



Virological and pathological findings in Bluetongue virus serotype 8 infected sheep

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ABSTRACT

Twenty-seven sheep of the four most common Swiss breeds and the English breed Poll Dorset were experimentally infected with a northern European field strain of bluetongue virus serotype 8 (BTV-8). Animals of all breeds developed clinical signs, viremia and pathological lesions, demonstrating that BTV-8 is fully capable of replicating and inducing bluetongue disease (BT) in the investigated sheep. Necropsy performed between 10 and 16 days *post-infectionem* (d.p.i.) revealed BT-typical hemorrhages, effusions, edema, erosions and activation of lymphatic tissues. Hemorrhages on the base of the *Arteria pulmonalis* and the left *Musculus papillaris subauricularis* were frequently present. Histology confirmed the macroscopical findings. Using a score system, clinical manifestation and pathology were found to be significantly related. Furthermore, clinical signs and fever were shown to be indicative for the concurrent presence of high amounts of viral ribonucleic acid (RNA) in blood. Spleen, lung, lymph nodes and tonsils from all animals were analyzed regarding viral RNA loads and infectivity using real-time reverse transcriptase PCR (rRT-PCR) and virus isolation in cell culture, respectively. The highest amount of viral RNA was detected in spleen and lung and rRT-PCR revealed to be a more sensitive method for virus detection compared to virus isolation. A long-term follow-up was performed with three sheep showing that BTV-8 viral RNA in blood was present up to 133 d.p.i. and in certain tissues even on 151 d.p.i. No significant breed-related differences were observed concerning clinicopathological picture and viremia, and the Swiss sheep were as susceptible to BTV-8 infection as Poll Dorset sheep, demonstrating a remarkably high virulence of BTV-8 for indigenous sheep breeds.

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1. Introduction

Bluetongue virus (BTV) is an arthropod-transmitted orbivirus of the family *Reoviridae*. There are 24 recognized

different BTV serotypes (Erasmus, 1975). A tentative 25th BTV serotype found in goats has recently been described (Hofmann et al., 2008b). Presently, BTV is considered to be endemic in geographical areas between latitude 34°S and 53°N in Africa, Middle East, Australia, Asia and America (Mellor and Boorman, 1995). Over the past 10 years the global distribution of BTV has profoundly changed by spreading to previously unaffected parts of the world such as most of Europe (MacLachlan et al., 2009). In 2006 BTV serotype 8 (BTV-8) emerged in northern Europe followed

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by a still ongoing epidemic affecting Switzerland amongst other countries (Hofmann et al., 2008a; Saegerman et al., 2008).

A broad spectrum of domestic and wild ruminant species is susceptible to bluetongue (BT) infection, although the disease severity and the clinicopathological picture may vary significantly between different species and even between different breeds of the same species (Gard, 1984). Sheep and certain deer species in particular are regarded as hosts showing the most pronounced disease manifestation (Gibbs and Greiner, 1994; MacLachlan, 1994). Furthermore, the susceptibility of sheep to BTV can be dependent on breed, as indigenous breeds in BTV-endemic areas show a natural resistance in contrast to introduced breeds (Gibbs and Greiner, 1994; Koumbati et al., 1999). Fine-wool breeds, such as Poll Dorset and Merino sheep, usually develop more severe clinical disease compared to other breeds (Gibbs and Greiner, 1994; Hamblin et al., 1998).

Transmission of BTV to susceptible hosts occurs through bites of infected female midges of *Culicoides* (*C.*) spp (Mellor, 1990). Viremia is highly cell-associated as BTV first replicates in dendritic cells, endothelial cells and mononuclear phagocytes (Barratt-Boyes and MacLachlan, 1994; Hemati et al., 2009). However, during the later stages of viremia, BTV is exclusively associated with the red blood cell fraction which results in a prolonged, but not persistent viremia (Barratt-Boyes and MacLachlan, 1995; Singer et al., 2001). In sheep viremia is first detected approximately 3 days *post-infectionem* (d.p.i.) and shows a biphasic peak on 6 and 10 d.p.i. (Foster et al., 1991). Infectious virus can be isolated for 11 d.p.i. from sheep blood using cultured cells and embryonated chicken eggs and up to 21 d.p.i. by feeding laboratory-reared *C. sonorensis* on BTV-infected animals (Bonneau et al., 2002). According to Koumbati et al. (1999) infectious virus from sheep blood can even be isolated until 54 d.p.i. using *in vitro* methods. However, detection of viral ribonucleic acid (RNA) by polymerase chain reaction (PCR) is possible beyond 100 d.p.i. (Katz et al., 1994; Bonneau et al., 2002). For BT pathogenesis the replication of BTV in endothelial cells represents an important key factor, as it causes virus-mediated injury to small blood vessels leading to vascular thrombosis, tissue infarction and hemorrhages (Pini, 1976; MacLachlan et al., 2009). Thus, varying disease expression (as described above) may also be explained by different susceptibility of microvascular endothelium to BTV which may show a different production of and response pattern to vasoactive inflammatory mediators, thereby leading to a varying degree of endothelial injury (DeMaula et al., 2002). The resulting macroscopical lesions, typical for BTV infection, therefore reflect virus-mediated injury to small blood vessels (MacLachlan et al., 2009) and usually are first observed 8 d.p.i. (Pini, 1976). Consequently, BT in sheep is manifested as hemorrhagic disease including pyrexia, apathy, congestion, edema, hyperemia and hemorrhages of mucous membranes and skin, oral erosions and ulcers, lameness, respiratory disorders like serous to bloody nasal discharge and in cases of fulminant BT acute lung edema and respiratory distress (Darpel et al., 2007; MacLachlan

