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Viraemia and clinical disease in Dorset Poll sheep following vaccination with live attenuated bluetongue virus vaccines serotypes 16 and 4

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ABSTRACT

The spread of bluetongue virus (BTV) is most successfully controlled by vaccination of susceptible ruminant populations. Currently two different types of BTV vaccines are used for this purpose; inactivated, mostly monovalent vaccine formulations and modified live virus vaccines (MLVs). Clinical signs and viraemia in Dorset Poll sheep vaccinated with BTV-4 and BTV-16 MLVs or inoculated with homogenates of midges (*C. sonorensis* and *C. nubeculosus*) previously infected with BTV-4 MLV are presented. All sheep vaccinated with the two MLVs mounted an infectious viraemia lasting for a minimum of 9 up to 23 days post vaccination and developed a range of clinical signs associated with BTV infection. Peak viraemia titres recorded in individual sheep ranged from 3.5 to 6.83 $\log_{10}$ $TCID_{50}$/ml indicating a high potential for infection of vector insects and onward transmission. The implications of these results are discussed with reference to the current outbreaks of BTV occurring in northern Europe and in relation to the future development of vaccines for this virus.

1. Introduction

Bluetongue (BT) is a non-contagious disease of domesticated and wild ruminants whose aetiological agent is bluetongue virus (BTV), an orbivirus spread primarily by *Culicoides* biting midges (Diptera: Ceratopogonidae). Since 1998, Europe has experienced its worst ever recorded outbreak of BT, involving at least 12 incursions of strains belonging to a total of 9 multiple different serotypes (for review see [1,2]). The first outbreaks of BT ever recorded in northern Europe started with the arrival of BTV serotype 8 (BTV-8) in the Netherlands and Belgium during the summer of 2006. The virus spread to Germany, France and Luxemburg, then 'overwintered' (2006–2007) allowing it to spread even further across the whole of Europe during 2007–2008 (for review see [1,2]). This BTV-8 strain inflicted severe economic damage to sheep and cattle on both dairy and beef holdings, which were not only affected directly by clinical disease, but also had to operate under strict movement restriction regimes as part of the control measures implemented to reduce spread of the virus [3].

It became clear following the overwintering of BTV-8 in northern Europe during 2006–2007 and the huge number of holdings subsequently infected during the summer and autumn of 2007 that, control of BTV spread and reduction of its clinical impact, could only be achieved via a coordinated vaccination campaign. However, at the time of this incursion in 2006 the only vaccine available for BTV-8 was an attenuated/modified live virus vaccine (MLV). The advantages of MLV vaccines are that they provide protection against the same serotype for at least 1 year, they are cheap to produce and have been used successfully for many years to protect sheep in endemic regions (such as South Africa). Other serotypes of MLV vaccines have been used to control clinical disease in the southern Mediterranean region by several countries [4]. However, their use also has several inherent risks, some of which have been substantiated in the laboratory and field, and others of which remained largely theoretical at that time.

There are real concerns over the transmission of BTV-MLV in the field and their virulence in naïve animal populations. While it has been reported that a MLV against BTV-2 did not induce clinical signs or infectious viraemia in vaccinated sheep of a local French breed, these results were at variance to those in a later study on BTV-2 and BTV-9 MLVs in Dorset Poll sheep from a UK origin [5]. In the latter study, moderate to severe clinical signs and an infectious viraemia were recorded, which for the BTV-9 MLV persisted for up to 19 days, reaching a peak titre of 6.25 $\log_{10}$ $TCID_{50}$/ml. Moreover,

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MLV strains replicated in adults of the vector species *C. sonorensis*, reaching virus titres considered sufficient for virus transmission to a mammalian host [5–7]. These results support the findings of Ferrari et al. [8] who, using RT-PCR technology, detected the MLV of BTV-2 in pools of field collected *Culicoides* in Italy, illustrating the potential for circulation of MLVs in the field [8]. Moreover, virus replication of BTV MLVs strains in South African livestock associated *Culicoides* species, including *C. imicola* which is also present in southern Europe, was previously shown [9].

Indeed all of the recent BTV-16 isolates from Southern Europe (since 1998) show a close phylogenetic relationship with the BTV-16 MLV [10]. This suggests that they were recently derived from a common source, possibly originating from MLV vaccination programmes in the region, including the annual BTV-vaccination campaign in Israel that included BTV-16 (now discontinued) as part of a multivalent vaccine preparation [11].

