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Clinical disease in sheep caused by bluetongue virus serotype 8, and prevention by an inactivated vaccine

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ABSTRACT

The ability to reduce clinical signs, induce neutralizing antibodies, and perhaps most importantly, to prevent or reduce viraemia (and therefore virus-transmission), represent primary criteria for assessment of bluetongue virus (BTV) vaccine efficacy. Identification of BTV challenge-strains that reliably induce viraemia and clinical signs comparable to those in naturally infected animals, is therefore important for vaccine evaluation. Texel cross-breed and Dorset Poll sheep vaccinated with inactivated BTV-8 vaccine ('Bovilis® BTV8' from MSD Animal Health), were challenged with low-passage BTV-8 (Northern European strain) grown in either insect (*Culicoides*) or mammalian cell-cultures. The severity of clinical signs was recorded (using a modified numerical scoring-system, which is described) along with viraemia and serum neutralizing (SN) antibody levels. Low level SN-antibodies were detected at the time of challenge (three weeks after vaccination). All unvaccinated control animals became infected after challenge, developing high SN-antibody titres by 21 days post challenge (dpc). Vaccinees showed faster increases in SN-antibody titres ('booster' response), with significantly higher titres at 6 dpc than unvaccinated controls. Although only limited clinical-signs could be attributed to BTV in younger animals infected with the mammalian-cell-culture derived virus, both BTV-8 challenge preparations induced severe clinical signs comparable to 'bluetongue' observed during natural outbreaks in older unvaccinated animals. Challenge with BTV-8 grown in *Culicoides* cell-cultures seemed to induce greater severity of clinical-scores and 'post-mortem lesions' than the mammalian-derived BTV-8 strain. Vaccination reduced clinical signs, fever, and viraemia equally well after challenge with either virus preparation.

1. Introduction

Bluetongue (BT) is an infectious viral disease of domestic and wild ruminants, caused by bluetongue virus (BTV). '*Bluetongue virus*' is also the 'type' species of the genus *Orbivirus*, within the family *Reoviridae* [1]. Twenty-four BTV serotypes have long been recognized, however Toggenburg virus (from Switzerland) and a BTV strain from Kuwait, have recently been recognized as BTV-25 and BTV-26 [2,3].

BTV is thought to be transmitted between its ruminant hosts primarily by biting midges (*Culicoides* spp.). Although it can also be transmitted via an oral route [4] or vertically from dam to offspring [5,6]. The world distribution of BTV is limited to geographical areas where 'vector-competent' *Culicoides* spp. are present and weather conditions allow the virus to replication in and be transmitted by these insects. Historically Europe has only suffered occasional BTV incursions into its southern countries, e.g. Spain/Portugal, and Cyprus [7]. However, recently there have been dramatic changes, with new BTV incursions into southern Europe every year since 1998, and the first BT outbreaks ever recorded in Northern Europe, starting with arrival of BTV-8 in the Netherlands and Belgium during 2006 [8,9].

The European BTV-8 epidemic caused major economic damage to livestock industries, due to high morbidity and mortality in ruminants (particularly sheep), losses in productivity, reproductive success and milk yield (in cattle), as well as restrictions to animal movement and trade in many countries that had no prior record of bluetongue disease (BT) (including the Netherlands, Germany, Belgium and France) [10].

Since the start of the BTV-8 outbreaks in the Maastricht region of the Netherlands in 2006, several inactivated BTV vaccines (against BTV-8 and other serotypes) have been developed by different veterinary pharmaceutical companies. These vaccines were applied on a massive scale, starting in 2008 [11], helping to remove the virus from most of Europe.

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Although a high percentage of sheep infected with BTV-8 showed severe clinical signs and pathology during the European BT outbreaks, many animals were less severely affected or infected sub-clinically [12]. Laboratory studies have also shown significant variations in the severity of clinical disease after experimental infection with BTV-8 [13–17]. The World Organization for Animal Health (OIE) specifies that efficacy testing of BTV vaccines should include a demonstration that they will control clinical BT disease in animals. This can be difficult to achieve in the absence of reliable methods to induce the disease.

A number of factors that might influence the clinical outcome of experimental infection with BTV have been considered and discussed, including the way in which the challenge-inoculum is prepared and administered, the breed of animal, and individual animal susceptibility [17]. The identification of a challenge strain that reliably induces disease in control animals, comparable to the clinical signs observed in naturally infected animals, is therefore of high importance to fully assess the potential of vaccines to protect against disease manifestation.

An additional objective of this study was to determine the protective efficacy of an inactivated BTV-8 vaccine against the clinical signs of BT, after experimental infection with a virulent challenge strain. A virulent challenge strain of BTV-8 was identified (involving comparisons of virus grown in mammalian cells (MC) or propagated in *Culicoides sonorensis* cells (KC)). Variables that may contribute to development of clinical disease were also explored, including age (1 year old, or at least 3 years old) and breed (Dorset Poll and Texel).

2. Materials and methods

2.1. Animals and experimental design

Two vaccine-challenge studies were performed (see Table 1). In the first study, twenty-two, one-year-old Texel cross-bred sheep were randomly assigned to two vaccination groups of 8 animals each (groups 1A and 1B), or to a control group (6 animals). Animals in groups 1A and 1B were vaccinated with two different 'standard' batches (batch I and batch II respectively) of inactivated 'Bovilis® BTV8' vaccine (MSD Animal Health). The sheep in the third group (group 1C) remained unvaccinated.

