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Live attenuated bluetongue vaccine viruses in Dorset Poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae)

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Abstract

The aim of this study was to address concerns associating with the use of BTV attenuated commercial vaccines in European sheep. These concerns include development of viraemia, possibility of transmission by vectors, reversion to virulence and re-assortment with wild-type viruses.

The two vaccine viruses (BTV2 and 9) replicated in two species of *Culicoides* subsequent to oral infection reaching titres suggesting transmission would occur.

Viraemia in Dorset Poll sheep inoculated with either vaccine or insect passaged vaccine viruses persisted for up to 17 days, recording titres that ranged from 2.5 to 6.25 $\log_{10}$TCID$_{50}$/ml, which is easily sufficient to infect vector *Culicoides.* Moderate to severe clinical signs of BT, albeit short lived, were observed in sheep following vaccination. However, to date there is no evidence of increasing virulence following two sequential passages through the vectors.

Keywords: Bluetongue; Vaccine; *Culicoides*

1. Introduction

Bluetongue (BT) is a non-contagious, infectious arthropod-borne viral disease of domesticated and wild ruminants, which is transmitted by the bites of certain vector species of *Culicoides* biting midges (Diptera; Ceratopogonidae) [1,2]. To date, 24 bluetongue virus (BTV) serotypes have been identified [3] and together they comprise the type species of the genus O*rbivirus* within the family Reoviridae. The BTV genome comprises 10 dsRNA segments enclosed within two protein shells. Each segment encodes at least one polypeptide, which together makes up the seven viral proteins, VP 1–7, and the three non-structural proteins, NS 1–3 [4,5].

Although BTV is an arbovirus, the virus can occasionally be transmitted from vertebrate to vertebrate in seminal fluid and by crossing the ruminant placenta [6]. Apparently tissue culture attenuated vaccine viruses are transmitted more frequently by this route than are field viruses.

Clinical signs of BT are usually only observed in certain breeds of sheep and some species of deer [7–9]. Cattle and goats usually experience sub-clinical infections but these species can serve as important reservoirs of the virus [10]. Viraemia is usually detected between 5 and 12 days post infection (dpi), although infectious virus has been detected in sheep blood for as long as 54 dpi [11,12] and in cattle blood for up to 100 days [13,14]. *Culicoides imicola* is the main European vector although other vectors, specifically *C. pulicaris* and *C. obsoletus* groups have recently been implicated [15–18]. The global distribution of BTV is traditionally considered to be between latitudes 35°S and 44°N, although in parts of western North America and China the virus may extend up to approximately 50°N [19–21]. The recent (1998–2004) incursions of BTV into Europe have involved 12 countries and 5 BTV serotypes (including at least seven different strains) and are unprecedented in their severity and extent. They have caused severe animal health and

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trade problems, and to date well over a million sheep have either died or been killed.

Attenuated vaccines produced by Onderstepoort Biological Products (OPB), Onderstepoort, South Africa have long been used to control clinical disease in sheep in southern Africa and more recently they have also been used in the face of the BTV incursions into Corsica, the Balearic Islands and Italy. The advantages of these vaccines are that they provide protection for at least one year, they are cheap to produce and they have been used successfully for many years to protect sheep in endemic regions. However, some authorities consider that there are a number of risks associated with the use of attenuated virus preparations in epidemic situations. The concerns are:

1. The disease susceptibility of untested breeds of sheep to vaccine viruses is unknown.
2. The introduced vaccine viruses may stimulate a viraemia in vaccinated animals that is sufficient to be ingested by blood-feeding vector *Culicoides*.
3. The vaccine viruses may be able to replicate in and be transmitted by vector *Culicoides*.
4. The vaccine viruses may revert to virulence on passage through vector *Culicoides*.
5. The vaccine viruses may reassort with wild-type viruses in dually infected, vaccinated animals or vector *Culicoides*, giving rise to novel BTV strains with modifies virulence characteristics.

In a recent study [22], reported the isolation of serotype-2 vaccine virus from non-vaccinated cattle following vaccination of sheep in central Italy. These studies go some way to substantiate some of the concerns outlined above.