MLVs also have the potential to exchange genome segments/reassort with other BTV strains, leading to the emergence of progeny viruses that may have novel biological characteristics, potentially including increased virulence. There is clear evidence for reassortment between BTV-2 and BTV-16 vaccine strains, leading to emergence of novel strains of BTV-2 in Italy during 2002 [10,12]. Consequently, BTV MLVs have not been licensed for use in northern Europe and authorities in affected countries decided to wait for pharmaceutical companies to produce inactivated BTV-8 vaccines. Partially because of the initial absence of a market which would make BTV-8 vaccine production economically viable, almost 2 years elapsed from the initial BTV-8 incursion in northern Europe until the first field vaccinations in the UK during 2008. This was despite the fact that commercially prepared inactivated vaccines against BTV-2 and BTV-4 had already been used successfully during earlier vaccination in southern Europe [4]. The efficacy of inactivated vaccines was demonstrated not only in these scenarios, but also eventually in 2008 when a voluntary vaccination campaign in England and Wales prevented re-emergence of BTV-8 in the UK, following over 1000 detected cases in 2007 [13].

Several issues, however, remain with the use of inactivated vaccines. Firstly, polyvalent or cross-serotype inactivated BTV vaccines are not widely available and, in some areas, up to 4 different vaccines may be required to provide effective protection in some areas of Europe [2]. Bivalent inactivated vaccines against BTV serotypes 1 and 8 have only recently been introduced to the market (http://www.fortdodge.eu). Secondly, the incursion of different serotypes into new locations is unpredictable; hence, the production of these vaccines is largely 'reactive', potentially resulting in significant periods of time elapsing between incursion and the availability of a vaccine against the homologous type.

As a consequence of such delays in the availability of appropriate inactivated vaccines some countries may consider the use of the easily available MLVs. Indeed, two distinct MLVs (BTV-6 and BTV-11) were detected in animals in Belgium, the Netherlands and Germany during late 2008, suggesting that they may have been used illegally and were then transmitted by northern European *Culicoides* [12,14–16].

To further evaluate the suitability of MLVs for use in UK breed of sheep, this study investigates the duration of viraemia and severity of clinical signs caused in Dorset Poll sheep following vaccination with the commercially available BTV-4 and BTV-16 MLVs. In order to investigate if the vaccine strains revert to virulence following insect passage, the sheep were also inoculated with the BTV-4 vaccine virus subsequent to passage in *Culicoides*. The results are discussed with reference to the current outbreaks of BTV strains in northern Europe and the potential use of MLVs (illegally or otherwise) in the region.

2. Material and methods

2.1. Modified live virus vaccines (BTV-4 and BTV-16 MLVs)

Modified live virus vaccines used in these experiments were commercial, freeze-dried attenuated, BTV-4 and BTV-16 MLVs both produced by Onderstepoort Biological Products Ltd., Onderstepoort, South Africa. Reference codes are available at http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-4/btv16 as virus collection number RSAvvv1/04 (BTV-4) and RSAvvv1/16 (BTV-16). These vaccines were reconstituted in 100 ml of sterile diluent according to the manufacturer's instructions. The infectious titre of each vaccine virus strain was determined in BHK-21 cells as described by Veronesi et al. [5]. An indirect sandwich ELISA was also used to confirm virus titres [17].

2.2. Modified live vaccine infection studies

Adult Dorset Poll sheep (>2 years of age) originating in the UK were used in groups of either 3 or 4 animals for 3 separate infection studies. The sheep were housed in a high containment animal facility at IAH Pirbright, fed twice a day with grain pellets and with *ad libitum* access to water throughout the experiment. Two uninfected 'control' sheep (sheep coded VJ84 and VJ86) were housed in the same pen as some of the sheep inoculated with BTV-16 MLV.

Experiment 1: Four sheep were subcutaneously inoculated with 1 ml of the BTV-4 MLV according to the manufacturer's standard vaccination protocol (sheep coded VJ93, VJ94, VJ95, VJ96).

Experiment 2 and 3: Groups of 4 and 3 sheep (respectively) were inoculated as above but with BTV-16 MLV (experiment 2: sheep coded VJ85, VJ87, VJ88, VJ89/experiment 3: sheep coded VM04, VM05 and VM06).

Rectal temperatures and clinical signs were recorded daily for 14 days post vaccination (p.v.). A clinical reaction index (CRI) was calculated for most of the sheep (with the exception of experiment 3) based on observed clinical signs using a modification of the method of Huismans et al. [18]. The clinical signs were scored as follows: Fever—1 point for each day of temperature $\geq$40 °C; anorexia 1 point for each day of anorexia; lesions—face lesions (such as rhinitis, conjunctivitis, hyperaemia and ulcers of the mucosa, facial oedema and tongue oedema), foot lesion (lameness or recumbency due to inflamed feet), respiratory tract lesions (bronchitis and/or pneumonia) all scored from 0 to 4 depending on the severity. Veterinary intervention was scored as 6 points when treatments with antibiotics and/or anti-inflammatories were required. If an animal was euthanized, the score was 8 points, natural death from BT was scored at 30 points. For experiment 3, clinical signs were summarised as mild, mild-moderate, moderate and severe.

