more closely mimic midge-delivered virus, would affect clinical disease outcome in a BTV-8 sheep susceptibility study. Eight sheep were intradermally inoculated with BTV-8; 4 received virus mixed with secreted *Culicoides* salivary proteins (BTV-8 + Cu SP), 4 received virus alone. Clinical signs were monitored daily for type, severity and duration. In sheep receiving the BTV-8 + Cu SP inoculum, clinical signs were more varied, more severe, and duration was three times longer compared to sheep receiving virus alone. These results suggest that *Culicoides* salivary proteins may play a contributing role in BTV pathology and that use of these proteins in experimental animal infections may allow development of a more robust target-host animal model.

Effetto delle proteine salivari di Culicoides sonorensis *sull'esito clinico di infezioni sperimentali con il sierotipo 8 del virus della Bluetongue in pecore Dorset*

Parole chiave
Bluetongue,
Culicoides sonorensis,
Moscerino,
Pecora,
Saliva,
Sierotipo 8 del virus della Bluetongue,
Vettore.

Riassunto
La sintomatologia clinica della Bluetongue (BT) varia notevolmente a seconda del sierotipo/ceppo del virus responsabile, della specie/razza e dello stato immunitario dell'animale infettato. Per valutare il rischio di infezione da parte dei 26 sierotipi del virus della Bluetongue (BTV) fino ad oggi identificati si infettano sperimentalmente animali suscettibili. Negli Stati Uniti la pecora risulta la specie più sensibile. Si è potuto constatare che l'infezione sperimentale di razze domestiche difficilmente riproduce i quadri clinici osservati nelle infezioni naturali quando il virus è trasmesso da *Culicoides*. Le valutazioni sul rischio epidemico basate sui risultati delle infezioni sperimentali possono pertanto indurre a sottovalutare la pericolosità di una potenziale epidemia. In questo studio, per riprodurre più fedelmente il contagio da *Culicoides,* le proteine salivari di *Culicoides* sono state iniettate nell'animale

contemporaneamente al virus. L'infezione sperimentale ha avuto l'obiettivo di determinare se le proteine salivari di *Culicoides* incidano sugli esiti clinici della BT nelle pecore. Mediante iniezione intradermica, a 8 pecore è stato pertanto inoculato il sierotipo 8 del virus (BTV-8), a 4 il virus mescolato con le proteine salivari di *Culicoides* (BTV-8 + Cu SP), ad ulteriori 4 pecore è stato somministrato il solo BTV. I sintomi clinici sono stati monitorati giornalmente per tipo, gravità e durata. Negli ovini inoculati con BTV-8 + Cu SP, i sintomi clinici sono risultati più eterogenei, più gravi e di una durata 3 volte superiore a quella delle pecore trattate con il solo virus. I risultati ottenuti suggeriscono che le proteine salivari di *Culicoides* possono influenzare la virulenza del BTV e che l'uso di queste proteine nelle infezioni sperimentali potrebbe consentire lo sviluppo di modelli animali più conformi alle infezioni naturali.

Introduction

In the US, 5 serotypes of Bluetongue virus (BTV) are considered endemic or 'domestic' (BTV-2, BTV-10, BTV-11, BTV-13, BTV-17). Each serotype results in varying disease levels in target species such as white-tailed deer, cattle, and sheep. However, since 1999, 10 incursive or 'exotic' BTV serotypes have been detected, some repeatedly, in wildlife and livestock (Corn *et al.* 2013, Ostlund 2009, Ostlund 2013, Stallknecht 2009). This suggests BTV serotype introductions are quite routine and consistent in North America. One serotype of concern to livestock producers is BTV-8, which resulted in unprecedented geographic spread and livestock morbidity and mortality in Northern Europe during the 2006-2007 *Culicoides* vector seasons. In the US, sheep are the most susceptible livestock species, and Polled Dorsets have become the second largest white-faced sheep breed. Clinical disease can include: fever, ulcers on the tongue, mouth and nostrils, ocular and nasal discharge, conjunctivitis, facial edema, excessive salivation, cyanotic (blue) tongue, respiratory distress, coronitis, loss of appetite, and depression. Severity and form of clinical disease in a specific species vary greatly depending on the outbreak serotype or specific strain within a serotype and on the immune status of herds during the vector season. A recent example of this variation in pathology was seen in the 2007 BTV-17 outbreak in Wyoming, US, with sheep flocks suffering up to 36% morbidity and 35% mortality. This was in contrast to prior outbreaks of this serotype, which were historically considered relatively mild (Miller *et al.* 2010).

