Clinical signs and pathology shown by British sheep and cattle infected with bluetongue virus serotype 8 derived from the 2006 outbreak in northern Europe

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Four poll Dorset sheep and four Holstein-Friesian cattle were infected with the northern European strain of bluetongue virus (BTV), BTV-8, to assess its pathogenicity in UK breeds. The time course of infection was monitored in both species by using real-time reverse transcriptase-PCR (RT-PCR), conventional RT-PCR and serology. Two of the sheep developed severe clinical signs that would have been fatal in the field; the other two were moderately and mildly ill, respectively. The cattle were clinically unaffected, but had high levels of viral RNA in their bloodstream. Real-time RT-PCR detected viral RNA as early as one day after infection in the cattle and three days after infection in the sheep. Antibodies against BTV were detected by six days after infection in the sheep and eight days after infection in the cattle. Postmortem examinations revealed pathology in the cattle that was more severe than suggested by the mild clinical signs, but the pathological and clinical findings in the sheep were more consistent.

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BLUETONGUE virus (BTV) is an arthropod-borne pathogen that is transmitted almost exclusively by the bites of *Culicoides* midges, which act as vectors for the virus between its ruminant hosts. The virus can infect most species of domestic and wild ruminants although the clinical signs are most common in sheep and some species of deer. Bluetongue is listed by the World Organisation for Animal Health (OIE) as a 'notifiable disease' and any affected countries should inform the OIE of any new outbreaks. Bluetongue is also a notifiable disease at a national level. Under section 88 of the Animal Health Act 1981 (in the UK) a person recognising signs of disease associated with notifiable diseases in animals under their care must inform the DEFRA Divisional Veterinary Manager (DEFRA 2007). Control measures in non-endemic areas include several restrictions on animal movements, which have a significant socioeconomic impact.

In recent years the distribution of BTV in Europe has changed considerably. New outbreaks have occurred each year from 1998 to 2006 in southern and central Europe, involving several strains from five different serotypes (Mellor and Wittmann 2002, Mertens and Mellor 2003, Purse and others 2005). In 2006 a sixth BTV serotype (BTV-8) caused an outbreak approximately 5° further north in Europe than ever before, infecting ruminants in a wide area across the Netherlands, Belgium, Germany, northern France and Luxembourg (Anon 2006a, b, c, d). Bluetongue is primarily regarded as a disease of sheep, and the severity of clinical signs depends on both the breed of sheep and the strain of virus. Some of the European fine wool and mutton sheep breeds are particularly susceptible, whereas indigenous breeds from endemic regions may only be affected subclinically (Howell 1963, Jeggo and others 1983, 1987, Veronesi and others 2005). For the BTV-8 strain in northern Europe the morbidity has been reported to be less than 10 per cent of animals becoming clinically ill as proportion of the population on a holding, but the case fatality, that is, the proportion of clinically ill sheep that die, can reach between 30 and 50 per cent (International Society for Infectious Diseases 2006). In cattle the infection is frequently described as sub-clinical (Howell 1963, Parsonson 1993, MacLachlan 1994, Melville and others 1995), although clinical cases have been reported, particularly during the initial stages of a new incursion of the virus into a serologically naive cattle population (Lopez and Sanchez 1958, Howell 1963, Parsonson 1990, 1993). Severe clinical signs and mortality were observed in cattle infected with BTV during outbreaks in previously unaffected areas of Israel, Spain and Portugal between 1955 and 1980 (Lopez and Sanchez 1958, Parsonson 1990). During the 2006 outbreak of BTV-8 in northern Europe less than 10 per cent of cattle in affected areas developed clinical signs, but there was a case fatality rate of up to 10 per cent in these animals (International Society for Infectious Diseases 2006).

There are 24 serotypes of BTV recognised on the basis of the specificity of reactions between the outer coat proteins of the virus and neutralising antibodies. However, there is also considerable variation within each serotype, associated with the geographical origins of the virus from around the world, and differences in the virulence characteristics of individual strains of the same serotype (Howell 1963, MacLachlan 1994, Maan and others 2007). At present there are no data concerning the susceptibility of UK breeds of sheep and cattle, or the nature of the clinical signs that could be expected, if animals were infected with the northern European strain of BTV-8 during an outbreak in the UK.

The close proximity of infected animals on the Belgian and Netherlands coastline to the UK, the high summer temperatures that have been recorded in recent years, the large numbers of *Culicoides* midges in spring and summer, and the existence of vector-competent populations of *Culicoides* species in the UK (Carpenter and others 2006), all indicate that BTV is a real threat, with the possibility for bluetongue to be introduced into the UK this year (Gloster and others 2007a, b). The ability to recognise the clinical signs of BTV infection is an important aspect of surveillance, as was demonstrated in northern Europe during 2006. It is therefore vital that veterinarians involved in surveillance are familiar with the clinical signs of infection caused by this strain of BTV-8 in UK populations of cattle and sheep.

This paper describes a study designed to provide data on the susceptibility, clinical signs and pathology that might be expected should poll Dorset sheep and Holstein-Friesian cattle be infected with BTV-8. The infected animals were

TABLE 1: Clinical scores* of the four sheep and four calves infected experimentally with serotype 8 of bluetongue virus

Animal	Fever score (0-13)	Anorexia score (0-5)	Lesion score: Face (0-4)	Lesion score: Feet (0-4)	Respiratory tract (0-4)	Veterinary intervention score (6)	Euthanasia score (8)	Total score	Comments
Sheep 1	4	1	4	4	4	6		23	Severe coronitis/ bleeding
Sheep 2†	5	3	4	3	4	6	(8)	25 (33)	Severe clinical signs
Sheep 3	4	0	3	3	2	0		12	
Sheep 4	4	0	2	2	0	0		8	
Calf 1	0	0	1	0	0	0		1	Ulcer on gum
Calf 2	0	0	0	0	0	0			
Calf 3	0	0	0	0	0	0			
Calf 4	0	0	1	0	0	0		1	Conjunctivitis