The object of this study was to investigate some of these concerns under controlled laboratory conditions.

2. Material and methods

2.1. Viruses

2.1.1. Wild-type field viruses

Isolates of BTV2 and BTV9 were supplied by Onderstepoort Veterinary Institute (OVI), South Africa as freeze dried, sheep blood (BTV 2, no. 3032/0 stored in OCG (15/7/92) and BTV 9, no. 3080/2 stored in OCG (13/7/92)).

Both viruses were reconstituted in 1 ml of sterile distilled water and then passaged by intravenous inoculation of 11-day-old embryonated hen eggs [23] to ensure the viability of the virus in the freeze dried blood and to amplify the virus titre. Hearts were removed from embryos that died between 2 and 7 dpi and homogenised as described by Parker et al.[24] A ten-fold dilution series of each egg homogenate were then titrated in embryonated hen eggs [23].

2.1.2. Attenuated vaccine viruses

Two BTV attenuated vaccine viruses serotypes were used.

Commercial freeze dried BTV2 attenuated, monovalent vaccine virus produced by Onderstepoort Biological Products (OPB), S. Africa (batch no 2 expiry date 1/9/02); Commercial freeze dried BTV9 attenuated, monovalent vaccine virus labelled as 'IZS Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale—Via Campo Boario 64100 TERAMO (Italy) Batch N. 006 Exp. Date 06/04'. The reference collection number ITLvvv1/09, passage history and reference code for this vaccine virus is available on http://www.iah.bbsrc.ac.uk/dsRNA_virus_proteins/ReoID/btv-9.

2.1.3. Passage and titration of viruses

BT commercial vaccine virus serotypes 2 and 9 were reconstituted in 100 ml of sterile diluent according to the manufacture's instructions. One millilitre of a 1:10 dilution of each of the two commercial vaccines prepared in Glasgow MEM was inoculated into separate 175 cm^2 monolayer cell cultures of BHK-21 cells. The inoculum was adsorbed for 20 min at ambient temperature. Flasks were subsequently overlaid with 50 ml of BHK-21 medium containing L-glutamine and 3% tryptose phosphate broth (maintenance medium). Cell cultures were examined daily for the presence of cytopathic effects (CPE). Virus was harvested when showing 100% CPE (2–3 days). The titres of the viruses were determined by titrating serial ten-fold dilutions of each sample (0.1 ml/well, using four replicates per dilution) on monolayer cultures of BHK-21 cells in 96-well microtitre plates previously overlaid with 100 μl/well of maintenance medium.

Plates were sealed, incubated at 35–39 °C and examined microscopically on days 3, 5 and 7 for CPE.

The presence of virus in the cell culture plates was confirmed by indirect sandwich ELISA using methods similar to those described by Thevasagayam et al. [25] Briefly, the supernatant fluids from the cell culture plates containing the virus titrations were transferred onto ELISA plates pre-coated with rabbit anti-BTV antibody. The presence and identity of virus in the test samples was then confirmed following the stepwise addition of guinea pig anti-BTV antibody, anti-guinea pig enzyme conjugate and then chromogen/substrate. Plates were read spectrophotometrically at 492 nm. Individual wells were recorded positive if they gave an optical density (OD) >0.15 over at least 3 consecutive dilutions and titrated from the strongest to the weakest dilution. Virus titres were determined as the dilution giving an OD value of 0.15.

Titres obtained in cell culture microplates and by ELISA were calculated as the last dilution recorded positive at the 50% end-point and expressed as $\log_{10}TCID_{50}$/ml [26].

2.2. Sheep

Dorset Poll sheep were used in these studies because previous work [27] has shown that they are highly susceptible to the development of BT disease.

Groups of either 2 or 4 animals were used at a time. The sheep were housed in an insect-free, high security animal facility. They were given water and food ad libitum before and after infection.

2.2.1. Control sheep infection

Each group of 2 Dorset Poll sheep were infected separately with the wild-type virulent strains of either BTV 2 or 9. Sheep were inoculated with 1.0 ml of the reconstituted virus by the intravenous route and 1.0 ml of the first passage, egg homogenate to ensure as near a field infection as possible. The virus titres of the latter inoculums were 5.5 $\log_{10}$ ELD_{50}/ml for BTV2 and BTV9.