Experimental animal infections are typically used to understand pathogenesis and predict disease severity of future outbreaks with endemic and incursive BTV serotypes. The value of such studies can be limited, however, because unlike natural infections, routine experimental infection of domestic US sheep breeds by inoculation only rarely produces clinical disease beyond a transient fever and mild clinical signs depending on the infectious dose, inoculation route, and the BTV serotype used. This is contrary to what is usually observed in natural infections where virus is transmitted by the bite of a *Culicoides* midge and may result in severe clinical disease with 30-50% mortality. It has been hypothesised that the more severe clinical disease levels seen in natural, midge-transmitted outbreaks is due to intradermal route of virus delivery, as well as interactions of the vector's saliva with the virus, with the animal's immune responses, or with both. This 'vector-enhanced'transmission has been reported for several arboviruses (Bergman *et al.* 2000, Cupp and Cupp 1997, Darpel *et al.* 2011, de Moura *et al.* 2007, Gillespie *et al.* 2000, Hajnicka *et al.* 1998, Hajnicka *et al.* 2000, Kocakova *et al.* 1999, Limesand *et al.* 2003, Ribeiro 1987, Tabachnick 2000, Urioste *et al.* 1994). The inability to replicate BT clinical disease in North American sheep by experimental inoculation can result in risk assessments underestimating the severity posed by a given virus serotype or strain. The aim of this study was to determine whether artificially mixing BTV inocula with secreted salivary proteins of *Culicoides sonorensis*, the primary BTV vector in the US, would result in a vector-enhanced Bluetongue clinical disease sheep model mimicking more precisely what is observed in natural bite-transmission infections of sheep in the US.

Materials and methods

Culicoides sonorensis salivary proteins

Salivary proteins were collected from Arthropod-Borne Animal Diseases Research Unit (ABADRU)-maintained *Culicoides sonorensis* colony biting midges (Jones 1960) using an artificial membrane feeder system as previously described (Langner *et al.* 2007, Lehiy and Drolet 2014).

Bluetongue virus inocula

The virus (BTV-8/NET2007/01) was obtained from the Central Veterinary Institute of Wageningen

University (Lelystad, the Netherlands). The BTV-8 inoculum was EDTA-blood harvested from Holstein Frisian cow NL441689187 from Bavel (the Netherlands); this was the first detected case of BTV-8 after overwintering (GenBank GQ506451-GQ506460) (Backx *et al.* 2007). The blood inoculum titer was previously determined to be 6 x 10^5 particles/ml by quantitative real time polymerase chain reaction (qRT-PCR) and RNA/virus particle determinations (Akita *et al.* 1992, Drolet *et al.* 2013). Each experimental sheep received 1 ml of the blood inoculum in 2 locations for a total of 1.2 x 10^6 particles. For the BTV-8 inocula containing *Culicoides* salivary proteins (BTV-8+Cu SP), 1 ml of the above blood inoculum was combined with 250 ul (100 ug) *C. sonorensis* salivary proteins, resulting in a total inoculum volume of 1.25 ml per injection location.

Sheep inoculations

A total of 8 BTV-seronegative American Poll Dorset yearlings were sedated with xylazine (0.05-0.1 mg/kg) and injected with BTV-8/NET2007/01 bovine blood stock virus intradermally (cluster of 10, 0.1ml injections) in the neck and inner left leg. Four of these sheep were inoculated with BTV-8 (Group A, 1 ml total volume/site), and 4 with BTV-8 + Cu SP (Group B, 1.25 ml total volume/site). Two negative control sheep were sham inoculated with uninfected bovine blood and housed with infected animals to examine direct contact transmission (1 per each experimental group). Body temperatures and clinical signs were scored daily on a scale of 0-3 with 0 being normal and 3 being severe. Two challenged animals (1 per group) were euthanized with pentobarbitol at the anticipated peak of viremia (day 8) and the remaining were held until day 28.

Results

Clinical signs of Bluetongue disease in groups A (BTV-8) and B (BTV-8 + Cu SP) were monitored daily. Sheep were characterised as febrile when rectal temperatures were > 39°C. Both groups showed similar degree ranges. However, the temperatures of sheep in Group B peaked 1 day earlier than the temperatures of sheep in Group A (Figure 1). Visible clinical signs were recorded daily using a scale of 0-3 (0 = normal, 1 = mild, 2 = moderate, 3 = severe) and included ocular discharge, nasal discharge, conjunctivitis, excessive salivation, oral ulcers, facial edema, coronitis, diarrhea, and respiratory distress (Table I). In both groups, excessive salivation directly correlated with the presence of oral ulcers was observed. Animal to animal variation was seen in both groups; yet, Group B (BTV-8 + Cu SP) showed more types and more severe clinical disease.

In Group A (BTV-8), the duration of clinical signs lasted from day 7 to day 9, moderate to severe ocular and nasal discharge being the most common signs observed. At the peak of clinical disease, 3 sheep had ocular and nasal discharge, 1 had mild conjunctivitis, 1 had moderate oral lesions and excessive salivation, 2 had moderate facial edema and 1 had moderate coronitis.