* Clinical scores were calculated for the clinical signs developed between one and 14 days after inoculation as follows: Fever score One point for each day of 40°C or above; Anorexia score One point for each day of anorexia; Lesion score: Facial lesions (rhinitis, conjunctivitis, hyperaemia and ulcers of mucosa, facial oedema, tongue oedema), foot lesions (lameness, recumbency due to inflamed feet), respiratory tract lesions (bronchitis, pneumonia) each scored from 0 to 4 depending on severity; Veterinary intervention score Six points if treatment with antibiotics and/or anti-inflammatories was required; Euthanasia score Eight points if euthanased
† Sheep was euthanased eight days after inoculation

monitored regularly to establish how soon after infection viral RNA could first be detected by conventional (targeting genome segment 7) or real-time (targeting genome segment 1) reverse transcriptase-PCR (RT-PCR) assays (Anthony and others 2007, Shaw and others 2007) and how soon antibodies could be detected by competitive ELISA (cELISA) (Gumm and Newman 1982, Anderson 1984, Afshar and others 1987, 1992). The implications of the experimental results for the transmission of the virus and disease control are discussed.

MATERIALS AND METHODS

Virus

Virus was isolated from blood samples from animals that were infected during the 2006 outbreak of bluetongue in the Netherlands.

Animals and experimental design

Four, two-year-old female poll Dorset sheep and four six-month-old male Holstein-Friesian calves were kept in the insect-secure isolation unit at the Institute for Animal Health (IAH), Pirbright, and observed daily by veterinarians for the duration of the experiment.

The sheep were inoculated with 1 ml of NET2006/01 (KC1BHK1) subcutaneously in the neck, and with 0·5 ml BTV-8-E1 intradermally into the inner left leg. The calves were inoculated with 2 ml of NET2006/01 subcutaneously and with 0·5 ml BTV-8-E1 intradermally along the back/flank. The temperature of each animal was recorded daily for 21 days, and they were also examined daily for clinical signs for 14 days, including recording with a digital camera. Their clinical signs were scored using a clinical reaction index (CRI) modified from that of Huismans and others (1987) (Table 1).

Blood samples were taken daily from each animal for 10 days, and then on alternate days until the end of the experiment. The sheep and the calf that were showing the most severe clinical signs at the peak of the disease were euthanased and examined postmortem eight and 10 days after inoculation respectively. The remaining animals were euthanased 28 to 33 days after they had been inoculated, and examined postmortem.

Molecular analyses

RNA extraction A volume of 0·4 ml blood collected in EDTA was added to 0·6 ml of lysis/binding buffer (Roche) and mixed by vortexing. RNA was extracted from 500 µl of this solution on a MagNA Pure LC robot (Roche) using the protocol 'total NA/External_lysis' according to the manufacturer's instruction.

Conventional RT-PCR Denatured RNA was reverse transcribed and cDNA was amplified using a one-step RT-PCR kit (Qiagen) containing the primer pairs BTV/S7/01/BTV/S7/02 and BTV/S7/03/BTV/S7/04 described by Anthony and others (2007).

Real-time RT-PCR The real-time RT-PCR was based on the method described by Shaw and others (2007). The superscript III/Platinum *Taq* one-step RT-PCR kit (Invitrogen) was used for all the real-time RT-PCR assays. A reaction mixture of 19 µl per sample was prepared containing the following components: 12·5 µl 2X reaction buffer mix (kit), 20 pmol of each primer (BTVuni 291-113F, BTVuni 381-357R, BTVrsa 291-311F and BTVrsa 387-357R), 2·5 pmol of each probe (RSA-BTV 341-320 and BTV 346-323), 0·5 µl magnesium sulphate (kit), 0·5 µl ROX reference dye (kit) and 0·5 µl of the Superscript III/Platinum *Taq* enzyme mix (kit). The reaction mixture was added to the wells of reaction plates (Stratagene). Denatured RNA (6 µl) was added to the reaction mixture and the reaction capped with optical caps (Stratagene).

Amplification was carried out using Mx3005P (Stratagene) under the following conditions: 55°C for 30 minutes, one cycle (reverse transcription), 95°C for 10 minutes, one cycle (denaturation of the Superscript III and activation of the Platinum *Taq* DNA polymerase), and 50 cycles of 95°C for 15 seconds and 60°C for one minute. Fluorescence was detected at the end of the 60°C annealing/extension step. Cycle threshold (Ct) values for each sample were determined from the point at which the fluorescence breached a threshold fluorescence line. A cycle threshold of less than 35 was considered positive for BTV RNA.

Serological analyses Whole blood samples were centrifuged at 2400 *g* for five to 10 minutes. Approximately 1 to 2 ml of serum was then collected into sterile microfuge tubes and stored at –20°C. A competitive ELISA for the detection of BTV specific antibodies was then used on each sample (Gumm and Newman 1982, Anderson 1984, Afshar and others 1987, 1992). Fifty microlitres per well of BTV antigen, optimally diluted in PBS, was passively absorbed on to F96 Maxisorp immunoplates (Nunc) for one hour at 37°C. The plates were then washed with PBS and test sera diluted 1:5 in blocking buffer consisting of PBS supplemented with 0·1 per cent v/v Tween 20 (BDH) and 0·3 per cent v/v normal bovine serum (Sigma) were added to duplicate wells on the plate. Each plate contained conjugate control wells, negative control serum, strong positive control serum and weak positive control serum. Monoclonal antibody (mAb) raised against BTV optimally diluted in blocking buffer was added in competition. Bound mAbs were detected by using rabbit anti-mouse immunoglobulin conjugated to horseradish peroxidase. The