All sheep were bled every 2 days for the first 3 weeks followed by 3 times a week for the remaining duration of the experiment. Blood was collected in EDTA tubes, washed and lysed as described by Hamblin et al. [19], then stored at 4 °C. To confirm infectious viraemia and quantify virus titres, titration of the blood was carried out on monolayer cultures of BHK-21 cells in microplates and virus titres calculated as described by Finney [20]. Some samples with low virus titres were additionally titrated in 11-day-old embryonated hens' eggs according to Parker et al. [21]. Positive eggs were confirmed by the indirect sandwich ELISA described by Thevasagayam et al. [17].

2.3. Culicoides infection (BTV-4 MLV)

The experiment was carried out in order to assess if transmission of MLVs between sheep and *Culicoides* vectors results in rever-

sion to virulence. However, to reduce animal use and to ensure infection of vector insects and sheep, *Culicoides* spp. were fed on a blood/virus mixture in an artificial feeding device. Infected insects were later processed to provide inoculums for sheep infection. Colonised adults of *Culicoides sonorensis* Wirth & Jones, a BTV vector in N. America, and *Culicoides nubeculosus* Meigen, a north-western Palaearctic species were used during the study. BTV-4 MLV was amplified once in BHK-21 cell culture and virus was harvested after showing 100% cytopatic effect (CPE). The MLV titre on BHK-21 cells was determined as described by Finney [20]. Approximately 250, 2–3-day-old female *Culicoides*, held in cardboard pillboxes (Watkins and Doncaster, UK), were starved of both sugar and water for 24 h before infection. Midges were orally fed on 1:1 mixture of un-clotted horse blood and BTV-4 MLV through a parafilm membrane as described by Mellor [22]. *Culicoides* were then immobilized using CO_2 fully engorged females were selected and transferred into clean pillboxes for an incubation period of 8–10 days at $23 \pm 1\,^{\circ}C$ and 80% relative humidity (R.H.). Following this incubation period, surviving *Culicoides* were sorted from dead individuals under CO_2 anaesthesia and stored at $-80\,^{\circ}C$ until attempting virus isolation.

2.4. Homogenisation and titration of infected Culicoides

Virus isolation was carried out from both species of *Culicoides* infected with BTV-4 MLV as described above. Pools of at least 100 surviving females of MLV-fed *C. sonorensis* or *C. nubeculosus* were placed in sterile 1.8 ml Eppendorf tubes with 1.0 ml of Glasgow MEM containing 0.6% antibiotics (2.0 μg/ml fungizone, 1000 IU/ml penicillin, 50 μg/ml neomycin and 1000 IU/ml polymyxin) and homogenised using a battery-operated microtissue grinder (Kontes, Vinelan, NY). Each tube was then centrifuged at $13{,}000 \times g$ for 3 min and the supernatant transferred into a bijou bottle; the remaining pellets of *Culicoides* were re-suspended in a further 1 ml of media. This procedure was repeated until a final volume of about 10 ml of samples was collected to ensure the maximum release of the virus. After filtration of each 10 ml pool through a 0.2 μm pore mini-filter (Sartorious, UK), samples were titrated in BHK-21 cells as described above.

2.5. Sheep inoculation with insect passaged BTV-4 MLV

Two separate groups of 4 Dorset Poll sheep (coded VJ97, VJ98, VJ99, VK00 and VK01, VK02, VJ82, VJ83) were inoculated with supernatant from the BTV-4 infected and homogenised pools of *C. sonorensis* and *C. nubeculosus*, respectively. Each sheep received 1.5 ml subcutaneously into the side of the neck as well as 0.5 intradermally (distributed between 5 sites on the inner thigh) of a standardised dose of virus at a concentration of between 4.5 and 5.0 $\log_{10}$ $TCID_{50}$/ml.

Blood samples, rectal temperatures and clinical signs from all sheep were collected, recorded and processed as described in Section 2.2 for the MLV infection studies. The clinical reaction index was determined as described earlier.

All the animal experiments were performed according to the Home Office Licence PPL70-5793.

3. Results

3.1. Infectious titre of the commercial modified live virus vaccines (BTV-4 and BTV-16 MLVs)

The infectious titres recorded in BHK-21 cell culture for BTV-4 and BTV-16 MLVs were 6.5 and 4.75 $\log_{10}$ $TCID_{50}$/ml respectively.

Table 1
Viraemia titres and duration recorded in Dorset Poll sheep after inoculation with commercially available or *Culicoides* passaged modified live attenuated vaccine viruses.

