Seroconversion, neutralising antibodies and protection in bluetongue serotype 8 vaccinated sheep

C.A.L. Oura\textsuperscript{a,∗}, J.L.N. Wood\textsuperscript{b}, A.J. Sanders\textsuperscript{a}, A. Bin-Tarif\textsuperscript{a}, M. Henstock\textsuperscript{a}, L. Edwards\textsuperscript{a}, T. Floyd\textsuperscript{b}, H. Simmons\textsuperscript{c}, C.A. Batten\textsuperscript{a}

\textsuperscript{a} Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, UK
\textsuperscript{b} Cambridge Infectious Diseases Consortium, Department of Veterinary Medicine, University of Cambridge, Madingley Road, Cambridge CB3 0ES, UK
\textsuperscript{c} VLA Weybridge, New Haw, Addlestone, Surrey KT15 3NB, UK

ABSTRACT

Bluetongue virus serotype 8 (BTV-8) has caused a major outbreak of disease in cattle and sheep in several countries across northern and western Europe from 2006 to 2008. In 2008 the European Union instigated a mass-vaccination programme in affected countries using whole virus inactivated vaccines. We evaluated vaccinal responses in sheep and the ability of the vaccine to protect against experimental challenge. Sheep vaccinated 10 months previously under field conditions were challenged with BTV-8. One of 7 vaccinated sheep became infected, as evidenced by detection of viral RNA by real-time RT-PCR and by virus isolation. The remaining 6 sheep appeared fully protected from virus replication. None of the vaccinated sheep showed clinical signs of BTV and there was a good correlation between the presence of neutralising antibodies on challenge and protection. Commercially available ELISAs were evaluated for their ability to detect antibodies in sheep vaccinated on a single occasion. The sandwich (double antigen) ELISA assays were found to be more sensitive at detecting antibodies in vaccinated sheep than the competitive ELISAs.

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1. Introduction

Bluetongue serotype 8 (BTV-8) successfully overwintered in many countries in northern and western Europe over the winter of 2006/2007 and emerged in the summer of 2007 to cause devastating effects to the livestock industries of many countries including the Netherlands, France, Belgium and Germany\cite{1,2}. BTV-8 arrived on the south-eastern shores of the UK in the summer of 2007\cite{3,4}. Once the ability of the virus to overwinter in northern Europe was confirmed the need for rapid and immediate control of the disease became apparent before it spread to all corners of Europe and beyond.

After much debate about the advantages and disadvantages of the use of live attenuated and inactivated vaccines against BTV\cite{5}, countries in northern and western Europe decided to use inactivated as opposed to live attenuated vaccines in their BTV-8 control plans. In the autumn of 2007 countries started to place orders for inactivated vaccine against BTV-8. In October 2007 the UK put in an order for 22.5 million doses of the Intervet-manufactured Bovilis-BTV-8 vaccine. The first 9 million doses of the vaccine were released to farmers in the high risk areas of the east and south of the UK at the end of April 2008. As more doses of vaccine became available to the UK farmers, the protection zone (PZ) moved northwards and westwards until it covered the whole of England and Wales by September 2008. The combination of high levels of vaccine coverage in areas where BTV-8 circulated in 2007 before the 2008 transmission season and the cold wet summer provided an explanation for the lack of BTV circulation detected in the UK throughout 2008\cite{6}.

The BTV-8 inactivated vaccines used across Europe in 2008 were produced rapidly and were licenced for emergency use without any associated efficacy guarantees. Although many millions of animals in Europe have now been vaccinated, there remain some unanswered questions about the immunological mechanisms resulting in vaccine efficacy (ability to protect clinically and to prevent onward transmission), the antibody responses to the products, particularly in sheep which only receive one dose, the duration of immunity, and both the extent and length of colostral antibody protection in lambs and calves born from vaccinated dams. This is the first published study detailing the efficacy of the BTV-8 Bovilis vaccine in sheep and the first study evaluating the long-term (10 months) protective properties of any BTV-8 inactivated vaccine on the market.