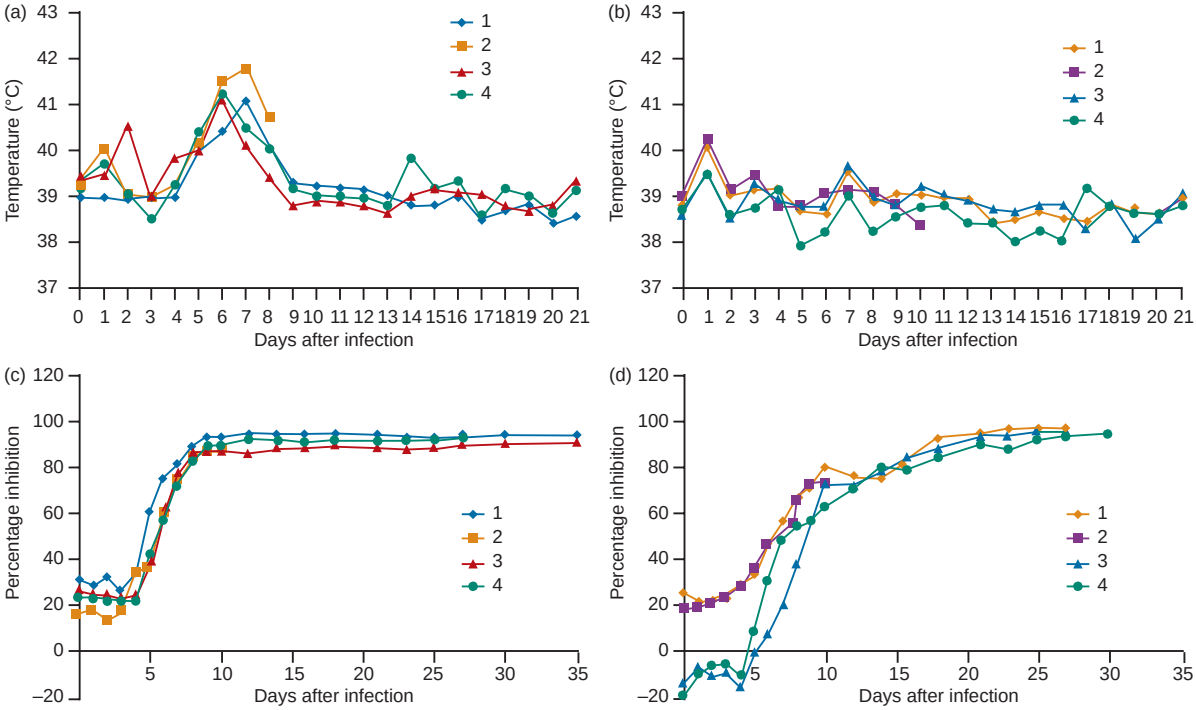

FIG 1: Temperatures of (a) four sheep and (b) four calves, and antibody responses of (c) four sheep and (d) four calves, at intervals after experimental inoculation with bluetongue virus serotype 8

plates were developed with orthophenylenediamine (Sigma) at a concentration of 0·04 mg/ml and containing 0·05 per cent hydrogen peroxide (30 per cent v/v). The reaction was stopped after 10 minutes by the addition of 1·0M sulphuric acid and the plates read spectrophotometrically at a wavelength of 492 nm. Each serum sample was run twice and the mean value of the results was calculated. The percentage inhibition (PI) values were calculated for each sample, and a cut-off of 65 PI was used to distinguish between positive and negative sera.

RESULTS

Virus isolation

Initial attempts to isolate the virus directly in BHK-21 cells from the blood of an animal that was positive by ELISA and real-time RT-PCR (sample number A83/0612) failed. However, two alternate isolation methods were used. First, washed blood was injected into embryonated chicken eggs; the harvested material was designated BTV-8-E1. Secondly, washed blood was added directly to KC cell-cultures, a *Culicoides sonorensis* cell line, which show no obvious cytopathic effect (cpe) after infection with BTV. Seven days after inoculation the culture supernatant was used to inoculate BHK-21 cells. Four days later the BHK cells showed 100 per cent cpe, and virus was harvested and identified as isolate NET2006/1 (KC1BHK1) (IAH 2007). No virus (or cpe) was detected when BTV-8-E1 was titrated on BHK cells, although BTV-8-E1 was positive for BTV-RNA by real-time RT-PCR (Ct 18·05). NET2006/01 was titrated successfully on BHK-21 cells at a titre of $1 \times 10^{6.5}$ TCID50 ml, using the calculation method of Kärber (1931).

Clinical observations

Pyrexia was recorded in all four sheep from five days after inoculation, their temperatures exceeding 40°C (Fig 1a). After six to seven days their temperatures had reached 41°C and because of the severity of the clinical signs, two of them were treated with flunixin meglumine (Finadyne; Schering-Plough Animal Health). No pyrexia was recorded in any of the cattle at any stage of the experiment (Fig 1b).

The clinical scores of the animals during the study are shown in Table 1. Clinical signs were first observed in three of the four sheep five days after inoculation; they had hyperaemia of the buccal, labial and nasal mucosa, facial oedema (most prominently in the lips), and conjunctivitis was starting to develop (Fig 2a). They also developed early signs of coronitis resulting in a reddening of the area around the coronary band, and warm, painful feet (Fig 2c). All four sheep developed a range of clinical signs from seven to 14 days after inoculation.

Sheep 1 developed severe oedema and swelling of the face, particularly the lips (Fig 2b). The hyperaemia of the nasal, buccal and gum mucosa intensified and small petechial haemorrhages developed on the mucosal membranes of the lips and the tongue. Its breathing was rapid and shallow (tachypnoea), and fluid was detected in the lung and bronchial regions of the chest cavity by auscultation. It developed severe coronitis, resulting in bleeding around the coronary band (Fig 2d), and it became severely lame and disinclined to move.

Sheep 2 developed facial oedema similar to sheep 1. It had very severe breathing difficulties, including tachypnoea, leading to abnormally rapid breathing (hyperpnoea) and severe respiratory distress. A combination of very swollen lips and tongue, severe nasal discharge and dyspnoea, prevented it from closing its mouth, and it breathed continuously through its mouth (Fig 3). Auscultation of the lung detected noises that strongly suggesting the presence of large amounts of fluid. The sheep became anorexic and depressed and it was euthanased eight days after it had been inoculated. Although it was lame and developed coronitis, there was no bleeding around the coronary band.