Clinical signs and rectal temperatures were recorded for 14 dpi. The clinical signs were expressed as a clinical reaction index (CRI), modified from Huismans et al. [28]. The CRI equals the sum of the scores allocated for the following:

1. Fever: score 1 point for each day of pyrexia (40 °C or above).
2. Anorexia: score 1 point for each day of anorexia (maximum score permitted = 5).
3. Lesions: facial lesions (rhinitis, conjunctivitis, facial oedema and tongue oedema), foot lesions (coronitis-lameness, recumbency due to inflamed feet), respiratory tract (bronchitis, pneumonia) each category scores 0–4 points.
4. Veterinary intervention: score 6 points if antibiotics and/or anti-inflammatory were required.
5. Spontaneous death and euthanasia: score 10 and 8, respectively.

2.2.2. Sheep vaccination

Groups of four sheep were each vaccinated subcutaneously, according to manufacturer's instructions, with a 1.0 ml dose of one of the two reconstituted BTV vaccine viruses, containing either 5.25 $\log_{10}TCID_{50}$/ml of BTV 2 or 5.0 $\log_{10}TCID_{50}$/ml of BTV 9. Rectal temperatures and clinical signs were recorded daily for 14 days post vaccination (dpv). The percentage of attenuation of the vaccine viruses relative to the disease caused by the corresponding wild type virulent virus serotype was calculated as 100−[mean test score/virulent virus score] × 100.

2.3. Midges

Colonised *C. sonorensis (=variipennis)*, a proven BTV vector from N. America and *C. nubeculosus,* a potential European vector were used for this study [29–31].

2.3.1. Oral infection

Midges were deprived of a sugar meal for 24 h before infection. Cages of approximately 250 female midges of each species were fed separately for approximately 30 min, with an infectious blood meal through a parafilm membrane maintained at 36 °C as described previously [32]. The blood meal contained equal volumes of un-clotted horse blood and a BTV vaccine virus amplified in BHK-21 cells at a concentration of 6.7 and 7.75 $\log_{10}TCID_{50}$/ml, respectively, for serotype 2 and 9. After feeding, the midges were immobilised with CO_2 and the fully engorged females removed and placed in clean cages, and then incubated for 8–10 days at 23 °C at 80% relative humidity (R.H.). Cotton pads moistened with a 5% sugar solution were provided as a food source. Surviving midges were collected after incubation and killed by freezing at −80 °C.

2.3.2. Homogenization and titration of infected midges

Insect passage 1: pools of at least 100 *C. sonorensis* and *C. nubeculosus* for each serotype blood meal were homogenized separately in sterile 1.8 ml Eppendorf tubes for 5 min with 1.0 ml of Eagles maintenance medium using a motor driven plastic pestle. After grinding, the homogenates were clarified for two minutes by centrifugation at 12,000 × *g* and the supernatant fluids decanted into separate bijoux bottles. The insect pellets were re-homogenised and centrifuged as before on a further five occasions to ensure complete homogenisation of the flies and maximum release of virus. The supernatant fluids obtained for each virus serotype were pooled separately to provide a final volume of approximately 6 ml. Each insect-virus pool was filtered through a 0.2 μm Sartorious mini-filter and then titrated on monolayer cultures of BHK-21 cells as described earlier. The virus titres recorded for each pooled batch of midges infected with BTV 2 was 6.0 and 5.5 $\log_{10}TCID_{50}$/ml, respectively, for *C. sonorensis* and *C. nubeculosus* and for midges infected with BTV 9 was 5.75 and 4.25 $\log_{10}TCID_{50}$/ml, respectively, for *C. sonorensis* and *C. nubeculosus*.

Insect passage 2: an aliquot of each midge homogenate of passage 1 was then mixed with an equal volume of blood and these were used to oral feed corresponding batches of either *C. sonorensis* or *C. nubeculosus*. After 8–10 days incubation, the surviving midges were homogenised and titrated in BHK-21 cells as described above. The virus titres recorded for the homogenates following the second passage of BTV 2 and 9 through *C. sonorensis* were 5.0 and 5.5 $\log_{10}TCID_{50}$/ml, respectively. No virus was detected in the pooled homogenates of *C. nubeculosus* after oral feeding with BTV 2 and 9 passage 1 viruses.