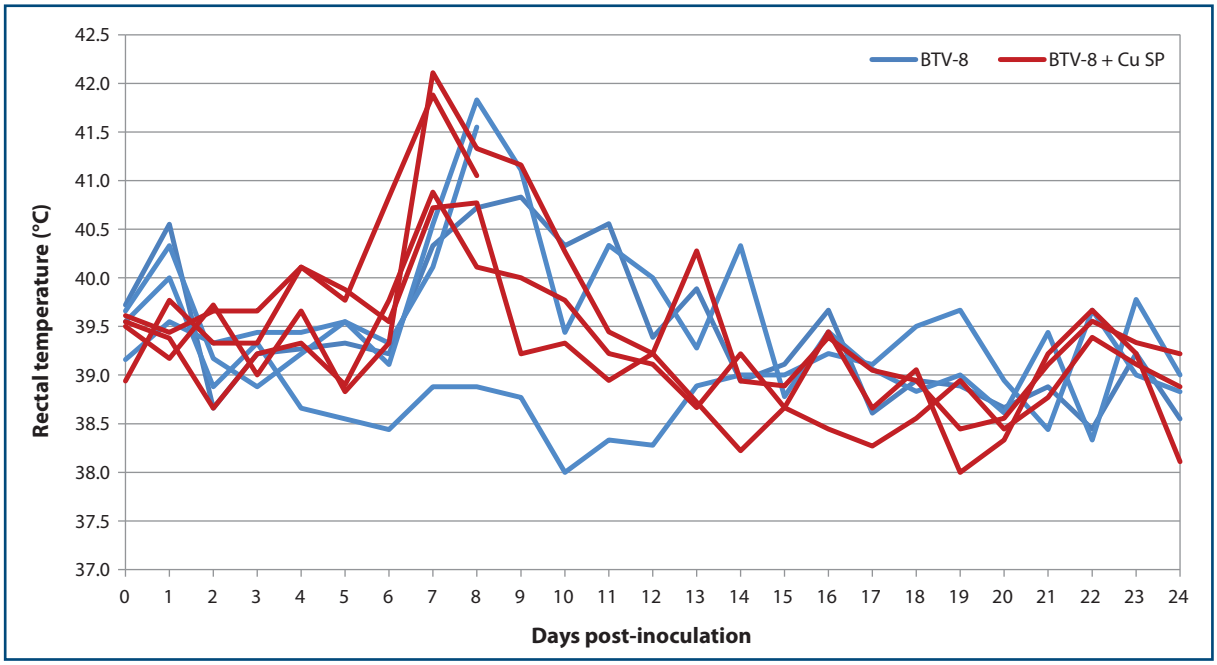

Figure 1. *Febrile response to Bluetongue virus serotype 8 (BTV-8) inoculation.* Rectal temperatures (°C) of sheep inoculated with BTV-8 (blue line) or BTV-8 mixed with *Culicoides sonorensis* midge salivary proteins (red line). Average normal temperature of negative controls for 28 days was 39°C.

Table I. *Heat map representation of peak clinical Bluetongue disease in sheep inoculated with either Bluetongue virus serotype 8 (BTV-8) or BTV-8 mixed with* Culicoides sonorensis *salivary proteins.*

	BTV-8				BTV-8 + *Culicoides* salivary proteins			
	Sheep 1	Sheep 2	Sheep 3	Sheep 4	Sheep 6	Sheep 7	Sheep 8	Sheep 9
Ocular discharge	2	0	3	2	2	3	3	3
Nasal discharge	2	0	2	3	3	3	2	3
Conjunctivitis	0	0	1	0	1	3	3	3
Excessive salivation	0	0	2	0	0	2	0	0
Oral ulcers	0	0	2	0	0	2	0	0
Facial edema	2	0	2	0	0	3	0	3
Coronitis	0	0	0	2	3	2	0	2
Diarrhea	0	0	0	0	3	2	0	0
Respiratory distress	0	0	0	0	0	0	0	2

0 = 0, normal; 1 = 1, mild; 2 = 2, moderate; 3 = 3, severe.

In Group B (BTV-8 + Cu SP), the duration of clinical disease lasted from day 3 to day 11, 3 times longer than Group A. Moderate to severe ocular and nasal discharge were the most common clinical signs observed (Figure 2). At the peak of clinical disease, all 4 sheep had ocular and nasal discharge and conjunctivitis. One sheep had moderate oral ulcers and excessive salivation, 2 had severe facial edema, 3 had moderate to severe coronitis, 2 had moderate to severe diarrhea and 1 had moderate respiratory distress.