Soon after the vaccination campaign started in the UK it was found that many sheep that had received one dose of vaccine did not have detectable antibodies when tested with competitive ELISAs.
that had been validated for the detection of BTV antibodies in naturally infected animals [7,8]. In this paper the sensitivities of the commercially available ELISAs (sandwich and competitive formats) in detecting BTV antibodies following vaccination in sheep vaccinated on one and two occasions have been compared.

2. Materials and methods

2.1. Animals and experimental design

Fifty-three (53) adult healthy Cheviot and poll Dorset sheep were selected for the study from a farm in East Anglia in the UK. All animals were tested negative for BTV antibodies by serology and for viral RNA by real-time RT-PCR prior to inclusion in the study. The sheep were vaccinated for the first time in May 2008 and a group of 33 sheep was sampled (whole blood) the day before vaccination and at 7, 14 and 56 days post-vaccination (dpv). At 56 dpv only 28 out of the 33 sheep were presented for bleeding by the farmer. A second group of 20 sheep, vaccinated in May 2008, was sampled 82 dpv. These sheep were vaccinated for a second time in September 2008 and sampled again 24 days after the second vaccination. Samples were tested throughout the study by competitive ELISA (cELISA), sandwich ELISA (sELISA) and samples from the second group of 20 sheep were tested by Serum Neutralisation Test (SNT). EDTA blood samples were taken from the 33 sheep at both 1 and 7 dpv and were tested for viral RNA by real-time RT-PCR.

Vaccine/Challenge Study Group 1: Three (3) unvaccinated poll Dorset sheep between 6 and 9 months of age, born from unvaccinated dams, were used as unvaccinated controls in the study.

Vaccine/Challenge Study Group 2: Seven (7) vaccinated poll Dorset sheep older than 12 months of age were selected from a farm in East Anglia in the UK. The sheep were vaccinated subcutaneously according to the manufacturers instructions in May 2008, 10 months prior to challenge.

One week prior to challenge the sheep in groups 1 and 2 were transferred to the Veterinary Laboratories Agency (VLA) BSL-3 facility. The sheep were inoculated subcutaneously in the neck with 1 ml of the Netherlands 2006 strain that was passaged twice in KC (Culicoides variipennis) cells (NET2006/02). The BTV challenge protocol used avoids the growth of virus on mammalian cells as this has been found to reduce viral virulence. Due to a lack of cytopathic effect (cpe) in KC cells it was not possible to provide a viral titre for the inoculum although, when tested by real-time RT-PCR, it gave a Ct of 15.

EDTA and whole blood (serum) samples were taken from all the sheep on days—1, 2, 4, 6, 8, 10, 14, 18 and 22 post-challenge infection (dipi). Samples were tested throughout the study by real-time RT-PCR, virus isolation, ELISA and SNT. Throughout the study the body temperature of the animals was monitored daily and the sheep were examined by a veterinarian daily for clinical signs.

2.2. Vaccine

The sheep were vaccinated with the Intervet-manufactured Bovilis-BTV-8 inactivated vaccine (Batch number 00018201, expiry 02/2009) according to the manufacturer’s instructions.

2.3. Molecular tests

2.3.1. Real-time RT-PCR

RNA was extracted from EDTA blood using either the MagnaPure (Roche) extraction robot using the ‘total NA/External Lysis’ protocol. Real-time RT-PCR was performed using a modified version of the procedure described by Shaw et al. [9].

2.4. Serology tests

2.4.1. ELISA assays

Whole blood samples were centrifuged at 2400 × g for 5–10 min to obtain serum. The detection of BTV specific antibodies in serum was carried out using three competitive ELISA assays (Pourquier C-ELISA kit (IDEXX, UK), ID-Screen Bluetongue Competition ELISA (ID-Vet, France), Bluetongue antibody test kit C-ELISA (VMRD, USA) and two sandwich (double antigen) ELISA assays (ID-Screen Bluetongue Early detection ELISA, ID-Vet, France) and INGEZIM BTV DR 12.BTV.K0, Ingenasa, Spain) according to the manufacturer’s instructions.