2.4. Inoculation of sheep with insect passaged viruses

The supernatant fluids from the infected *C. sonorensis* and *C. nubeculosus* first and second insect passaged homogenates were diluted separately to give a standard dose of between 4.5 and 5.0 $\log_{10}TCID_{50}$/ml. Each sample was then inoculated into separate groups of four Dorset Poll sheep such that each sheep received 0.1 ml of the inoculum intradermally into five sites on the inner thigh (0.5 ml) and 1.5 ml subcutaneously into the side of the neck. Clinical signs and rectal temperature were recorded each day for 2 weeks.

Table 1
Percentage attenuation relative to wild-type virus before and after passage through vector midges

Serotypes	Inoculum	Passage	Total CRI score (four sheep)	Mean CRI	% Attenuation (relative to virulent virus CRI)
BTV 2	Vaccine		12	3	81.25%
	C. sonorensis	1st	4	1	94%
		2nd	7	1.75	88%
	C. nubeculosus	1st	7	1.75	89%
		2nd	nvd[a]	nvd[a]	nvd[a]
BTV 9	Vaccine		28	7	73%
	C. sonorensis	1st	22	5.5	79%
		2nd	16	4	85%
	C. nubeculosus	1st	13	3.25	88%
		2nd	nvd[a]	nvd[a]	nvd[a]

[a] nvd: no virus detected.

2.5. Isolation and detection of virus in sheep blood

Whole blood in EDTA was collected every 2 days for 3 weeks and then three times a week for a further 3 weeks following inoculation of either vaccine or vaccine-derived viruses. The red blood cells were washed and lysed as described by Hamblin et al. (1992) [33] and stored at +4 °C. Confirmation of viraemia and virus titre was determined by titration in microplates on monolayer cultures of BHK-21 cells as described earlier and/or by intravenous inoculation of 11-day-old embryonated hen eggs [23]. In the latter case, hearts were removed from embryos that died between 2 and 7 dpi and homogenised as described by Parker et al. [24]. Confirmation of BTV in embryos that died and virus titres achieved were made by indirect sandwich ELISA. Virus titres were calculated as the last dilution recorded positive at the 50% end-point and expressed as $\log_{10}$TCID$_{50}$/ml. The highest values achieved by either egg inoculation or cell culture were recorded. Samples recorded negative at a dilution of 1:10 in both eggs and microplate cell cultures, that is $\leq$1.5 $\log_{10}$TCID$_{50}$ of virus/ml, were retested by inoculating a 1.0 ml aliquot of the undiluted blood onto a 25 cm^2 monolayer flasks of BHK-21 cells as described earlier for the passage of virus. The presence of BTV in these flasks was confirmed by indirect ELISA and positive results (evidence of BTV) were recorded as titres $\leq$1.5 $\log_{10}$TCID$_{50}$/ml.

3. Results

3.1. Control sheep infection

Two sheep each were used to determine the CRI for the wild-type virulent BTV 2 and 9, and moderate to severe disease was recorded for each serotype. Clinical signs, including pyrexia, facial oedema, conjunctivitis, rhinitis and laminitis, persisted for several days. The highest scores allocated for clinical signs observed were 17 and 26 for BTV 2 and 9, respectively.

3.2. Sheep vaccination

Some of the clinical signs attributable to BT (including pyrexia, conjunctivitis, facial oedema, hyperaemia, rhinitis and coronitis) were observed in all the sheep inoculated with either vaccine or insect passaged vaccine viruses. Although these signs were severe in most cases, they were of short duration and therefore only low CRIs were recorded (mean CRI 3 and 7 for BTV 2 and 9, respectively) (Table 1). Two of the four sheep given the BTV2 vaccine developed a transient pyrexia greater than or equal to 40 °C on day 7, whereas all the four sheep vaccinated with BTV9 developed a more prolonged pyrexia commencing on either day 7 or day 8 and persisting for up to 5 days (Table 2). Tables 3 and 4 record the level and duration of viraemia recorded following mono-