Inoculum	Mean viraemia titre[a] (maximum)	Mean viraemia duration in days (range)
BTV-4 MLV Experiment 1	3.8 (4.25)	14 (11–16)
BTV-16 MLV Experiment 2	6.0 (6.83)	All animals still viraemic at 21 days post vaccination when the experiment was terminated. Depending on the onset, viraemia was therefore detected for 17 or 19 days
BTV-16 MLV Experiment 3	5.8 (6.0)	16 (10–23)
BTV-4 MLV *C. sonorensis* pass.	3.6 (4.25)	13 (11–16)
BTV-4 MLV *C. nubeculosus* pass.	3.4 (4.25)	12 (9–14)

[a] $\log_{10}$ $TCID_{50}$/ml.

3.2. Modified live vaccine infection studies

Experiment 1 (*BTV-4 MLV*): All sheep developed viraemia from day 5 or day 7 post vaccination (p.v.) onwards, which lasted for up to 16 days (sheep VJ93). The highest viraemia titre of 4.25 $\log_{10}$ $TCID_{50}$/ml was observed 9 days p.v. in sheep VJ96 (Table 1). Pyrexia (temperature $\geq 40\,^{\circ}C$) was recorded in 2 out of 4 vaccinated sheep (VJ94 and 96) for at least 1 day. Typical BT clinical signs (including conjunctivitis, facial oedema, hyperaemia, rhinitis and coronitis) were also observed in all of the vaccinated sheep (Table 2).

Experiment 2 (*BTV-16 MLV*): All four sheep vaccinated with the BTV-16 MLV developed a viraemia commencing at day 3 or day 5 p.v. (Table 1). The maximum titre of 6.83 $\log_{10}$ $TCID_{50}$/ml was detected in sheep VJ85 at 7 days p.v. Infectious viraemia was still recorded in all 4 sheep at 21 days p.v., with titres ranging from 4.0 $\log_{10}$ $TCID_{50}$/ml (VJ85) to 1.75 $\log_{10}$ $TCID_{50}$/ml (both VJ87 and VJ89). The duration of viraemia beyond 21 days p.v. was not able to be investigated as the animal experiment was terminated at this time. Hyperthermia was detected in all 4 vaccinated sheep, especially sheep VJ88, which showed pyrexia $\geq 40\,^{\circ}C$ for up to 6 days. All of the vaccinated animals showed a general decline in health, with lethargy and apathy which curtailed movement, drinking and eating. Additionally, all animals demonstrated a reddening of the mucosal membranes in the eyes, nose and mouth. The faces were moderately oedematous. All sheep developed coronitis of all 4 ft. which, with the exception of VJ87, led to a varying degree of lameness and disinclination to move. Therefore three out of four vaccinated sheep developed moderate to severe disease (VJ85, VJ88, VJ89, Table 2), although acute pulmonary signs were absent. Sheep VJ88, which developed severe depression, apathy, and several consecutive days of fever, was treated with flunixin meglumine (Finadyne®/Schering-Plough) and antibiotics (Pen&Strep/Norbrook). None of the above clinical signs of BT were detected in the two unvaccinated control animals which were housed in the same box as the vaccinated animals.

Experiment 3 (*BTV-16 MLV*): Infectious viraemia was detected in all 3 vaccinated sheep starting at 5 days p.v. Viraemia titre in sheep VM06 reached a peak of 6.0 $\log_{10}$ $TCID_{50}$/ml at 9 days p.v. and persisted for 27 days p.v. (the end of the experiment) with a final titre of 1.75 $\log_{10}$ $TCID_{50}$/ml (Table 1). Two of the 3 vaccinated sheep developed transient pyrexia from day 7 to day 9. Overall, mild-moderate clinical signs typical of BT were recorded as for experiment two.

Table 2
Clinical scores of the sheep infected experimentally with live attenuated vaccine virus BTV-4 and BTV-16.

Inoculum	Animal	Fever	Anorexia (0–5)	Face (0–4)	Lesion feet (0–4)	Respiratory tract (0–4)	Veterinary intervention (6)	Euthanasia (8) Natural death (30)	Total score	Comments
BTV-4 MLV[a]	VJ 93	0	0	3	2	0	0	0	5	
	VJ 94	2	0	4	3	0	0	0	9	
	VJ 95	0	0	3	4	0	0	0	7	
	VJ 96	1	0	1	4	0	0	0	6	
	Total score								27	
	Mean								**6.75**	
BTV-4 MLV passaged once into *C. sonorensis*[a]	VJ 97	0	0	4	3	0	0	0	**7**	
	VJ 98	2	0	3	2	1	6	0	14	
	VJ 99	0	0	4	2	0	0	0	6	
	VK00	2	0	0	2	4	0	30	38	Acute respiratory dysfunction. Swelling and discolouring of lips and tongue.
	Total score								65	
	Mean								**16.25**	
BTV-4 MLV passaged once into *C. nubeculosus*[a]	VK01	0	0	2	4	0	0	0	6	
	VK02	0	0	3	2	0	0	0	5	
	VJ82	0	0	3	2	0	0	0	5	
	VJ83	1	0	3	2	1	6	0	12	
	Total score								28	
	Mean								7	
BTV-16 MLV	VJ85	2	1	3	4	0	0	0	10	
	VJ87	3	0	3	4	0	0	0	10	
	VJ88	6	2	4	4	0	6	0	22	
	VJ89	1	0	2	4	0	0	0	7	
	Total score								49	
	Mean								**12.25**	
Control Sheep VJ84	1	0	0	0	0	0	0	1		
Control Sheep VJ86	0	0	0	0	0	0	0	0		