2.4.2. Serum Neutralisation Test (SNT)

SNT was performed according to the method of Haig et al. [10] using the BTV-8 South African reference virus and serotype-specific BTV-8 positive control antisera. Briefly, sera were diluted (1:10–1:1280) and titrated against 100 TCID50 of the BTV-8 South African reference virus. Plates were incubated for 1 h at 37 °C and then transferred to 4 °C overnight. The following day 50 μl of a Vero cell (African green monkey kidney) suspension 2 × 105/ml were added per well and, after incubation for 4–7 days at 37 °C, the wells were scored for cytopathic effect (CPE) observed. The neutralisation titre was determined as the dilution of serum giving a 50% neutralisation end point SNT antibody titres were expressed as log10 reciprocal of the highest positive serum dilution.

2.5. Virus isolation

EDTA blood samples were washed 3 × with PBS and sonicated as described in the OIE manual [11]. KC (C. variipennis) cells were inoculated with 200–500 μl of washed blood and incubated overnight at 26 °C. The following day the inoculum was removed and replaced with fresh media (Schneiders (Sigma, UK), 1% pen/strep, 1% Amphoterin B, 10% PCS). Cells were incubated 26 °C for 7 days and then harvested by centrifugation (2400 × g for 5–10 min), supernatant was tested by real-time RT-PCR for the presence of BTV RNA as described in Section 2.3.

2.6. Statistical analyses

Proportions of sheep with detectable antibodies at different points in time following vaccination were compared using a standard χ2 approach. The levels of neutralising antibodies in sheep before and after booster vaccination were compared using a standard paired Student t-test approach.

3. Results

3.1. A comparison of the ability of commercial ELISA assays to detect antibodies in sheep following a single dose of inactivated BTV-8 vaccine

Comparison between the different ELISAs at different dpv (Table 1) show significantly lower percentage seroconversion with the cELISAs compared to the sELISAs at each of the 3 time-points (7 dpv: χ24df = 44.4, P<0.0001; 14 dpv: χ24df = 47.4 P<0.0001; 56 dpv: χ24df = 32.9, P<0.0001), consistent with the sELISAs being more sensitive than the cELISAs at detecting antibodies in single-vaccinated sheep. In addition all the sheep that were positive with the cELISAs were also positive with the sELISAs.

3.2. Seroconversion and neutralising antibody titres in vaccinated sheep

Of the 20 sheep vaccinated once, 16 had detectable neutralising antibodies measured by SNT with titres (log10) ranging from
Table 1
Rates of detection of antibodies in sheep at different times following administration of one dose of inactivated vaccine, using 5 commercially available ELISAs.

<table>
<thead>
<tr>
<th></th>
<th>−1 dpv</th>
<th>7 dpv</th>
<th>14 dpv</th>
<th>56 dpv*</th>
</tr>
</thead>
<tbody>
<tr>
<td>cELISA 1</td>
<td>0/33 (0%)</td>
<td>2/33 (6%)</td>
<td>10/33 (30%)</td>
<td>7/28 (25%)</td>
</tr>
<tr>
<td>cELISA 2</td>
<td>0/33 (0%)</td>
<td>3/33 (9%)</td>
<td>12/33 (36%)</td>
<td>13/28 (46%)</td>
</tr>
<tr>
<td>cELISA 3</td>
<td>0/33 (0%)</td>
<td>4/33 (12%)</td>
<td>22/33 (66%)</td>
<td>10/28 (36%)</td>
</tr>
<tr>
<td>sELISA 1</td>
<td>0/33 (0%)</td>
<td>17/33 (52%)</td>
<td>30/33 (90%)</td>
<td>21/28 (75%)</td>
</tr>
<tr>
<td>sELISA 2</td>
<td>0/33 (0%)</td>
<td>20/33 (60%)</td>
<td>30/33 (90%)</td>
<td>25/28 (89%)</td>
</tr>
</tbody>
</table>

sELISA—sandwich (double antigen) ELISA.
cELISA—competitive ELISA.
dpv—days post-vaccination.

*At 56 dpv only 28 out of the 33 sheep were bled.

Table 2
Rates of detection of antibodies in sheep at different times following administration of a first and second dose of inactivated vaccine, using 5 commercially available ELISAs.