Table 2
Pyrexia and viraemia duration recorded in Dorset Poll sheep after inoculation with vaccines and passaged vaccines

	Serotype	No. of sheep showing pyrexia	Pyrexia duration range (days)[a]	Max viraemia duration (days)	Min viraemia duration (days)
Vaccine	BTV2	2/4	1	17	17
	BTV9	4/4	1–5	15	7
C. sonorensis 1st passage	BTV2	0/4	0	15	5
	BTV9	4/4	1–5	9	5
C. sonorensis 2nd passage	BTV2	1/4	1	19	13
	BTV9	4/4	1–3	13	7
C. nubeculosus 1st passage	BTV2	0/4	0	15	9
	BTV9	2/4	1–4	17	9

[a] No. of days/sheep.

Table 3
Viraemia ($log_{10}TCID_{50}$/ml or $log_{10}ELD_{50}$/ml) recorded in Dorset Poll sheep inoculated with BTV2 attenuated vaccine virus

BTV2	Days post infection											
	0	2	4	6	8	10	12	14	16	18	20	22
Vaccine												
Sheep1	nvd	1.8	2.5	3.5	**4.5**	3.5	2.8	2.5	1.8	nvd	ns	nvd
Sheep2	nvd	2.1	3.5	3.8	**4.75**	3.5	2.8	2.8	2.1	≤1.5	ns	nvd
Sheep3	nvd	1.8	2.5	3.2	**4.5**	3.8	3.1	2.8	2.5	2.1	nvd	nvd
Sheep4	nvd	2.5	2.5	3.5	**3.9**	2.5	3.1	3.1	1.8	2.1	nvd	nvd
C. son. 1st passage												
Sheep1	nvd	ndv	ndv	nvd	nvd	**4.5**	3.25	≤1.5	ndv	ndv	ns	ns
Sheep2	nvd	ndv	1.83	3	3.75	**4.5**	1.83	2	nvd	nvd	ns	nvd
Sheep3	nvd	ndv	nvd	≤1.5	**3.5**	2.16	≥2.5	≤1.5	≤1.5	≤1.5	ns	nvd
Sheep4	nvd	1.75	≤1.5	3.5	**4**	2.16	2.16	≤1.5	≤1.5	nvd	ns	ns
C. son. 2nd passage												
Sheep1	nvd	1.75	2.8	2	**2.75**	2.5	2	≤1.5	≤1.5	nvd	ns	nvd
Sheep2	nvd	2.25	2.1	3	**3.75**	2.25	2.1	≤1.5	≤1.5	nvd	ns	nvd
Sheep3	nvd	nvd	2.5	≤1.5	nvd	≤1.5	**2.75**	≤1.5	≤1.5	≤1.5	ns	≤1.5
Sheep4	nvd	nvd	1.8	2.1	**3.75**	3.25	2.25	≤1.5	≤1.5	nvd	ns	nvd
C. nub. 1st passage												
Sheep1	nvd	nvd	2.75	**2.75**	1.75	2.16	1.83	nvd	nvd	nvd	ns	nd
Sheep2	nvd	nvd	**3.25**	3	2.5	1.83	1.83	≤1.5	≤1.5	≤1.5	ns	nd
Sheep3	nvd	nvd	2.16	**3.5**	3.25	2.5	2.16	1.75	≤1.5	nvd	ns	nd
Sheep4	nvd	nvd	3.5	3	**3.5**	2.5	2.5	≤1.5	≤1.5	≤1.5	ns	nd

nvd: no virus detected; ns: no sample; ≤1.5: virus detection in un-diluted washed and lysed blood assayed in BHK-21 cells; nd: not done; the peak titre recorded is given in bold.

valent vaccination against either BTV 2 or 9. The highest virus titres recorded in each group of sheep were 4.75 and 5.25 $log_{10}TCID_{50}$/ml for BTV 2 and 9, respectively, occurring at 8 dpv. The maximum duration of viraemia determined in either eggs or cell culture was 17 and 15 days for BTV 2 and 9, respectively.