In a second study, twenty-four Dorset Poll sheep (≥3 years old) were randomly allotted to four groups of six animals (groups 2A to 2D). Six, two-year-old Texel sheep constituted a fifth group of animals (2E). Animals in groups 2A and 2B were vaccinated with batch III of 'Bovilis® BTV8' (MSD Animal Health), whilst animals in the control groups 2C, 2D and 2E were treated with 1 ml of saline.

Before vaccination, all animals were shown to be free of BTV specific antibodies, as detected by a competitive ELISA (VMRD, USA) and all animals were free of BTV as detected by RT-PCR [18] before the challenge with the infectious virus.

2.2. Vaccine

Three different standard batches of the inactivated vaccine 'Bovilis® BTV8' (MSD Animal Health) were used (see Table 1). Vaccinations were performed in accordance with the manufacturer's recommendations, i.e. a 1 ml dose of the vaccine preparations was administered once subcutaneously in the axilla of the animals.

2.3. Challenge

Twenty-one days after vaccination all of the animals in both studies were inoculated subcutaneously (SC) in the neck with BTV-8 challenge virus. Study groups 1A, 1B, 1C, 2A and 2C were challenged with the MC-BTV-8, isolated in October 2006 from a BTV-8 infected clinically ill sheep in the South of the Netherlands (Heerlen). The virus was given one passage on embryonated chicken eggs, one passage on baby hamster kidney cells (BHK-21 cells) and one passage on Vero cells, resulting in MC-BTV-8 virus stock with a titre 7.3 log 10 $TCID_{50}$/ml (as determined on BHK-21 cells). In the first study, a total challenge dose of 7.3 $\log_{10}$ $TCID_{50}$ was given SC in 10 ml. In the second study, the animals of groups 2A and 2C were challenged SC with a total dose of 8.3 $\log_{10}$ $TCID_{50}$ of MC-virus, in 10 ml, with a Ct value of 14.0.

The animals of study groups 2B, 2D and 2E were challenged with a BTV-8 isolated in 2007 from a BTV-8 infected cow in the UK, passaged twice in insect cells (KC cells – derived from *C. sonorensis*) [19]. This insect-cell derived virus is stored in the Orbivirus Reference Collection (ORC) at the Institute for Animal Health, Pirbright, UK, as reference strain 'UKG2007/64'. Animals were challenged SC with 1 ml containing 7.25 $\log_{10}$ $TCID_{50}$ (with a Ct value of 13.1). The virus titre of the KC-BTV-8 preparation was determined on KC cells.

2.4. Body temperature and clinical signs

From two days before challenge until three weeks post challenge (pc), rectal body temperatures were monitored daily. The general 'aspect' of the animals (depression, anorexia) and BTV-specific clinical signs, such as lesions at the mouth, eyes, nose and feet were also monitored and quantified using a modified version of the clinical reaction index (CRI) scoring system developed by Huismans et al. [20] and Darpel et al. [17].

Clinical scores were based on signs developed from day 3 to 14 days post challenge (dpc), calculated as followed:

Table 1
Experimental design.

Group (number of animals)	Age (years)	Breed	Treatment	Challenge preparation[a] and dose ($\log_{10}$ $TCID_{50}$) at day 21 days post vaccination
Study 1				
Gr.1A (8)	1	Texel	Bovilis® BTV8 batch I	MC, 7.3
Gr.1B (8)	1	Texel	Bovilis® BTV8 batch II	MC, 7.3
Gr.1C (6)	1	Texel	None	MC, 7.3
Study 2				
Gr.2A (6)	>3	Dorset Poll	Bovilis® BTV8 batch III	MC, 8.3
Gr.2B (6)	>3	Dorset Poll	Bovilis® BTV8 batch III	KC, 7.25
Gr.2C (6)	>3	Dorset Poll	Saline	MC, 8.3
Gr.2D (6)	>3	Dorset Poll	Saline	KC, 7.25
Gr.2E (6)	>2	Texel	Saline	KC, 7.25

[a] MC: virus grown on mammalian cells; KC: virus grown on insect cells.

- Fever score: 1 point for each day of temperature >40 to 41 °C; 5 points per day of temperature >41 °C.
- Anorexia score: 1 point per day, up to 5 days. On the fifth day the animal would be euthanized to prevent suffering.
- Facial lesions: conjunctivitis, hyperaemia and oedema each scored 0–2 points per day, depending on severity – ulcers scored 0–3 depending on severity.
- Feet lesions: lameness and coronitis each scored 0–2 depending on severity.
- Respiratory tract lesions: rhinitis, coughing and dyspnoea, each scored 0–2 depending on severity.
- Veterinary intervention: 20 points independent of the number of interventions. These points were not taken in account if the animal had to be euthanized.
- Death (natural or euthanasia): 40 points.

The duration of clinical signs was also taken into account by adding daily scores, from 3 to 14 dpc.

2.5. Preparation of serum samples

Whole blood samples were collected from all animals just before vaccination (−21 dpc), before challenge (at 0 dpc), and at weekly intervals post challenge. The serum fraction was collected after centrifugation at ~3000 × *g* for 10 min. Complement was inactivated by incubation at 56 °C for 30 min. The presence of BTV-specific antibodies was assessed by serum neutralization tests (SNTs).