Sheep 3 developed more moderate clinical signs, including generalised swelling of the face, hyperaemia of the nasal, buccal and labial mucosa and a mucocatarrhal nasal discharge. It also developed moderate coronitis on all four feet, and became lame. On auscultation there was some evidence of fluid in the lungs, but it showed only mild signs of respiratory distress (slight tachypnoea).

Sheep 4 was the least affected. Its clinical signs included mild conjunctivitis, moderate facial oedema and mild hyper-

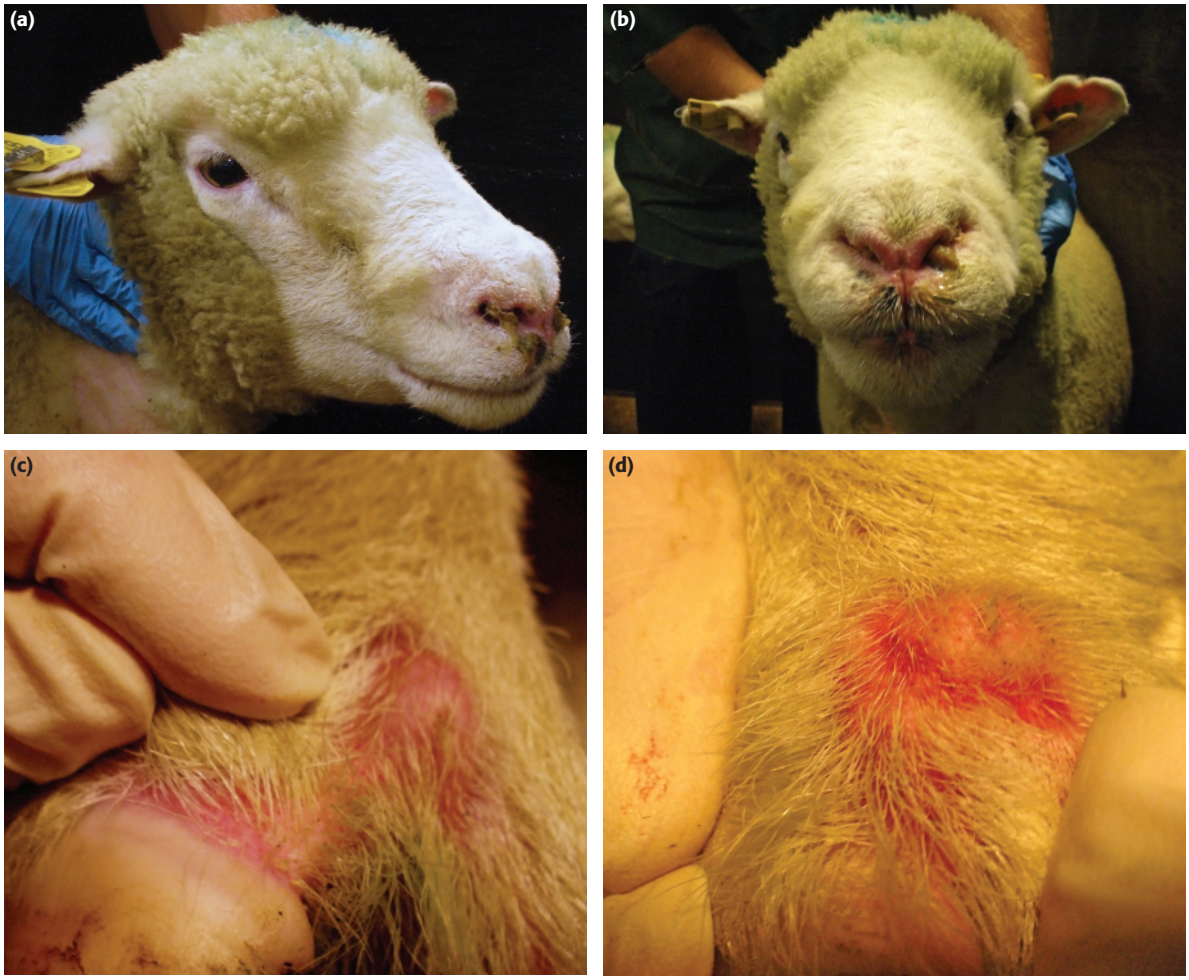

FIG 2: Clinical signs of bluetongue in sheep 1 infected with bluetongue virus serotype 8. (a) Five days after inoculation hyperaemia was observed in the mucous membranes of the face, with oedema of the lips, nose and mouth, which was accompanied by a purulent nasal discharge; (b) these changes had progressed by seven days after inoculation. (c) Five days after inoculation coronitis and hyperaemia was observed in the skin above the coronary band; (d) by seven days bleeding due to severe endothelial cell damage to the blood vessels in the skin above the coronary band was also observed

aemia of the nose and mouth. It developed only mild coronitis and its respiratory system appeared to be unaffected.

The calves were almost entirely clinically unaffected by infection with the BTV-8 strain. Calf 1 developed mild conjunctivitis, and calf 4 developed ulcers on its upper gum that were not associated with any salivation or detectable anorexia. These signs may have been incidental and not directly associated with the BTV infection. They would probably not have been detected under field conditions. Calf 2 was euthanased 10 days after it had been inoculated.

Pathology

Sheep 2 Sheep 2 showed severe subcutaneous oedema in the dermis of its face and lips, causing the dermal layer to develop a gel-like consistency. Multiple petechial haemorrhages were visible on the gums, lips, buccal and nasal mucosa and tongue, which was also moderately swollen. There was generalised, severe, lymphadenopathy in its mandibular, prescapular, pharyngeal, inguinal, bronchial and jejunal lymph nodes, and petechial haemorrhages developed throughout the lymph nodes, which in some cases were filled with blood (Fig 4a). There was similarly severe pathology in the tonsils.