3.3. Sheep inoculation with "insect passaged vaccine viruses"

Pyrexia was observed in 10 of the 12 sheep inoculated with BTV 9 vaccine-derived virus compared with only 1 of 12 sheep inoculated with similarly derived BTV 2 vac-

Table 4
Viraemia ($log_{10}TCID_{50}$/ml or $log_{10}ELD_{50}$/ml) recorded in Dorset Poll sheep inoculated with BTV9 attenuated vaccine virus

BTV9	Days post infection											
	0	2	4	6	8	10	12	14	16	18	20	22
Vaccine												
Sheep1	nvd	2	2.17	4.17	**5.25**	4.25	3.75	2.0	1.83	nvd	nvd	nvd
Sheep2	nvd	1.85	3.75	**5.17**	4.5	3.5	2.75	nvd	nvd	nvd	nvd	nvd
Sheep3	nvd	nvd	3.75	4.5	**5.25**	3.8	3.25	1.75	nvd	nvd	nvd	nvd
Sheep4	nvd	nvd	nvd	2.5	**3.75**	2.8	1.75	nvd	nvd	nvd	nvd	nvd
C. son. 1st passage												
Sheep1	nvd	nvd	nvd	**2.75**	nvd	2.25	nvd	nvd	nvd	nvd	nvd	nvd
Sheep2	nvd	1.75	3.5	**5**	4.75	3.5	nvd	nvd	nvd	nvd	nvd	nvd
Sheep3	nvd	nvd	nvd	1.83	**2.5**	1.8	2.5	nvd	nvd	nvd	nvd	nvd
Sheep4	nvd	nvd	4.75	**5.5**	**5.5**	3.5	nvd	nvd	nvd	nvd	nvd	nvd
C. son. 2nd passage												
Sheep1	nvd	nvd	4.4	**6**	5.25	4.75	3.75	nvd	nvd	nvd	nvd	nvd
Sheep2	nvd	2	2.25	**5.5**	5	3.25	3	nvd	nvd	nvd	nvd	nvd
Sheep3	nvd	nvd	2.16	3	3.5	4.5	**5.5**	4.5	3.75	nvd	nvd	nvd
Sheep4	nvd	2.75	**3.1**	nvd	2	nvd	nvd	nvd	nvd	nvd	nvd	nvd
C. nub. 1st passage												
Sheep1	nvd	2.75	nvd	**2.75**	1.75	≤1.5	nvd	nvd	nvd	nvd	nvd	nd
Sheep2	nvd	2.25	2	**3.25**	≤1.5	≤1.5	3.25	2.5	nvd	nvd	nvd	nd
Sheep3	nvd	1.85	5.5	**6.25**	4.83	4.5	4.25	2	nvd	nvd	nvd	nd
Sheep4	nvd	2.25	3.5	4.5	**5.5**	4	2.1	2	2	2.25	nvd	nd

nvd: no virus detected; ≤1.5: virus detection in un-diluted washed and lysed blood assayed in BHK-21 cells; nd: not done; the peak titre recorded is given in bold.

cine virus (Table 2). The level and duration of viraemia recorded in sheep inoculated with the 2 BTV vaccine viruses after first and second passage in vector midges is given in Tables 3 and 4. Both vaccine viruses replicated in Dorset Poll sheep after 1st and 2nd passage through *C. sonorensis* and 1st passage though *C. nubeculosus*. The highest levels of viraemia (4.5 and 6.25 $\log_{10}$TCID$_{50}$/ml) were recorded at days 10 and 6 dpi in sheep inoculated with BTV2 and 9, respectively (Tables 3 and 4). The duration of a detectable viraemia using embryonated hen eggs and/or cell culture ranged from 7 to 21 days (Table 2).

3.4. Determination of the clinical reaction index (CRI) in sheep inoculated with vaccine and vaccine derived viruses

The mean CRI scores generated for each group of four sheep inoculated with vaccine viruses and midge passaged vaccine viruses are given in Table 1. The degree of attenuation of these vaccine virus preparations relative to a homologous wild-type virus infection, was calculated as 100−[mean test score/virulent virus score] × 100.