2.6. Serum neutralization test (SNT)

The SNT was developed at MSD Animal Health and validated in accordance with the VICH guide lines 1 and 2. Serial twofold dilutions of sera were made in micro-titre plates and incubated with live MC-adapted BTV-8 virus (100 $TCID_{50}$ per well) for 1 h at 37 °C. After this 1 h incubation period, Vero-cells (1.6×10^4 cells per well) were added in each well. After incubation at 37 °C for 4 days, the monolayer was examined for cytopathogenic effect (CPE). The number of positive wells (containing monolayers with CPE) and negative wells were recorded for each dilution. The SN titres of the test samples were given as $\log_2$ of the reciprocal of the highest dilution where all virus particles were neutralized (no CPE).

2.7. Collection and preparation of EDTA-blood samples

EDTA treated blood samples were collected every 2 days, from 0 dpc (just before challenge) to 21/22 dpc. The presence of the virus was assessed in fresh EDTA treated blood samples or in washed blood samples. EDTA treated blood samples were washed in accordance with the method developed by Clavijo et al. [21] with some minor modifications. Briefly, blood samples were diluted by adding 25 ml of physiological salt solution. After gently mixing, the diluted blood samples were centrifuged at 420 × *g* for 10 min at 4 °C. Supernatants were removed and the sedimented red blood cells (RBC) were gently resuspended in physiological salt solution (30 ml). The procedure was repeated twice. After the last centrifugation, RBC were resuspended in an equal volume of RBC resuspension buffer, which consisted of 0.01 M PBS supplemented with 0.5% BSA and gentamycin (112.5 μg/ml). The washed blood samples were stored at 4 °C.

2.8. Detection of the BTV viral RNA

The presence of virus in blood samples was assessed by real-time Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR). Viral RNA was extracted from the blood samples using the NucleoSpin® RNA Virus Kit (Macherey-Nagel), according to the manufacturer's instructions.

In the first study, a one-step RT-PCR protocol was applied, as reported by Agüero et al. [22], using primers specific for the NS1 coding region, as described by Katz et al. [23]. The amplification products were analysed by electrophoresis on a 2% agarose gels, stained with ethidium bromide, and scored as 0 (negative) or 2 (positive). In the second study, a pan-BTV real-time RT-PCR developed by Shaw et al. [18], targeting genome segment 1 (VP1 gene) was used with minor modifications.

Individual 'cycle threshold' (Ct) values were determined from the point at which the level of fluorescence passes the auto calculated or user defined 'threshold value'. PCR Base Line Subtracted Curve Fit method (Bio-Rad iQ5 software) was used for data analysis. Ct values were averaged and categorized into 3 groups: viraemia negative ($Ct \geq 35$); doubtful ($30 \leq Ct < 35$); viraemia positive ($30 < Ct$), with the scores 0, 1 and 2 respectively. The average RT-PCR scores per group were calculated in both studies.

2.9. Macroscopic examination

A post mortem examination was performed on animals of the second experiment. The buccal, thoracic and abdominal cavities were opened and the internal organs were inspected in situ for abnormalities. More specifically, aorta, trachea, main bronchi and bronchioles were opened and inspected. Animals showing pain and discomfort that were considered to be non-transient and likely to become more severe, were euthanized before the end of the studies for animal welfare reasons. The remaining animals were euthanized at the end of the experiment.

2.10. Statistical analyses

The presence of virus neutralizing antibodies, body temperatures, clinical signs and RT-PCR scores indicative of viraemia was evaluated. Both experiments were used to evaluate the effect of vaccination and in the second experiment the effect of challenge stock and breed were investigated. Prior to analysis by ANOVA, the clinical scores (CRI) were rank transformed [24]. SNT were evaluated using repeated measures ANOVA [25]. Finally in the second experiment, the RT-PCR scores were evaluated using generalized estimating equations (GEE) methods [26]. In the case of missing RT-PCR score due to the unplanned euthanasia of animals, a RT-PCR score of 2 was attributed at each time point following the death of the animals until the end of the experiment. The RT-PCR scores obtained from experiment 1 were not statistically analysed due to the absence of sufficient variability in the results. All statistical analyses were performed in SAS 9, SAS Institute Inc., Cary, NC, USA with the level of significance of 5%.

3. Results

3.1. BTV-8 specific antibody response

The presence of BTV-8 specific serum neutralizing (SN) antibodies was monitored throughout both experiments using SNT. Neutralizing antibodies were not detected at the start of either experiment, but were detected in the unvaccinated control animals (groups 1C, 2C, 2D and 2E) at 6 dpc (Fig. 1). In contrast, low levels of neutralizing antibodies were detected in the vaccinated animals prior to challenge (at 0 dpc in groups 1A, 1B, 2A and 2B). The SN titres measured in the vaccinated animals just before challenge (at 0 dpc) were significantly higher than in the control animals (*p*-value of 0.0002 and 0.009 for experiments 1 and 2 respectively). The vaccinated animals also showed a faster increase in SN antibody titre