[a] All sheep during this trial were treated with Ivermectin as maggots of the nasal botfly (*Oestrus ovis*) were discovered in the animal holding facility.

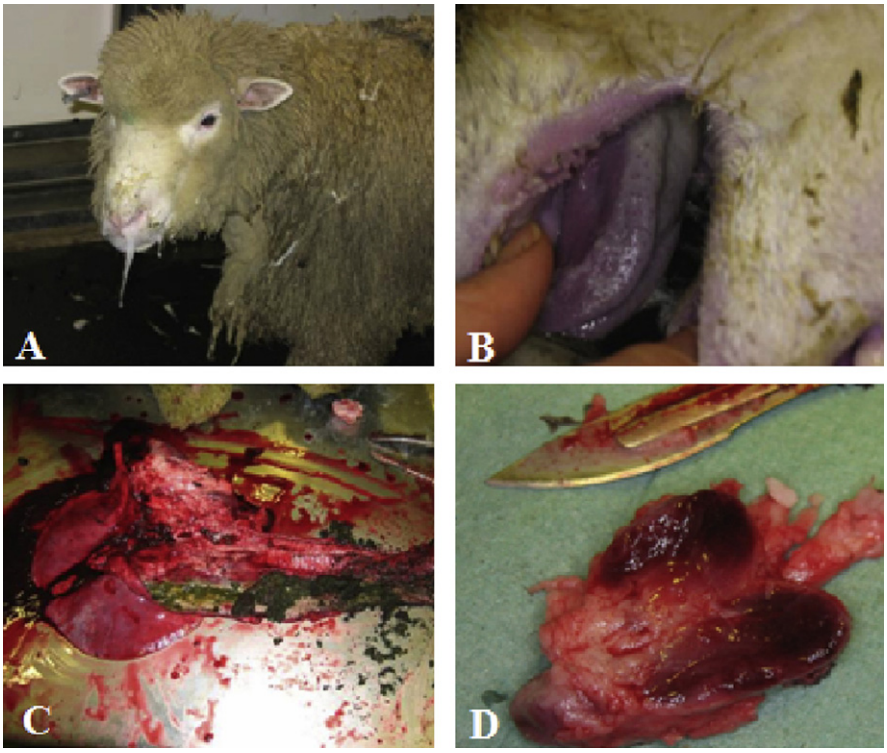

Fig. 1. Unusual clinical findings in a Dorset Poll sheep infected with BTV-4 vaccine virus (*C. sonorensis* 1st passage). One of the four sheep (VK00) infected with BTV-4 vaccine virus (RSAvvv1/04/*C. sonorensis* 1st passage) showed severe salivation and frothing (A) at 6 d.p.i. The condition progressed rapidly and the animal experienced severe respiratory distress and breathing problems. Asphyxia caused the mouth cavity including the tongue (B) to turn cyanotic. The animal was euthanized prior to imminent natural death. The post mortem revealed obstruction of the oesophagus and food aspiration into the trachea (C), as well as haemorrhagic mandibular (D) and prescapular lymph nodes.

3.3. *Culicoides* infection (BTV-4 MLV)

The titre of the BHK-21 cell-passaged BTV-4 MLV used to feed the midges on the first passage was calculated as 8.0 $\log_{10}$ $TCID_{50}$/ml. Following the oral infection, homogenates of pooled *C. sonorensis* or *C. nubeculosus* contained a virus titre of 5.25 and 6.0 $\log_{10}$ $TCID_{50}$/ml respectively.

3.4. Sheep inoculated with insect passaged BTV-4 MLV

All of the sheep inoculated with insect passaged MLV, from either of the *Culicoides* species, developed a viraemia which persisted for 6–14 days (Table 1). The maximum titre recorded was 4.25 $\log_{10}$ $TCID_{50}$/ml. Two sheep (VJ98 and VK00) inoculated with homogenised infected *C. sonorensis*, showed temperatures above 40 °C but this only lasted for no more than 2 days.