4. Discussion

4.1. Disease and protection

The development of clinical signs in 32 out of 32 Dorset Poll sheep subsequent to their vaccination with commercial freeze dried BTV2 attenuated, monovalent vaccine virus produced by Onderstepoort Biological Products (OPB), S. Africa and the commercial freeze dried BTV9 attenuated, monovalent vaccine virus labelled as 'IZS Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise G. Caporale—Via Campo Boario 64100 TERAMO (Italy) Batch N. 006 Exp. Date 06/04', suggests that these vaccine viruses are not fully attenuated. In particular, one out of four sheep vaccinated with the commercially available BTV-9 vaccine, originating from Italy, experienced the longest duration of pyrexia of any of the experimental animals used in this work (6 days), including the ones infected with wild-type virulent strain BTV serotype 9 (4 days). The observation that some BT vaccine viruses are themselves able to cause a BT disease in Dorset Poll sheep is worrying and as this phenomenon is unlikely to be restricted to a single breed of sheep, suggests that such vaccines may require safety testing in a series of the target breeds before release into new geographical areas.

CRI scores and the percentage reduction in observed clinical signs in sheep inoculated with the vaccine viruses or insect passaged vaccine viruses (1st and 2nd passages), as compared with wild-type viruses of the same serotype, were similar and provided no evidence of increasing vaccine virus virulence on vector passage. However, because the numbers of animals used was relatively low further work in this area is recommended to confirm this point.

4.2. Viraemia in sheep

Viraemia was detected in all 32 Dorset Poll sheep inoculated either with attenuated vaccine virus or vaccine virus passaged through *C. sonorensis* or *C. nubeculosus*. The duration of detectable viraemia in the experimental animals, ranged from 9 to 17 days. Because of the inherent limitations of these tests in terms of sensitivity, it is probable that the actual viraemia may extend for even longer. The peak titres recorded here, ranged from 2.75 to 6.25 $\log_{10}$TCID$_{50}$/ml. Previous workers have shown that BTV titres as low as 2.4 $\log_{10}$TCID$_{50}$/ml are sufficient to infect vector species of *Culicoides* [34]. Our findings are therefore highly significant and strongly support the conclusions of Ferrari et al. [18,22] that vaccine virus is replicating in vaccinated sheep to sufficiently high titres to permit infection of midges and subsequent transmission in the field.

4.3. Vaccine virus infection and replication in vector Culicoides

The results presented in this paper also confirm that both BT vaccine viruses (serotypes 2 and 9) are able to replicate in vector species of *Culicoides* after infection by the natural, oral route. Although virus titres in individual midges were not determined, the titres recorded for BTV 2 and 9 in pools of 156 and 278 *C. sonorensis*, respectively, and 459 and 390 *C. nubeculosus*, respectively, after the first oral infection were 6.8 and 6.55 for *C. sonorensis* and 6.3 and 5.05 $\log_{10}$TCID$_{50}$/pool for *C. nubeculosus*. Based on earlier work [35,36] transmission will occur if BTV titres are in excess of 2.5 $\log_{10}$TCID$_{50}$/midge. Again our findings corroborate those of Ferrari et al. [22] in Italy.

4.4. Reassortment between vaccine and field strains of BTV

Reassorment between vaccine and field virus or between different serotypes of vaccine virus were not investigated in this study. However, the fact that vaccine virus will replicate in vector midges and that these viruses can then be transmitted to vertebrate hosts must provide the opportunity for reassortment to occur. In fact, previous workers have already reported dual infections in sheep and cattle's blood [37] and a high frequency of reassortment of genome segments in *C. variipennis* experimentally infected with BTV 10 and 17 [38].

The effect of such reassortments between BTV serotypes in dually infected vectors and/or hosts could result in altered virus characteristics. Perpetuation of such reassorted viruses in the field could have a significant impact on the serological status of animals [39] and could also result in modification of the virus virulence [40].

The data reported in this paper address many of the concerns that are associated with the use of attenuated BTV vaccines, particularly in epizootic regions. On the basis of these findings further work is clearly necessary to extend the

study to other breeds of sheep vaccinated with other monovalent and polyvalent attenuated BTV vaccines.

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