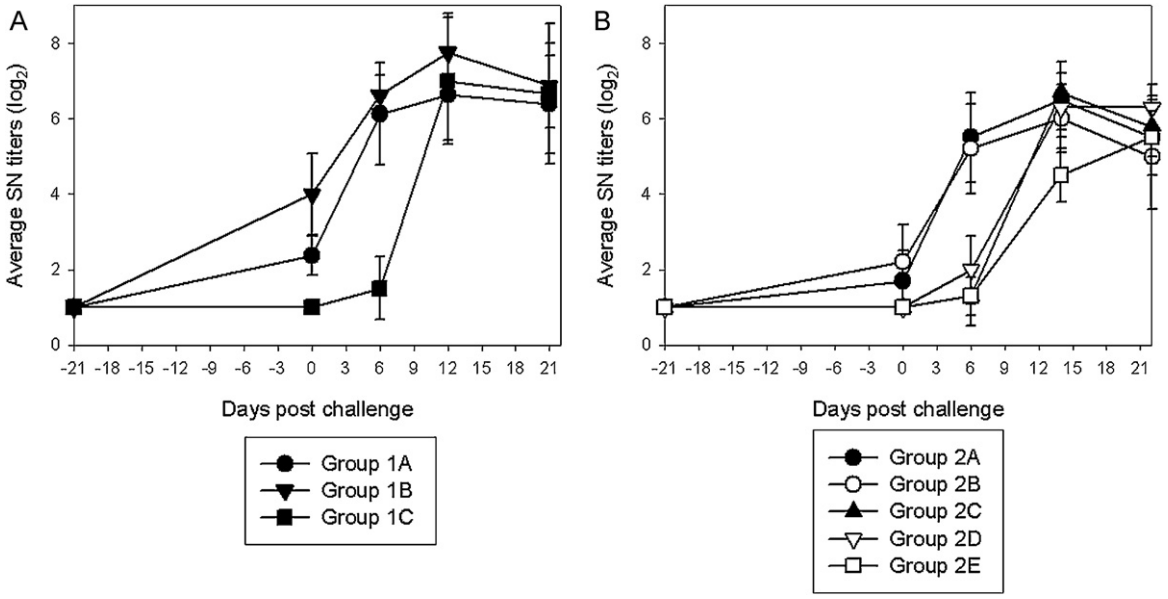

Fig. 1. Course of average neutralizing antibody titres. The left panel (A for experiment 1) shows the average SN titres of Texel cross-bred sheep. Groups 1A (circle) and 1B (triangle) were vaccinated respectively with Bovilis® BTV8 batches I and II at day −21 after challenge whilst animals of group 1C (square) were kept unvaccinated. All animals were challenged (at day 0) with MC-BTV-8 three weeks after vaccination. The right panel (B for experiment 2) shows the average SN titres induced in Dorset Poll (groups 2A to 2D) or Texel sheep (group 2E, open square). Dorset Poll were either vaccinated with Bovilis® BTV8 batch III (groups 2A, closed circle and 2B, open circle) or left unvaccinated (groups 2C to 2E) at day −21 post challenge. Animals of groups 2A and 2C (closed symbols) were challenged at day 0 with MC-BTV-8 and animals of groups 2B, 2D and 2E were challenged with KC-BTV-8 (open symbols). Serum blood from all animals was collected at several time points and examined for the presence of BTV-8 neutralizing antibodies. The SN titres were averaged per group and expressed in $\log_2$. The error bars indicate the group standard deviation.

(a 'booster' response), generating significantly higher SN antibodies titres at 6 dpc than the unvaccinated animals in control groups (p-values <0.0001 for both experiments). All of the challenged animals had SN antibody titres ≥5 by 21 dpc, and no significant differences were detected in average titres, between breeds or challenge preparations.

3.2. Body temperatures and clinical signs

All four control groups (1C, 2C, 2D and 2E) developed fever (body temperature above 40 °C – Fig. 2) for 2–3 days between 6 and 8 dpc (groups 2D and 2E). Although average body temperatures of the vaccinated animals remained below 40 °C for most of the experiments, vaccinated animals that were challenged with MC-BTV-8 (groups 1A, 1B and 2A) did show a slight temperature increase on 2 dpc.

Clinical scores established for both studies, are given in Table 2. Other than fever, only limited clinical signs that could be attributed to BTV were observed in the first experiment (younger animals infected with MC virus). Mild serous nasal discharge for one or more days was observed in one or more animals in all three groups (1A, 1B and 1C). A purulent nasal discharge was also observed in one of the unvaccinated control animals and anorexia for one or two days, was recorded in two control animals (group 1C).

In the second study including animals older than three years, unvaccinated control animals (groups 2C, 2D and 2E) displayed severe clinical signs comparable to clinical 'bluetongue' previously observed in naturally infected sheep [27]. These included ulceration of the nasal (Fig. 3A) and oral mucosa (Fig. 3B), as well as erosions at the coronary band (Fig. 3C). These signs were also significantly more severe (p-value = 0.014) after challenge with KC grown virus than after challenge with MC grown virus (group 2D versus group 2C). Three and four of the unvaccinated animals from groups 2D and 2E respectively had to be euthanized before the end of the study for animal-welfare reasons. No significant differences in clinical scores were observed between the two breeds (p-value = 0.32).

In both studies, vaccination with inactivated BTV-8 vaccines, significantly reduced the clinical scores post challenge (Fig. 2 and Table 2, p-values were <0.0001 and 0.0004 for experiments 1 and 2 respectively).