<table>
<thead>
<tr>
<th></th>
<th>82 days post first vaccine</th>
<th>24 days post second vaccine</th>
</tr>
</thead>
<tbody>
<tr>
<td>cELISA 1</td>
<td>4/20 (20%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>cELISA 2</td>
<td>9/20 (45%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>cELISA 3</td>
<td>7/20 (35%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>sELISA 1</td>
<td>20/20 (100%)</td>
<td>20/20 (100%)</td>
</tr>
<tr>
<td>sELISA 2</td>
<td>20/20 (100%)</td>
<td>20/20 (100%)</td>
</tr>
</tbody>
</table>

sELISA—sandwich (double antigen) ELISA.
cELISA—competitive ELISA.

1.0 to 1.9 (Fig. 1). When these sheep were sampled 24 days after a second dose of vaccine, all animals had antibodies detectable by all assays (Table 2). Significantly higher levels of neutralising antibodies (Fig. 1) were present after the second compared to the first vaccination (paired t-test, P < 0.0001). The four sheep that had undetectable levels of neutralising antibodies after the first vaccination had high titres (log10) of neutralising antibodies (2.4, 2.8, 3.0, >3.1) after the second vaccination, consistent with a strong anamnestic response.

3.3. Absence of BTV genome in sheep post-vaccination

In order to determine whether BTV RNA (measured by real-time RT-PCR) persists in animals vaccinated with inactivated BTV vaccines, the group of 33 sheep were vaccinated and then tested by real-time RT-PCR [9] at 1 and 7 dpv. No viral RNA was detected in any of the 33 sheep at either time-point.

3.4. Protection of sheep following virulent BTV-8 challenge 10 months after immunisation with BTV-8 inactivated vaccine

Following challenge, viral RNA was detected in the control unvaccinated animals from 2 dpi (Fig. 2a) and antibodies were detected from 6 dpi (Table 3). The levels of BTV RNA as measured by the threshold cycle (Ct) peaked at around 4–6 dpi and then slowly reduced up to 21 dpi when the study was terminated (Fig. 2a). Virus was detected in blood from all 3 unvaccinated controls from 4 up to 21 dpi. These control sheep showed mild to moderate signs of BTV, including inappetance, facial oedema, hyperaemia of the gums, ulcerous lesions in the mouth, nasal discharge and laboured breathing and all had elevated body temperatures (Fig. 3).

Six of 7 vaccinates were seropositive by both sELISA and SNT at the time of challenge (Table 3). Not all these sheep had cELISA antibodies. All 6 seropositive vaccinated sheep were completely protected both clinically and virologically in that no viral RNA was detected in any of them (Fig. 2b). The seronegative vaccinated sheep (sheep 4: Table 3) did not show any clinical signs of BTV following challenge and its temperature remained below 40 °C, but did develop a viraemia from 2 dpi up to the end of the study (Fig. 2b). Slightly lower levels of viral RNA (as measured by Ct values) were seen in this animal compared to the sheep in the unvaccinated control group (Fig. 2a and b). Interestingly when samples from this animal were tested by both cELISA, sELISA and SNT at various times post-vaccination (7, 14, 21, 28 and 56 dpv) the animal did not seroconvert and no neutralising antibodies were detected at any of these time-points. Neutralising antibodies increased significantly in all vaccinated sheep following challenge (Table 3).
4. Discussion

Inactivated vaccines have been used in many countries across Europe to combat outbreaks of disease and to control virus circulation caused by various BTV serotypes. A double dose of monovalent inactivated vaccine directed against BTV-2, 4 and 16 and a bivalent vaccine for BTV-2 and 4 have been shown to protect sheep from both viral replication and clinical disease [12,13]. There has been some debate as to whether a single dose of inactivated vaccine is sufficient to provide long-term protection. However, a single dose of BTV-2 inactivated vaccine was shown to fully protect sheep against both clinical signs and viraemia for at least 12 months [14].