Petechial haemorrhages were also present on several of the major organs, including the heart, kidneys and throughout the intestines, particularly at the iliocaecal junction. The dermal side of the skin showed only minor petechiation. There was a severe pleuritis and pericarditis associated with a substantial quantity of fluid in the thoracic cavity and the pericardium (Fig 4b). The lungs were severely swollen and oedemic. The lungs, airways and alveoli were completely filled with either serum-like fluid or froth (Fig 4c to e). There was a circular haemorrhage, about 2 cm in diameter, on the outer wall of the aorta; the wall of the aorta itself looked unaffected but small blood vessels branching off the aorta appeared to have leaked blood into the surrounding area.

Calf 2 Calf 2 had a generalised, severe lymphadenopathy, with petechial haemorrhages distributed throughout the lymph nodes, as in sheep 2 (Fig 5c), and its tonsils showed a similar pathology (Fig 5d). There were petechial haemorrhages on several other organs, including the root of the tongue (Fig 5a), spleen and kidney. No fluid was observed in any of the body cavities and the lungs seemed macroscopically normal; however, there was an area of haemorrhage on the outside of the wall of the aorta, similar to that observed in sheep 2 (Fig 5b).

Remaining animals All the remaining sheep and cattle showed signs of lymphadenopathy. There were petechial haemorrhages throughout the lymph nodes and tonsils, and in some of the lymph nodes there was purulent lymphadenitis (Fig 6). The lungs of the calves looked macroscopically healthy, but the lungs of the sheep showed variable degrees of fibrotic changes and consolidation associated with unventilated areas. Sheep 1 had developed severe fibrosis throughout the whole of the left lobe, with fibrotic tissue filling the left side of the pleural cavity and preventing any air exchange. This animal also had severe enteritis of the small intestine, hyperaemia and multiple petechial haemorrhages over the jejunum, ileum and caecum. All the sheep and calves had some degree of haemorrhage on the outside of the wall of the aorta.

FIG 3: Severe respiratory distress in sheep 2 infected with bluetongue virus serotype 8. Seven days after inoculation the animal was in respiratory distress and auscultation of the chest cavity revealed sounds consistent with a significant amount of fluid in the lungs; the severe oedema and swelling of the lips, face and tongue caused it to breathe continuously through its mouth

Molecular analyses BTV RNA was first detected in all four sheep by real-time RT-PCR at three days after inoculation, and conventional RT-PCR detected viral RNA after three days in sheep 3 and after four days in the other three sheep. All four sheep remained positive for viral RNA by real-time RT-PCR throughout the rest of the experiment; however, no viral RNA was detected by conventional RT-PCR after 10 days in sheep 4 or after 25 days in sheep 3. The levels of RNA peaked in the blood of all four sheep at approximately five days after inoculation, as demonstrated by low Ct values in the real-time RT-PCR assay; lower Ct values indicate higher loads of RNA (Table 2).

BTV RNA was first detected by real-time RT-PCR at one day after inoculation in calf 1 and after two days in calf 4, but not until after six days in calves 2 and 3. The levels of RNA peaked in all four calves between seven and 14 days after inoculation. Conventional RT-PCR detected viral RNA after three days in calf 1 and after four days in calf 4. All four calves were positive by this method after six days (Table 2).

Intra-test variations in the conventional and real-time RT-PCR assays were greater when testing the calves' blood than when testing the sheep's blood. Variations in the conventional RT-PCR assay resulted in a failure to detect viral RNA after repeated analyses in some samples, with Ct values less than 30 (as measured by real-time RT-PCR) from calves 2 and 4.

Serological analyses Throughout the experiment BTV antibodies were measured by competitive ELISA in the sheep and calves (Fig 1c and d). All four sheep had seroconverted by seven days after inoculation, and the titres reached a plateau by 10 days. Calves 1 and 2 seroconverted after eight days and calves 3 and 4 seroconverted after 10 and 12 days respectively. The antibody titres in the calves reached a plateau by 18 days after inoculation.

DISCUSSION

The infection of four poll Dorset sheep and four Holstein-Friesian calves with the northern European outbreak strain of BTV-8 resulted in clinical disease in all four sheep, but the calves showed no clinical signs. Despite the use of identical doses of virus, the severity of the clinical signs varied significantly, with severe clinical signs in two of the sheep (which would have led to death under field conditions) but relatively mild signs in the other two. This high degree of variation, combined with the known interbreed variation in susceptibility to BTV (Parsonson 1993, MacLachlan 1994), illustrates the difficulty in reliably detecting bluetongue by clinical signs alone.

The clinical signs of infection in the sheep developed earlier than in previous studies at the IAH, in which sheep