3.3. Macroscopic examination

Four out of six unvaccinated Texel sheep (group 2E) that were infected with KC-BTV-8 had to be euthanized before the end of the study. Upon post-mortem examination, they revealed a range of serious pathological findings including ulcerations on the lateral surface of the tongue and the buccal mucosa, extensive subcutaneous oedema all over the carcasses, sometimes associated with yellowish gelatinous material and haemorrhage (Fig. 4A and B). Haemorrhages were also observed in the tunica media of the *A. pulmonalis* (Fig. 4C). No relevant pathological findings were reported in the two remaining Texel sheep euthanized at the end of the study.

Three out of six of the unvaccinated Dorset Poll animals that were challenged with KC-BTV-8 (group 2D) were also euthanized before the planned end point. Oedema and haemorrhages (e.g. in the *A. pulmonalis*) were observed and some lymph nodes were wet on the cut surface. Lesions found in the other three sheep in this group were less prominent. The unvaccinated Dorset Poll sheep challenged with MC-BTV-8 (group 2C) developed haemorrhages and/or oedema in the lymph nodes, thymus, tongue (three out of six sheep) and *A. pulmonalis* (two out of six sheep).

Some minor macroscopic findings were also observed in the vaccinees of the second study (mainly petechiae in the lymph nodes). One Dorset Poll sheep challenged with KC-BTV-8 (group 2B) showed bleeding on the *A. pulmonalis*. Localized bleeding on the tongue was also observed in two out of twelve sheep although they were not viraemic.

3.4. Viraemia

The presence of BTV RNA in blood was monitored by RT-PCR assays. No BTV RNA was detected in the animals before the

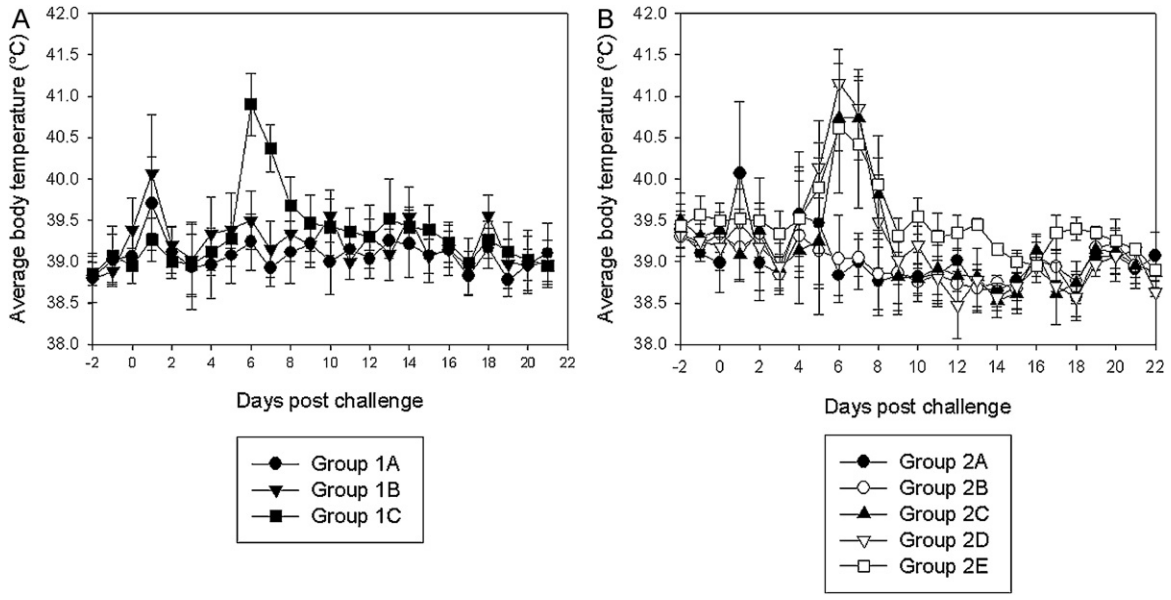

Fig. 2. Course of average body temperatures after challenge. The left panel (A for experiment 1) shows the average body temperature of Texel cross-bred sheep. Groups 1A (circle) and 1B (triangle) were vaccinated respectively with Bovilis® BTV8 batches I and II. Animals of group 1C (square) were kept unvaccinated. All animals were challenged (at day 0) with MC-BTV-8 three weeks after vaccination. The right panel (B for experiment 2) shows the average body temperatures of the Dorset Poll (groups 2A to 2D) or Texel sheep (group 2E, open square). Dorset Poll were either vaccinated with Bovilis® BTV8 batch III (groups 2A, closed circle and 2B, open circle) or left unvaccinated (groups 2C to 2E). Animals of groups 2A and 2C (closed symbols) were challenged at day 0 with MC-BTV-8 and animals of groups 2B, 2D and 2E were challenged with KC-BTV-8 (open symbols). Body temperature of all animals was measured from two days before challenge until the end of the experiment. The body temperature measurements were averaged per group. The error bars indicate the group standard deviation.

challenge (Fig. 5). All control animals (groups 1C, 2C, 2D and 2E) had RT-PCR scores of 2 (Ct value <30) for several days starting 2–4 days post challenge (see individual data in supplementary tables). No statistical differences could be established in RT-PCR scores between the different challenge preparations (group 2C versus group 2D, p-value = 0.11). Similarly no statistical difference between the Dorset Poll and Texel sheep could be estimated (p-value = 0.13). In both experiments, the vaccinated animals had in average lower RT-PCR scores than in the unvaccinated animals. Results from the first study showed that only one out of sixteen vaccinees developed viraemia. Moreover a significant difference between vaccinees (group 2A and 2B) and control animals (groups 2C and 2D) of the second experiment could be estimated (p-value <0.0001).