Because sheep were housed 5 per room and shared food and water, individual scores for loss of appetite were not possible, but an overall loss of appetite was noted in both groups on days 8 and 9. Depression was also seen in both groups and was characterised by listlessness, lowered heads, and slow or diminished avoidance of human contact. Clinical disease signs were not observed in the 2 sham inoculated negative control sheep which were housed with the inoculated sheep, confirming no direct contact transmission.

Discussion

Natural, *Culicoides*-transmitted BTV outbreaks in US sheep herds can result in severe clinical disease with 30-50% mortality. Conversely, routine experimental infection by virus inoculation of virulent field isolates rarely results in clinical disease beyond a transient fever. This phenomenon has been seen repeatedly for BTV by our lab over the past 30 years and reported by others with several serotypes of BTV (Backx *et al.* 2007, Darpel *et al.* 2007), and other viruses such as Epizootic haemorrhagic disease (Breard *et al.* 2013), Schmallenberg (Wernike *et al.*

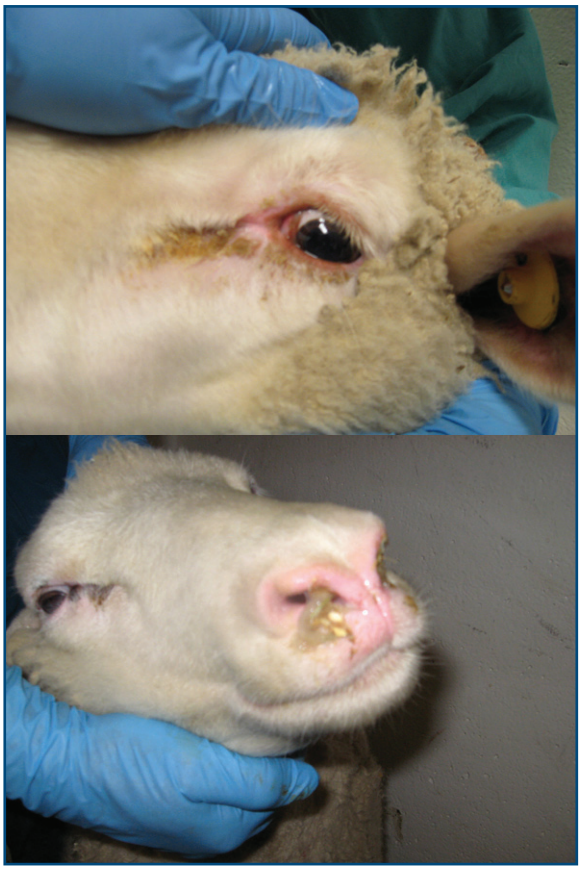

Figure 2. *Clinical Bluetongue disease.* Ocular and nasal discharge and conjunctivitis in sheep following inoculation with Bluetongue virus serotype 8 with *Culicoides sonorensis* midge salivary proteins.

2012, Wernike *et al.* 2013) and Rift Valley fever (Drolet *et al.* 2012). In order to perform evaluative research on pathogenesis, immune response, and vaccine candidate efficacy, there is a need for consistent, reliable and well-characterised BTV sheep challenge models. Salivary proteins have been linked to the efficient vector-to-animal transmission of a number of arboviruses including West Nile (Schneider *et al.* 2006), Sindbis (Schneider *et al.* 2004), Dengue fever (Espada-Murao and Morita 2011), and vesicular stomatitis (Limesand *et al.* 2000). Interestingly, a recent report involving *C. sonorensis* saliva has suggested that protein components may be involved in the efficient transmission of BTV from an infected host into the midge vector by interacting directly with the VP2 protein of the virus (Darpel *et al.* 2011). The question remains, however, as to how this would increase bite transmission efficiency from midge to animal since in the same study, viral infection of mammalian cell cultures decreased 2-6 fold. Whether by direct interaction with arboviruses or immunomodulation of the vertebrate host's response to those arboviruses, it is clear that insects play a critical role in arbovirus infection efficiency and resulting clinical disease. Thus, insects should be used for arbovirus transmission and infection

research; however, logistically this is difficult. The lack of colonized insect sources, access to facilities that allow insect/animal work, ensuring proper containment of insects during the experiment, and the inherent variability in virus dose delivery during insect feeding results in the continued use of injection versus insect transmission of virus to experimentally infect animals.

Results from this preliminary study show that sheep receiving virus and *C. sonorensis* salivary proteins exhibited more varied and severe clinical disease, and the duration of disease was 3 times longer compared to sheep receiving virus alone. This suggests that artificially mixing *C. sonorensis* salivary proteins with BTV in experimental inocula may offer an experimental target-host animal model that better represents natural bite transmission and allow for more accurate examination of factors contributing to susceptibility, pathogenesis, vaccine efficacy, and outbreak disease risk assessments.

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