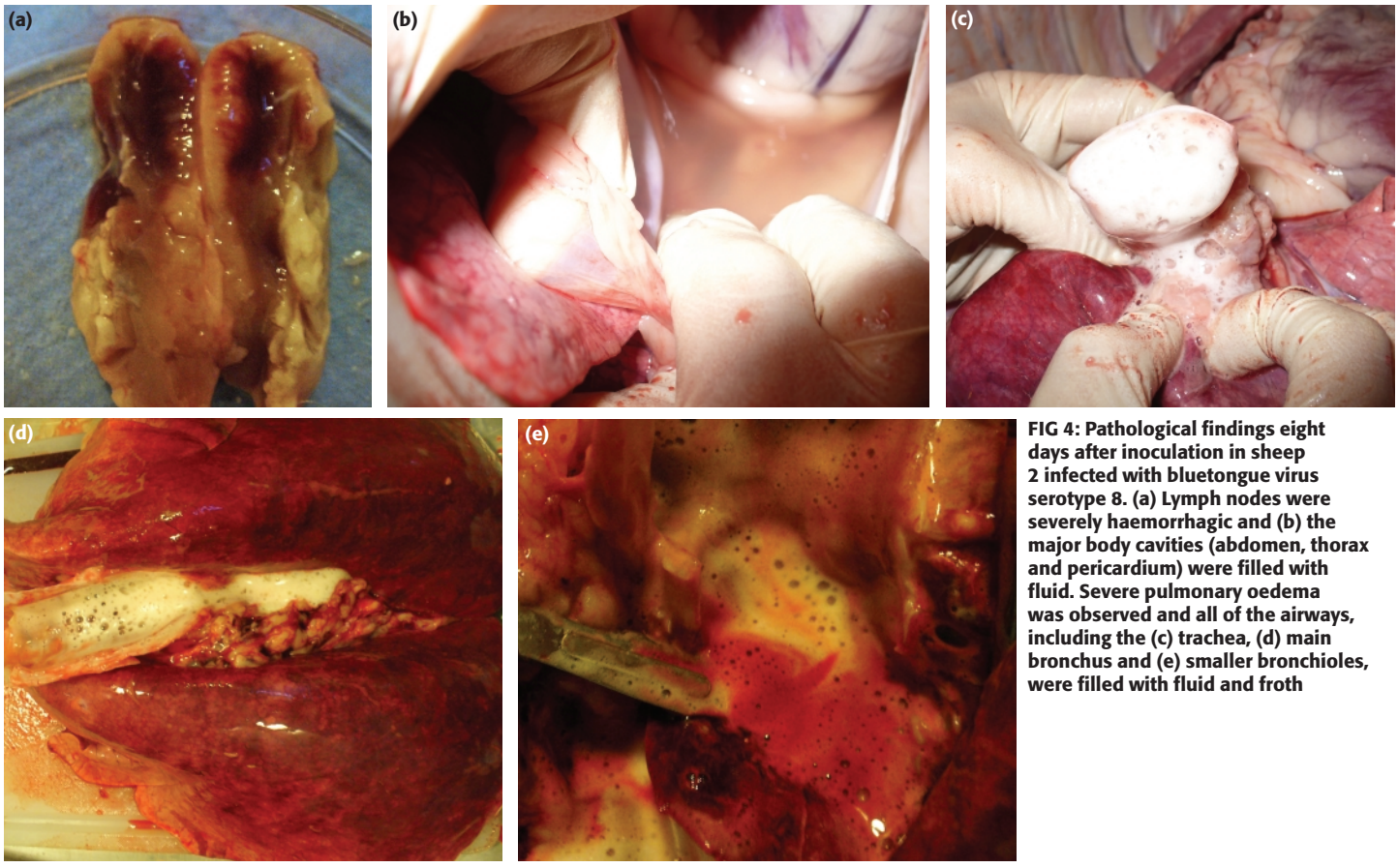

FIG 4: Pathological findings eight days after inoculation in sheep 2 infected with bluetongue virus serotype 8. (a) Lymph nodes were severely haemorrhagic and (b) the major body cavities (abdomen, thorax and pericardium) were filled with fluid. Severe pulmonary oedema was observed and all of the airways, including the (c) trachea, (d) main bronchus and (e) smaller bronchioles, were filled with fluid and froth

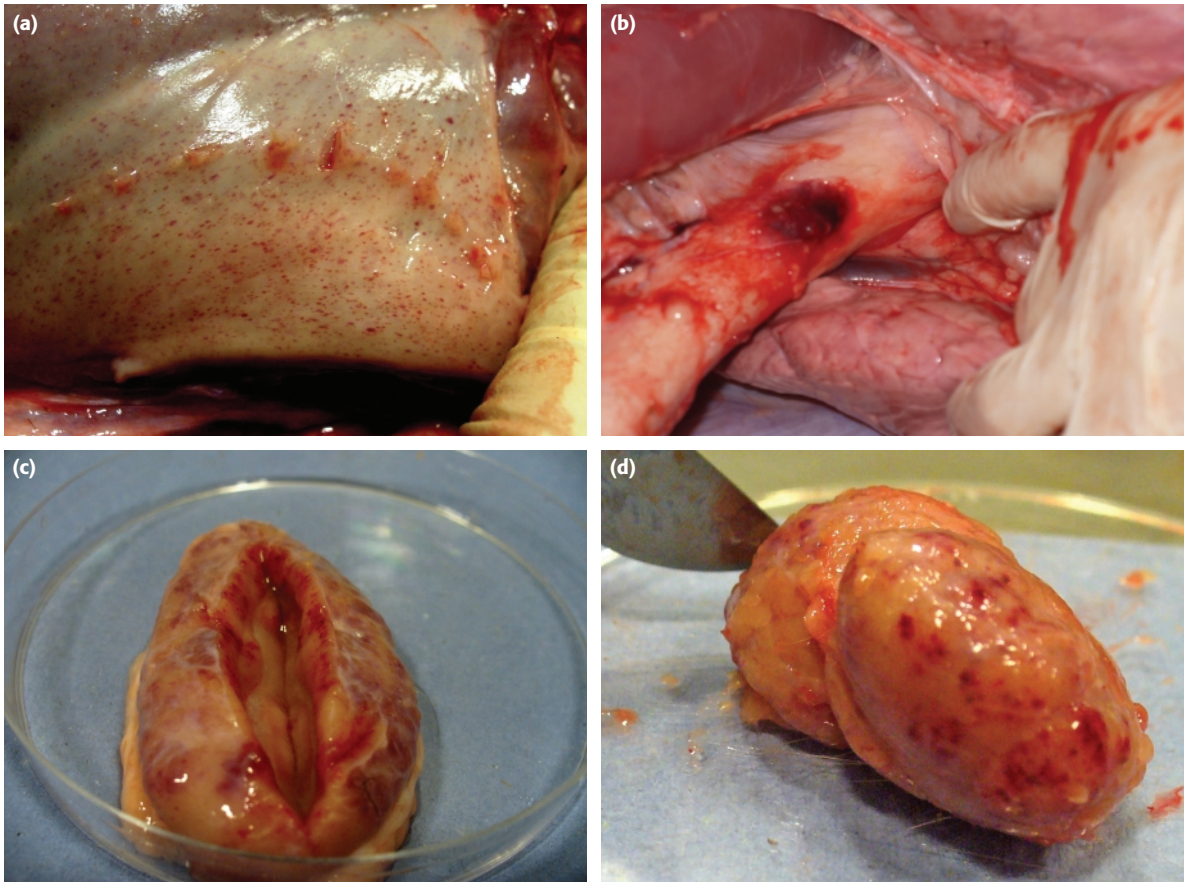

FIG 5: Pathological findings 10 days after inoculation in calf 2 infected with bluetongue virus serotype 8. (a) Petechial haemorrhages were detected beneath the root of the tongue, (b) there was a circle (approximately 2 cm in diameter) of severe haemorrhagic bleeding on the outside of the wall of the aorta, (c) the lymph nodes and (d) the tonsil were swollen and haemorrhagic