4. Discussion

According to European Community legislation concerning the production and control of inactivated mammalian bacterial and viral vaccines for veterinary use, any claim on the efficiency of a vaccine must be supported by clinical study data, allowing comparisons to unvaccinated control animals upon challenge with infectious virus [28]. Ideally vaccine validation trials would therefore use challenge inoculums which introduce clinical signs similar in nature and severity to those reported after natural infection. However, reproduction of clinical signs in unvaccinated control animals can be difficult to achieve under experimental conditions for a number of viruses, including respiratory syncytial virus [29] and bovine viral diarrhoea virus [30]. Mild clinical manifestations of BT disease have been reported after experimental infection of sheep and cattle with BTV-8 [14], whilst other studies have reported clear or variable clinical signs [13,31]. Herein two BTV-8 strains, both derived from the Northern European outbreak (2006/2007) but generated by two different isolation methods (mammalian or insect cell culture respectively), were assessed, to identify a virulent virus strain suitable for challenge studies.

The severity of clinical signs differed between the four control groups (1C, 2C, 2D and 2E), with only mild clinical signs observed in the younger sheep (first study, group 1C). In contrast older sheep (second study, 2C, D and E) developed clinical signs typical of BT disease as reported by others [16,17]. Greater severity (higher clinical scores) of the clinical signs and 'post mortem lesions' seemed to be induced after challenge with the KC-BTV-8 virus (Table 2). Slight differences were observed in the timing and the extent of pyrexia between the control groups 1C, 2C (MC derived virus), 2D and 2E (KC-insect cell derived virus).

OIE currently recommends the use of infected blood as a source of challenge viruses, since this avoids the possibility of attenuation caused by passage in mammalian cells [32]. Two recent publications show that a low passage BTV-8 inoculum can induce clinical signs [14,31] and pathological lesions [31] similar to those induced by infectious blood. Infected blood challenge procedures are difficult to standardize and present risk of contamination with extraneous agents, including other BTV serotypes/topotypes/lineages [33]. Our findings confirm that low passage BTV-8 preparations can provide a valid alternative to the use of infectious blood for vaccine challenge studies. Our observations that clinical signs were more pronounced after infection

Table 2
Mean clinical reaction index (CRI) after vaccination and challenge of sheep with BTV-8.

	Group							
	1A	1B	1C	2A	2B	2C	2D	2E
CRI	0.5	0.6	4.8	8.7	8	25.8	56	44.5

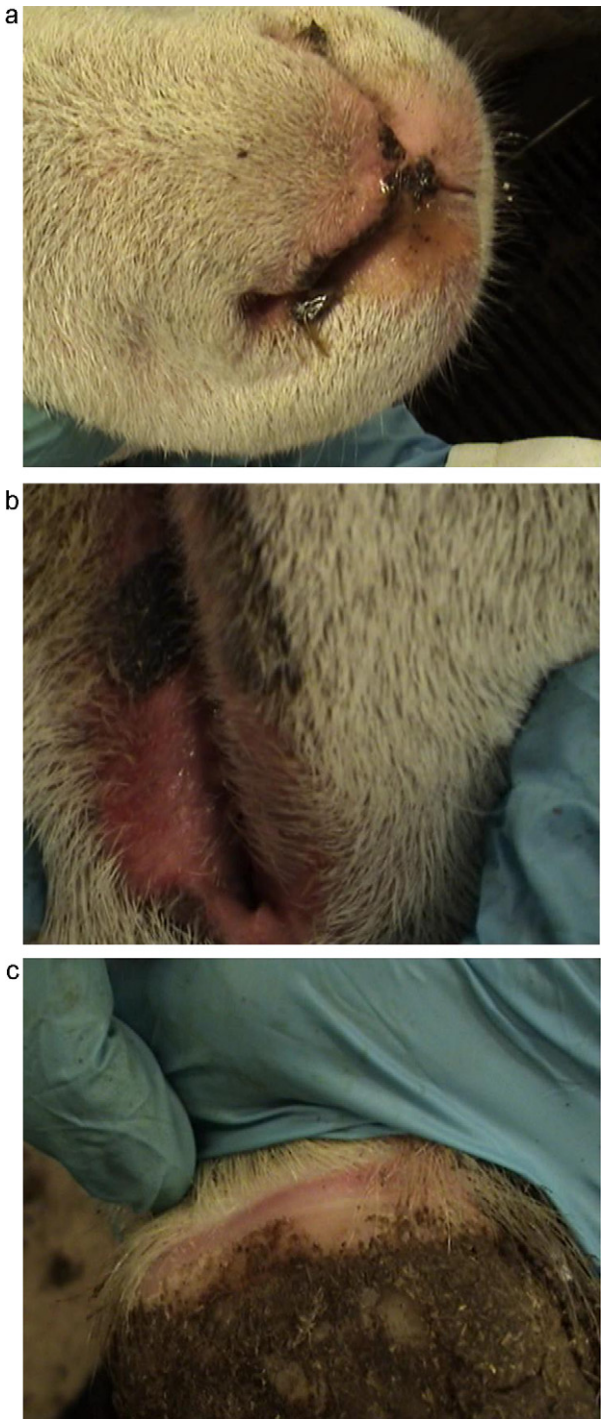

Fig. 3. Clinical signs observed after challenge infection of adult sheep with KC-BTV-8: ulcerations on the nasal mucosa (A, Dorset Poll) and the tongue (B, Texel sheep), erosions at the coronary band of the hoofs (C, Dorset Poll).