One of these sheep (VK00) showed a rise in body temperature as early as 4 days post infection (p.i.) (41 °C/4 days p.i. and 40.6 °C/5 days p.i.). On day 6 p.i., the same sheep showed extreme salivation with increasing respiratory distress. The condition progressed quickly and all mucosal surfaces, including the tongue, turned blue due to cyanosis (Fig. 1). The face and the lips were slightly swollen. In response to the acute respiratory dysfunction the animal was euthanized on humanitarian grounds but would have died naturally within the next hour (Table 2). A post mortem examination was conducted immediately following death. Food residues were found in the trachea and main bronchus. Acute oxygen deficiency led to the cyanosis of the whole oral cavity, including a 'blue tongue'. The food aspiration itself appeared to be caused by an oesophageal obstruction with food, making it impossible for the animal to swallow (Fig. 1). The only other pathological findings were haemorrhagic prescapular and mandibular lymph nodes, although these were not enlarged (Fig. 1).

One of the sheep (VJ83), injected with *C. nubeculosus* homogenate, developed pyrexia and respiratory dysfunction (e.g. coughing, shortness of breath and noise on lung auscultation) at day 13 p.i., and was treated with antibiotics (Pen&Strep/Norbrook). Sheep VJ98 (which had been inoculated with homogenised, infected *C. sonorensis*) also showed signs of bronchitis at 13 days p.i. and received the same therapy.

4. Discussion

All the Dorset Poll sheep vaccinated with either BTV-4 or BTV-16 MLVs showed clinical disease of a variable degree of severity, similar to that recorded in animals infected with wild-type strains [23–25]. In addition, significant periods of viraemia were recorded in sheep vaccinated with MLVs. Live virus was detected in pools of *Culicoides* that had been fed on BTV-4 MLV/blood mixtures and incubated for 10 days to allow completion of the extrinsic incubation period, indicating a high potential for MLV transmission. These findings are consistent with previous studies investigating the safety of BTV-2 and BTV-9 MLVs, which was also carried out in Dorset Poll sheep [5]. Both studies strongly indicate that, although effective in the endemic areas for which they were designed, the use of these MLVs vaccines in northern Europe, especially in sheep breeds in which they have not been tested, would be a matter of concern.

The differences in the severity of disease and viraemia observed in these studies, as compared to other field and laboratory investigations, may be explained by the breed of sheep used. Published studies clearly show that some sheep breeds are more susceptible to BT than others [26,27]. This is highlighted by the fact that a previous study with BTV-2 MLV reported the development of a viraemia in vaccinated animals of only 2.6 $\log_{10}$ $TCID_{50}$/ml or less for a duration of no more than 2–3 days (personal comment quoted

in [28]). In contrast, peak viraemias recorded in the current study for BTV-4 and BTV-16 MLVs were between 3.5 $\log_{10}$ $TCID_{50}$/ml and 6.83 $\log_{10}$ $TCID_{50}$/ml and in the later case persisted for up to 23 days p.v.

Among the groups of sheep vaccinated with MLVs in the current study, the BTV-16 MLV caused more severe clinical signs than BTV-4 MLV. It is noticeable, however, that every MLV serotype tested by us to date (2, 4, 9 and 16) has produced clinical disease in Dorset Poll sheep of UK origin. The particularly strong clinical reaction to BTV-16 is of interest, especially as the amount of MLV in the BTV-16 vaccine was almost 20 times lower than in the BTV-4 vaccine preparation. This suggests that the MLV of BTV-16 is significantly more virulent than that of BTV-4, highlighting the variability between different strains/MLVs. Disease and other side effects caused by the BTV-16 MLV, not only as monovalent formulation but also in polyvalent formulation (with MLV's serotypes 2, 4 and 9), have recently been reported from the field, leading to its use being discontinued [4,29].

All four of the MLVs that we have tested (as reported here and by [5]), developed viraemias that are considered sufficient for infection of feeding *Culicoides* indicating a serious risk of MLV's transmission 'to' and 'by' vector insects in the field. The RT-PCR-based detection of BTV-2 MLV in field-trapped *Culicoides*, in Italy during 2003 [8], also provides a strong indication that MLVs would be spread between vaccinated and unvaccinated stock if employed in northern Europe.

The investigation of reversion to virulence of insect passaged BTV MLVs in Dorset Poll sheep was inconclusive, due to the inherent virulence of the MLVs themselves. No significant differences in virulence, viraemic titre or viraemia duration were observed between sheep vaccinated with the MVLs, and those inoculated with the respective vaccine strain passaged in *Culicoides* spp. in the current or in our previous work [5]. Among the sheep injected with homogenates of BTV-4 vaccine virus-infected *Culicoides*, individuals injected with *C. nubeculosus* 1st passage demonstrated the lowest incidence of pyrexia (only one out of four sheep). The animal numbers used were too small to conclude if the passage of vaccine virus strains in vector insects has any significant effect on virus strain characteristics.