were infected experimentally with other BTV serotypes/strains. During those experiments, coronitis was normally observed only in the later stages of BTV-infection, most often at 10 to 12 days after inoculation, after the peak of pyrexia, whereas, in this study coronitis was observed as early as five days after inoculation. Severe cases of dyspnoea and respiratory distress had previously been recorded seven to 10 days after inoculation (Thomas and Neitz 1947, Erasmus 1975, Mahrt and Osburn 1986; K. E. Darpel, E. Veronesi, unpublished observations) whereas, in this experiment, signs of respiratory distress were observed as early as after five days. The early onset of severe vascular endothelial cell damage and the resulting clinical signs (haemorrhages) suggest that individual poll Dorset sheep may be highly susceptible to this BTV-8 strain.

The four calves remained clinically unaffected, although severe endothelial cell damage, particularly in lymphatic tissues, was observed. In the light of the severe clinical signs that were reported in some of the cattle infected during the northern European outbreak, it appears likely that BTV was able to infect and replicate in the endothelial cells of some animals more effectively than in this experiment. Individual susceptibility and associated pulmonary pathology could be increased by the presence of concurrent infections, such as lungworm, infections bovine rhinotracheitis (IBR) or bovine respiratory syncytial virus (BRSV). The pathology observed in the lungs seems to be a critical factor for the overall severity of the clinical signs in both sheep and cattle. It has been suggested that the differences in the severity of clinical signs between cattle and sheep may be related to a different susceptibility of the pulmonary endothelial cells to BTV (DeMaula and others 2001, 2002a, b). Direct damage to the endothelial cells by replicating virus, and subsequent pulmonary oedema, seem to be the most likely cause of the early and severe clinical signs and respiratory distress displayed by sheep 1 and 2. The early onset of these signs, and the absence of any obvious bacterial infection postmortem, suggest that a secondary bacterial infection was unlikely to have been the cause of the clinical signs, although bacterial infections may still be important in the outcome of BTV infections in the field.

No breed or age predisposition have been reported for cattle affected by BTV-8 in the field in northern Europe. None of the four Holstein-Friesian calves showed any significant clinical signs of infection, but owing to the small number of animals it is not possible to conclude that the breed has a low susceptibility to BTV-8. However, the BTV-8 strain did cause pathology in the calves, pathology that might result in the development of clinical signs in more susceptible animals, like those observed in the northern European outbreak. Other breeds may show more severe clinical signs of disease. Field data from the northern European outbreak indicate morbidity in cattle of approximately 10 per cent, with case fatality of up to 10 per cent. With such low rates it would be impractical to conduct farm animal experiments under high containment in the UK with sufficient animals to assess the susceptibility of different breeds. It is therefore important that the field data from the current BTV outbreak in northern Europe is analysed carefully.

Novel diagnostic techniques have recently been developed for BTV, including real-time and conventional RT-PCR assays (Anthony and others 2007, Maan and others 2007, Mertens and others 2007, Shaw and others 2007). These assays have been very useful for the detection of circulating virus before the peak of viraemia, seroconversion and the onset of clinical signs, and both of them detected BTV in the four sheep and four calves. These assays have also been used extensively, together with more established serological methods, as diagnostic and surveillance tools during the northern European outbreak. In general the authors consider that a Ct value of

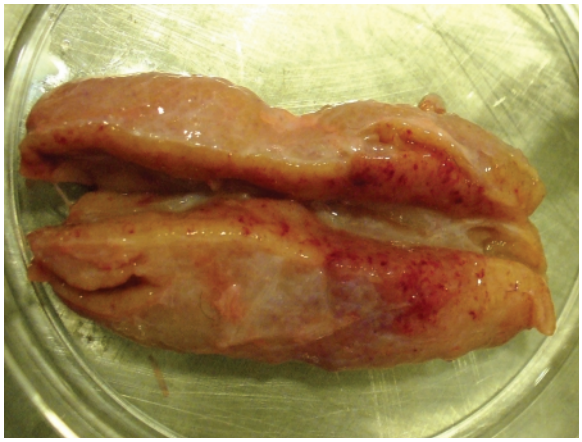

FIG 6: Lymph node from one of the other calves examined approximately 30 days after inoculation showing purulent lymphadenitis

less than 30 in the Segment-1-specific real-time RT-PCR is required for the reliable detection of BTV RNA by the Segment-7-specific conventional RT-PCR.

Reliance on a single assay method to monitor an outbreak would inevitably increase the risk of obtaining false positive or false negative results. Most diagnostic laboratories now combine RT-PCR and ELISA. In relation to the PCR assays, although real-time RT-PCR detected viral RNA earlier than the conventional RT-PCR, the latter assay would provide a useful confirmatory method for low-throughput applications, particularly the initial confirmation of an outbreak. This conventional gel-based technology also forms a basis for the current methods of serotyping BTV strains by RT-PCR, targeting genome segment 2 (Mertens and others 2007). Both the real-time and the conventional PCR assays gave higher within-test variations with cattle blood than with sheep blood, a finding that requires further investigation.