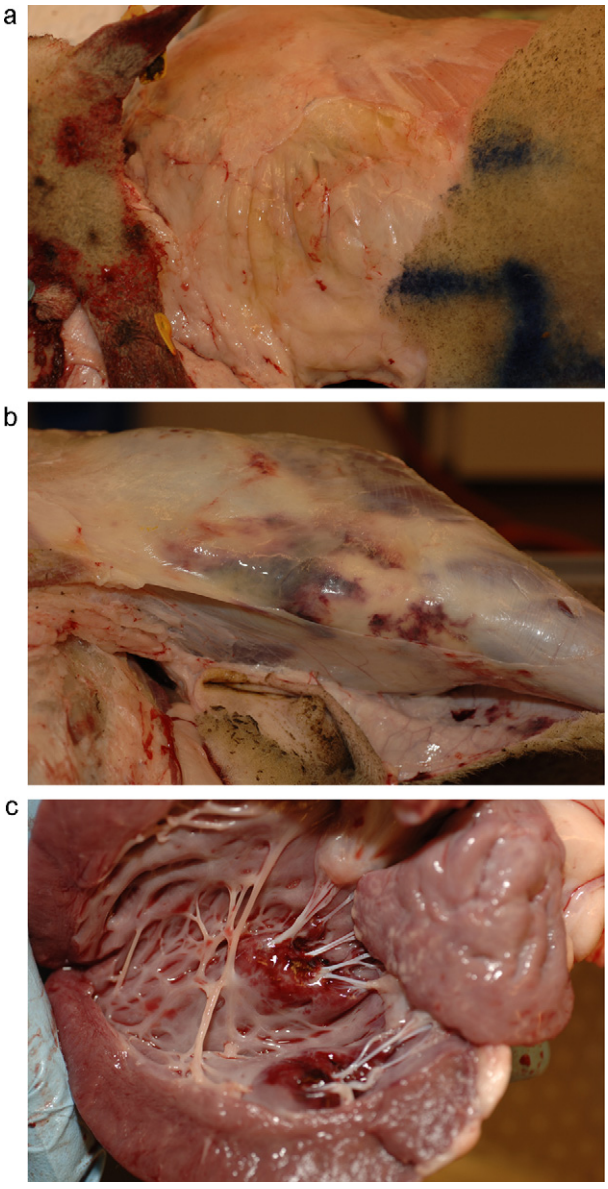

Fig. 4. Pathological lesions observed after challenge infection of adult Texel sheep with KC-BTV-8: subcutaneous oedema (A), subcutaneous haemorrhages (B) and haemorrhages in the heart (C).

with BTV-8 grown in insect cell cultures (KC cells), suggest that this procedure provides also a suitable method for production of an infectious inoculum for challenge studies. However, the dose of such an inoculum may need to be further optimized to avoid unnecessary suffering of the animals.

The two BTV-8 strains used for challenge in this study were isolated from different vertebrate hosts. Indeed the MC-BTV-8 was isolated in 2006 from a sheep infected in the Netherlands and the KC-BTV-8 was isolated from a cow in UK in 2007 (see UKG2007/64 in the dsRNA virus collection at IAH Pirbright). Full genome sequence data for the index case of BTV-8 from the Netherlands 2006 [34] and for the KC-BTV-8 challenge strain are available at IAH Pirbright (data unpublished). The comparison of the genome sequence between these two isolates shows that these two viruses are very similar with few nucleotide changes (~99.9% nt sequence identity) most of which are silent, strongly suggesting that these two BTV-8 strains represent close members of the same virus lineage, derived from a single point introduction. Unfortunately, sequence data for the MC-BTV-8 used in our study are not available, but based on the similarities between the Dutch and UK index cases and since no other BTV strains were circulating in Northern Europe in 2006/2007, it can be reasonably expected that the original isolates used to generate the two challenge viruses used herein may be genetically very similar. The possibility that genetic changes between the KC and MC preparations have an effect on the virulence is considered low but cannot be disclosed.

The MC- and KC-BTV-8 strains used for challenge have also different levels of adaptation to mammalian cell culture, making conventional 'virus titration' difficult to carry out and the virus titre