Clinical signs developing after 10 days p.i., as seen here in sheep VJ83 and VJ98, are likely to be the result of secondary bacterial infections. However, such secondary infections occur in wild-type infected animals contributing to the overall economic damage caused by BT [30]. Of particular clinical interest however was sheep VK00, which was inoculated with BTV-4 MLV passaged in the vector, *C. sonorensis*. This animal died at 6 days p.i. and necropsy revealed a food obstruction of the oesophagus and the subsequent aspiration of food into the respiratory tract as the most likely causes of the excessive salivation and asphyxia observed. The overall condition, including the food in the trachea, suggested pharyngeal or oesophageal paralysis and a subsequent defect in the swallowing reflex resulting in food aspiration. Erasmus [30] suggested that oesophageal paresis may occur more often in BTV infection than generally believed and it is interesting to note this manifestation in a MLV inoculated individual. Oesophageal obstruction can occur in ruminants for several reasons and it is therefore difficult to be certain that these clinical signs were caused solely by infection with BTV-4 vaccine virus. However, oesophageal obstruction in healthy ruminants is not usually such an acute or progressive condition as observed here and normally involves a stage of gas production in the rumen ("bloating") which was not seen in this animal [31].

Recent indications of the possible illegal use of MLVs for BTV 6 and 11 in northern Europe [32,33] has highlighted our lack of knowledge concerning the likely behaviour of these vaccines in sheep breeds present in the region [34]. The suspicion of a new BTV strain in the Netherlands, which was later identified as BTV-6, was originally raised in October 2008 due to clinical signs in BTV-8 vaccinated cattle, suggesting that this vaccine-derived BTV-6 strain can cause clinical disease [16]. More recently and, subsequent to a laboratory study, it was reported that cattle were only sub-clinically infected by either of the two newly identified MLVs' strains (BTV-6 and BTV-11) and consequently, the protection zones were lifted for both strains [35]. It should be noted, however, that these MLVs have, to date, only been detected in cattle, which are usually less affected clinically by BTV than sheep. It therefore remains uncertain what effect they would have in European sheep, including Dorset Polls.

It appears very likely that these BTV-6 and BTV-11 strains were also onward transmitted by northern Palaearctic species of *Culicoides* as infected cattle were detected on several farms in the Netherlands, Belgium and Germany. Finally, if the two new BTV strains overwinter, there is a risk that these vaccine virus-derived strains may reassort with the circulating wild-type strain of BTV-8. Indeed, there is already evidence for reassortment of the BTV-6 strain in 2008 in genome segments 7 and 10 [12].

An understanding of the likely impact of MLVs' strains on ruminant breeds in northern Europe and investigations of the origin of possible illegal vaccine importations into the EU would be beneficial in reducing the potential for MLV associated outbreaks in the region. The key for eventual control of BTV, however, relies both upon the more rapid provision of suitable inactivated vaccines (and potentially of even more effective next generation and multivalent or cross-reactive vaccines) and their commercial availability at a cost that makes their use economically viable/attractive to farmers.

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References

[1] Carpenter S, Wilson A, Mellor PS. Culicoides and the emergence of bluetongue virus in northern Europe. Trends Microbiol 2009;17(April (4)):172–8.
[2] Mellor P, Carpenter S, Harrup L, Baylis M, Wilson A, Mertens PPC. Bluetongue in Europe and the Mediterranean Basin. Elsevier: Paris; 2009.
[3] Hoogendam K. International study on the economic consequences of outbreaks of bluetongue serotype 8 in north-western Europe. Leeuwarden (Netherlands): Van Hall Institute; 2007.
[4] Savini G, MacLaclalan NJ, Sanchez-Vinaino JM, Zientara S. Vaccines against bluetongue in Europe. Comp Immunol Microbiol Infect Dis 2008;31(2–3):101–20.
[5] Veronesi E, Hamblin C, Mellor PS. Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae). Vaccine 2005;23(December (48–49)):5509–16.
[6] Fu H, Leake CJ, Mertens PP, Mellor PS. The barriers to bluetongue virus infection, dissemination and transmission in the vector, *Culicoides variipennis* (Diptera: Ceratopogonidae). Arch Virol 1999;144(4):747–61.
[7] Jennings DM, Mellor PS. Variation in the responses of *Culicoides variipennis* (Diptera, Ceratopogonidae) to oral infection with bluetongue virus. Arch Virol 1987;95(3–4):177–82.
[8] Ferrari G, De Liberato C, Scavia G, Lorenzetti R, Zini M, Farina F, et al. Active circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy. Prev Vet Med 2005;68(2–4):103–13.
[9] Venter GJ, Mellor PS, Wright I, Paweska JT. Replication of live-attenuated vaccine strains of bluetongue virus in orally infected South African Culicoides species. Med Vet Entomol 2007;21:239–47.
[10] Batten CA, Maan S, Shaw AE, Maan NS, Mertens PPC. A European field strain of bluetongue virus derived from two parental vaccine strains by genome segment reassortment. Virus Res 2008;137(1):56–63.
[11] Alpar HO, Bramwell VW, Veronesi E, Darpel KE, Pastoret PP, Mertens PP. Bluetongue virus vaccine past and present. Paris: Elsevier; 2009.
[12] Maan S, Maan NS, Potgieter AC, Wright I, Van Rijn P, Nomikou K, et al. Full genome characterization of bluetongue virus serotype 6 from the Netherlands 2008 and comparison to other strains; in preparation.
[13] Carpenter S, Wilson A, Mellor P. Bluetongue virus and Culicoides in the UK: the impact of research policy. Outlooks Pest Manage 2009:161–4.