With the rapid expansion of BTV-8 across Europe came the urgent need to develop safe and effective inactivated BTV-8 vaccines. Various pharmaceutical companies have produced vaccines that came onto the market throughout 2008. Some vaccine producers started to produce inactivated BTV vaccines for the first time, using different protocols and adjuvants, so it was essential that each individual vaccine underwent appropriate safety and efficacy testing prior to use. However, due to the urgency of the situation and the need to vaccinate animals early in 2008, prior to the BTV transmission season, many of these vaccines were licensed for use without the usual rigorous efficacy testing. Some vaccine companies recommended a single initial dose for sheep whereas other companies recommended an initial course of two doses. A very important question that needed to be addressed was whether sheep would be protected, following a single shot of vaccine, for the entire length of the midge season which is up to 10 months in some southern European countries.

A vaccine trial was carried out in Germany to assess the efficacy of three inactivated monovalent vaccines selected for the German compulsory vaccination programme in 2008 [15]. This study showed that all the vaccinated sheep that seroconverted were fully protected against clinical disease and viral replication and only one of the vaccinated animals was weakly positive in the real-time RT-PCR. However, the inactivated BTV-8 vaccine selected for use in the UK in 2008 was not included in the German study.

In this study, there was a poor correlation between the absence of antibodies, measured using a cELISA, and protection, as 3 out of 7 sheep that were negative in the cELISA after vaccination were protected fully both clinically and virologically. Antibodies detected by the ELISA kits are directed against the group-specific inner capsid protein (VP-7) whereas neutralising antibodies are raised against serotype-specific VP-2 and VP-5 [16]. There was however a good correlation between the presence of neutralising antibodies at the time of challenge and both clinical and virological protection. This study suggests that, if an animal has neutralising antibodies to a particular serotype present at the time of challenge, it is likely to be protected against that serotype. However, it has also been reported that animals are protected in the absence of
neutralising antibodies [13] and that animals which seroconvert following vaccination, although negative at the time of challenge, are still protected against both viraemia and clinical signs when challenged with the homologous virulent serotype [17]. In this study the vaccinated animal (sheep 4) that was not fully protected on challenge with BTV-8 showed no evidence of seroconversion or neutralising antibody titres at any time-point post-vaccination. This sheep was protected from clinical signs and its body temperature remained below 40°C (in contrast to the unvaccinated control sheep) however we cannot exclude the possibility that this animal was improperly vaccinated which could have resulted in only partial protection.

This study indicates (albeit with only 7 animals), that a single dose of the Bovilis-BTV-8 vaccine protects sheep for up to 10 months, which covers the complete vector season in northern Europe. This is an important finding for the British sheep industry as it suggests that sheep vaccinated in the spring, perhaps before turnout, do not need revaccinating before the winter.

Soon after the vaccination campaign started in the UK, testing of samples submitted to the Community Reference Laboratory (CRL) at IAH, Pirbright with cELISA assays revealed that many sheep did no seroconversion after a single dose of inactivated vaccine. This lack of apparent antibodies in vaccinated sheep precipitated many questions about the efficacy of the vaccine and its ability to protect sheep. The question that needed to be addressed was whether the lack of antibodies, as measured with the cELISA assays, reflected low assay sensitivity or a failure of one dose of vaccine to induce antibodies. As some farmers were making the decision to vaccinate their sheep twice it became important to measure the effect of a booster vaccine on antibody levels. The results reported here demonstrate that the sandwich (double antigen) ELISAs are more sensitive than the competitive ELISAs in the detection of antibodies in vaccinated animals, consistent with the German vaccine trial [15]. Thus, if the object of the ELISA testing is to verify the vaccinal status of the animal, sELISAs as opposed to cELISAs should be used. However, antibodies may not be detected using the sELISA in all vaccinated sheep and the lack of antibodies in vaccinated sheep does not necessarily mean that they will not be protected against disease.

In conclusion, sELISAs appear to be more sensitive than cELISAs at detecting antibodies in sheep vaccinated on one occasion with Bovilis-BTV-8 and so, sELISAs should be used to verify the vaccinal status of sheep. In an efficacy study, a single dose of inactivated vaccine against BTV-8 provided solid clinical protection at 10 months against experimental challenge and prevented virus replication in 6 out of the 7 sheep. There was a strong anamnestic response in the vaccinated sheep on challenge and a good correlation between the presence of neutralising antibodies at the time of challenge and protection from both virus replication and clinical disease.

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