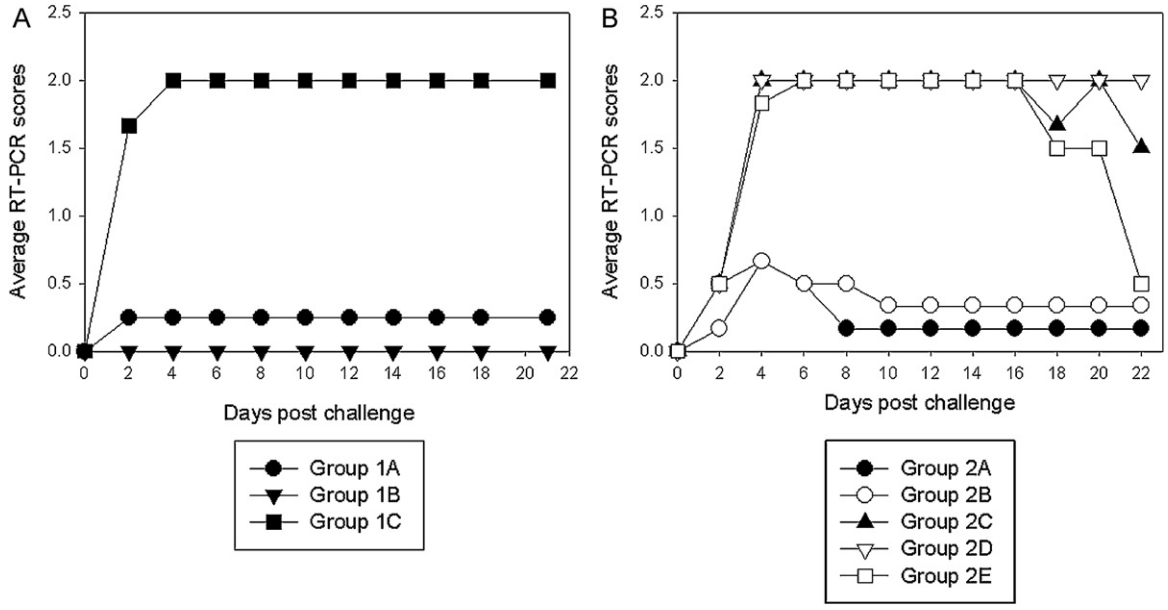

Fig. 5. Viraemia after challenge of adult sheep with BTV-8 virus. The left panel (A for experiment 1) shows the average RT-PCR scores of Texel cross-bred sheep. Groups 1A (circle) and 1B (triangle) were vaccinated respectively with Bovilis® BTV8 batches I and II. Animals of group 1C (square) were kept unvaccinated. All animals were challenged (at day 0) with MC-BTV-8 three weeks after vaccination. The right panel (B for experiment 2) shows the average RT-PCR scores of the Dorset Poll (groups 2A to 2D) or Texel sheep (group 2E, open square). Dorset Poll were either vaccinated with Bovilis® BTV8 batch III (groups 2A, closed circle and 2B, open circle) or left unvaccinated (groups 2C to 2E). Animals of groups 2A and 2C (closed symbols) were challenged at day 0 with MC-BTV-8 and animals of groups 2B, 2D and 2E were challenged with KC-BTV-8 (open symbols). BTV RNA was isolated and amplified by RT-PCR. RT-PCR scores of 0, 1 or 2 for respectively negative, doubtful or positive viraemia were attributed and averaged per group.

difficult to compare. As detected by RT-PCR, all of the control animals in our study were successfully infected, regardless of the dose or source of challenge virus used. The kinetics of neutralizing antibody and viraemia development were similar in sheep challenged with two different doses of the same virus preparation (group 1C versus group 2C – infected with 7.3 $\log_{10}$ or 8.3 $\log_{10}$ $TCID_{50}$ of MC-BTV-8 respectively). These data are consistent with recent studies in sheep and calves [14,31] showing that the kinetics of viraemia (as determined by RT-PCR) were similar after infection with different doses of different challenge preparations (i.e. low cell-passaged versus infectious blood). However, the doses used here should be considered as high (representing a severe and reliable challenge) and are certainly much greater than that delivered during feeding by systemically infected adult *Culicoides* in the field, even though such insects can reliably cause infection [35]. Even significantly lower doses of infectious BTV-8 (as low as 3.0 $\log_{10}$ $TCID_{50}$) can induce typical BT clinical signs and fever in ewes [6]. Furthermore, recent studies by Martinelle et al. [31] show that clinical signs of BT can be induced in calves inoculated with 4.5 $\log_{10}$ ELD50 of low cell-passaged BTV-8. It is therefore considered likely that the differences in clinical signs between different groups, that were observed in our study, were not simply related to the inoculum dose. We conclude that these differences are likely to be related to the age, breed and/or strain of challenge virus.

Although younger animals are often more susceptible to disease in general (possibly due to acquired immunity in older animals), reports from natural outbreaks of BTV-8 in the naive European ruminant populations, indicate that adult sheep developed more severe clinical signs than lambs [12], in agreement with our results.

It is well documented [36] that there are differences in BTV susceptibility between breeds of sheep. Fine-wool European breeds such as the Merino and Dorset Poll are usually regarded as highly susceptible to BT disease. Our results, showing more pathological lesions in Texel sheep than in Dorset Poll sheep, also suggest a 'breed-effect', with this particular lineage of BTV-8. However, a study using a larger number of animals would be required to confirm this conclusion, and different strains lineages or topotypes of BTV may generate different results with these or other breeds. Variation in the severity of clinical signs between individual Texel sheep may also be larger than between individual Dorset Poll sheep, suggesting that differences in the individual susceptibility have significant impact on the severity of diseases outcomes [17].

In both studies described here, vaccination with inactivated Bovilis BTV8 vaccines induced a significant reduction of fever, clinical signs and viraemia post challenge as compared to the respective control groups. This protection is especially significant after challenge with a clearly virulent challenge strain, as shown for the KC cell-culture generated BTV-8. As mass-vaccination campaigns against BTV-8 aim to eradicate of the virus, the efficacy of a vaccine to not only reduce clinical signs but more importantly to prevent or strongly reduce viraemia and therefore virus transmission, should be regarded as the main criterion for testing BTV-8 vaccine efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.11.100.

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