[14] De Clercq K, Mertens PP, De Leeuw, Oura CAL, Houdart P, Maan S, et al. Emergence of bluetongue serotypes in Europe, Part 2: the occurrence of a BTV-11 strain in Belgium. Transboundary Emerg Dis; in press.
[15] ProMED. Available on line at www.promedmail.org archive number 20090205.0516; February 2009.
[16] ProMED. Available on line at www.promedmail.org archive number 20081018.3301; October 2008.
[17] Thevasagayam JA, Wellby MP, Mertens PP, Burroughs JN, Anderson J. Detection and differentiation of epizootic haemorrhagic disease of deer and bluetongue viruses by serogroup-specific sandwich ELISA. J Virol Methods 1996;56(1):49–57.
[18] Huismans H, van der Walt NT, Cloete M, Erasmus BJ. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. Virology 1987;157(1):172–9.
[19] Hamblin C, Anderson EC, Mellor PS, Graham SD, Mertens PPC, Burroughs JN. The detection of African horse sickness virus-antigens and antibodies in young equidae. Epidemiol Infect 1992;108(1):193–201.
[20] Finney DJ. Statistical method in biological assay. London: Charles Griffin and Co. Ltd.; 1964.
[21] Parker J, Herniman KA, Gibbs EP, Sellers RF. An experimental inacrivated vaccine against bluetongue virus and observations on viraemia in experimentally infected sheep. Vet Rec 1975;96(13):284–7.
[22] Mellor PS. A membrane feeding technique for the infection of *Culicoides nubeculosus* Mg. and *Culicoides variipennis* sonorensis Coq. with *Onchocerca cervicalis* Rail. and Henry. Trans R Soc Trop Med Hyg 1971;65(2):199–201.
[23] Darpel KE, Batten CA, Veronesi E, Shaw AE, Anthony S, Bachanek-Bankowska K, et al. Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe. Vet Rec 2007;161(8):253–61.
[24] Hamblin C, Salt JS, Graham SD, Hopwood K, Wade-Evans AM. Bluetongue virus serotypes 1 and 3 infection in Poll Dorset sheep. Aust Vet J 1998;76(September (9)):622–9.
[25] Koumbati M, Mangana O, Nomikou K, Mellor PS, Papadopoulos O. Duration of bluetongue viraemia and serological responses in experimentally infected European breeds of sheep and goats. Vet Microbiol 1999;64(4):277–85.
[26] Jeggo MJ, Corteyn AH, Taylor WP, Davidson WL, Gorman BM. Virulence of bluetongue virus for British sheep. Res Vet Sci 1987;42(January (1)):24–8.
[27] MacLachlan NJ. The pathogenesis and immunology of bluetongue virus infection of ruminants. Comp Immunol Microbiol Infect Dis 1994;17(August–November (3–4)):197–206.
[28] Hammoumi S, Breard E, Sailleau C, Russo P, Grillet C, Cetre-Sossah C, et al. Studies on the safety and immunogenicity of the south African bluetongue virus serotype 2 monovalent vaccine: specific detection of the vaccine strain genome by RT-PCR. J Vet Med Ser B: Infect Dis Vet Public Health 2003;50(7):316–21.
[29] Savini G, Ronchi GF, Leone A, Ciarelli A, Mighaccio P, Franchi P, et al. An inactivated vaccine for the control of bluetongue virus serotype 16 infection in sheep in Italy. Vet Microbiol 2007;124(1–2):140–6.
[30] Erasmus BJ. Bluetongue in sheep and goats. Aust Vet J 1975;51:165–70.
[31] Radostis OM, Gay CC. Veterinary medicine a textbook for the diseases of cattle, sheep, goats and horses. W.B. Saunders Company Ltd.; 2000.
[32] ProMED. Available on line at www.promedmail.org archive number 20090218.0684; February 2009.
[33] ProMED. Available on line at www.promedmail.org archive number 20081028.3410; October 2008.
[34] ProMED. Available on line at www.promedmail.org archive number 20081031.3431; October 2008.
[35] ProMED. Available on line at www.promemail.org archive number 20090304.0888. March 2009.