Lower Ct values in the real-time RT-PCR assay indicate higher copy numbers of BTV RNA in the test sample. The lowest Ct values were detected between five and seven days after inoculation in sheep and between seven and 14 days in the calves. The two calves in which BTV RNA was detected by real-time RT-PCR after one and two days showed a slower rise in viral RNA levels in the blood, and gave lower overall Ct values than the sheep in later stages of infection. During this study the sheep with the lowest Ct values showed the most severe clinical signs of disease, but a correlation between the level of viraemia and the clinical signs is not always observed (Jeggo and others 1983, 1986, 1987, Veronesi and others 2005; K. E. Darpel, E. Veronesi, unpublished observations). The manifestation of clinical signs is a complex process that is dependent on many factors, including the strain of virus, breed of sheep, individual susceptibility, the immune response of the host and other environmental factors. It may therefore be unrealistic to expect a simple relationship between the manifestation of clinical signs and the level of viraemia.

TABLE 2: Comparisons of the times at which viral RNA was detected in the blood of four sheep and four calves infected experimentally with serotype 8 of bluetongue virus by a real-time reverse transcriptase-PCR (rRT-PCR) assay and by a conventional RT-PCR assay

Days after inoculation	rRT-PCR	Conventional	rRT-PCR	Conventional	rRT-PCR	Conventional	rRT-PCR	Conventional
Sheep	Sheep 1		Sheep 2		Sheep 3		Sheep 4	
0	No Ct	Negative	No Ct	Negative	No Ct	Negative	No Ct	Negative
1	No Ct	Negative	No Ct	Negative	No Ct	Negative	No Ct	Negative
2	No Ct	Negative	No Ct	Negative	No Ct	Negative	No Ct	Negative
3	32·26	Negative	30·15	Negative	31·02	Positive	35·62	Negative
4	26·30	Positive	23·23	Positive	24·82	Positive	29·65	Positive
5	20·71	Positive	20·83	Positive	23·38	Positive	25·99	Positive
6	20·63	Positive	21·74	Positive	24·53	Positive	24·22	Positive
7	21·00	Positive	21·60	Positive	23·31	Positive	25·04	Positive
8	21·63	Positive			25·72	Positive	25·83	Positive
9	23·26	Positive			28·72	Positive	28·73	Positive
10	25·08	Positive			29·46	Positive	31·74	Negative
12	26·58	Positive			30·32	Positive	31·06	Negative
14	26·83	Positive			31·05	Positive	31·71	Negative
16	26·66	Positive			29·38	Positive	32·49	Negative
18	27·26	Positive			32·76	Positive	32·86	Negative
21	28·33	Positive			29·90	Positive	32·12	Negative
23	27·50	Positive			29·53	Positive	32·53	Negative
25	28·56	Positive			30·15	Negative	32·82	Negative
27	28·40	Positive			31·63	Negative	32·66	Negative
30	28·50	Positive			30·34	Negative		
35	27·68	Positive			32·37	Negative		
Cattle	Calf 1		Calf 2		Calf 3		Calf 4	
0	No Ct	Negative	No Ct	Negative	No Ct	Negative	No Ct	Negative
1	32·99	Negative	No Ct	Negative	No Ct	Negative	No Ct	Negative
2	33·01	Negative	No Ct	Negative	No Ct	Negative	32·96	Negative
3	30·99	Positive	No Ct	Negative	No Ct	Negative	31·68	Negative
4	28·39	Positive	No Ct	Negative	No Ct	Negative	29·37	Positive
5	27·21	Positive	No Ct	Negative	No Ct	Negative	28·63	Negative
6	25·19	Positive	28·56	Positive	29·69	Positive	23·11	Positive
7	24·10	Positive	24·69	Positive	24·55	Positive	22·27	Positive
8	23·31	Positive	26·76	Negative	27·12	Positive	21·48	Positive
9	23·23	Positive	23·73	Positive	26·18	Positive	26·58	Negative
10	23·57	Positive	27·26	Negative	28·33	Positive	21·62	Positive
12	23·75	Positive			25·69	Positive	24·02	Positive
14	24·14	Positive			25·58	Positive	25·13	Positive
16	25·29	Positive			26·09	Positive	28·12	Negative
18	27·50	Positive			34·34	Negative	28·31	Negative
21	27·34	Positive			36·41	Negative	28·56	Positive
23	25·56	Positive			32·83	Negative	No sample	
25	31·35	Negative			38·14	Negative	No sample	
27	28·30	Positive					29·28	Positive
30	27·77	Positive					29·33	Positive

Ct Cycle threshold

The isolation of infectious virus from the blood of infected animals is a difficult and laborious task because the infective material needs to be inoculated into embryonated chicken eggs. It has been reported that bluetongue viral RNA (detected by PCR) remains in the blood for longer periods than infectious virus (Bonneau and others 2002); further work is therefore required to assess the relationship between the presence of infectious virus (virus isolation) and the presence of viral RNA (real-time RT-PCR) in the blood of infected ruminants, specifically for this BTV-8 strain.

This study was based on the infection of the sheep and calves with an arbovirus by needle injection. This is not a natural route of infection and it is not known how closely it might mimic the natural transmission of the virus by *Culicoides* midges. It is uncertain whether the early development of clinical signs in the sheep might have been partly due to the artificial route of infection, and how early the clinical signs might be observed after natural insect-associated transmission. The use of sentinel animals in the field may help to answer these questions.

It is clear that if BTV arrives in the UK, infected cattle may play an important role in its amplification and transmission. The lack of clinical signs associated with high levels of viraemia suggest that infected, viraemic cattle could remain undetected for some time, but would be an important source of infection for the local vector population of *Culicoides* species. The first clinical signs, possibly in sheep, may only be detected once the infection is well established and has spread to second or third infection-cycle hosts. This was certainly the case in Europe, where the northern BTV-8 strain is thought to have been circulating within the ruminant population for up to 10 weeks before it was detected by the observation of typical clinical signs in sheep. To avoid this happening in the UK, it is important that both veterinary practitioners and farmers can recognise the disease as quickly as possible (Dercksen and Lewis 2007). The tools for the rapid diagnosis of the disease in the early stages of an outbreak are available, and have been used in this study, but the limiting factor in the early detection of bluetongue in the UK will almost certainly be the recognition of the disease in the field.

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