et al., 2008; Worwa et al., 2008). In Switzerland there are no indigenous fine-wool sheep and most breeds are long-established. As sheep are the most sensitive species in terms of BT surveillance based on clinical disease, this study aimed to assess the pathogenicity of BTV-8 for indigenous Swiss sheep breeds. Therefore, sheep belonging to four most common Swiss and one English breed were experimentally infected and investigated with regard to macroscopical and histological lesions, viremia, virus load in organs and virus isolation. In three sheep a long-term follow-up was performed. Clinical and pathological manifestation was evaluated using score systems. Comparisons between fever, viremia, clinical and pathological score were investigated and breed differences are discussed.

2. Materials and methods

2.1. Animal experiments

2.1.1. Sheep

All animals were held according to the laws on care and use of laboratory animals in Switzerland. Experiments were approved by the state committee for animal experimentation and were performed in the biosafety level 4 containment facilities of the Institute of Virology and Immunoprophylaxis.

A total of 32 animals of the four most common Swiss sheep breeds namely six Swiss Black-brown Mountain sheep (animal numbers A1-5, 28), six Brown-headed Meat sheep (B6-10, 29), six Swiss White Alpine sheep (C11-15, 30), six Valais Black Nose sheep (D16-20, 31) and in addition, eight English Poll Dorset sheep (E21-27, 32) were used for this study. All animals originated from conventional holdings, were 6–30 months old and pure-bred. Prior to inoculation all animals were clinically examined, dewormed and tested negative for pestivirus and BTV RNA as well as BTV antibody.

2.1.2. Virus inoculation

According to the infection protocol used by Worwa et al. (2008) sheep E21, E22 and E23 were inoculated intradermally and subcutaneously with 2.1 mL of 1:2 diluted cattle blood containing the northern European field strain of BTV-8 from a German 2007 outbreak with cycle threshold value (Ct value) 24.9 (kindly provided by Bernd Hoffmann, Friedrich-Löffler Institute [FLI], Riems, Germany). Infectious blood with Ct value 25.0 was obtained from E21, E22, and E23 and used to inoculate five animals of each breed A, B, C and D and four animals of breed E using the same procedure as described above. The remaining sheep served as controls (F28-32) being mock-infected with BTV-negative sheep blood. All results obtained from E21, E22, and E23 were included in the study.

2.1.3. Clinical score system

Based on the observed clinical signs reported by Worwa et al. (2008), a clinical score system ranking from 0 until 3 was established: one point for each prominent clinical sign (fever, congestion, erosion, hemorrhage, lameness) was assigned and less pronounced, but multiple symptoms

such as swollen lymph nodes, hyperemia, cyanosis, warm clows, salivation, apathy and reduced digestion were accounted with 1 point. Clinical signs that were present prior to inoculation were discarded for the graduation. The severity of clinical disease observed per day was evaluated using the following scheme: score 0 = no symptoms (0 points), score 1 = mild BT (1–2 points), score 2 = moderate BT (3 points) and score 3 = severe BT (4 and more points). Lung edema automatically was ranked with score 3.

2.1.4. Blood sampling

Samples of EDTA blood were collected before inoculation and then regularly from 3 d.p.i. until the end of the trial at 16 d.p.i. except of sheep C11, D17, E27. From those sheep EDTA blood was taken as described above and thereafter weekly until necropsy at 151 d.p.i.

2.1.5. Serology

Plasma from all animals was tested with INGEZIM BTV DR1 (INGENASA, Madrid, Spain) in duplicate wells according to the manufacturers instructions.

2.2. Pathology

2.2.1. Necropsy

Animals were humanly euthanized with bolt and exsanguinated between 10 and 16 d.p.i., when they showed clear clinical signs of acute BT infection. Sheep C11, D17, E27 were euthanized at 151 d.p.i. Macroscopical lesions of all sheep were recorded and tissue specimens for histological and virological analysis were collected.

2.2.2. Pathological score system

A pathological score system with focus on lesions of the *Arteria pulmonalis* (*A. pulmonalis*) and the *Musculus papillaris subauricularis* (*M. papillaris*) was used to quantify the severity of macroscopical alterations observed at necropsy: score 0 = no lesions, score 1 = mild BT (hemorrhages on *A. pulmonalis* or *M. papillaris*), score 2 = moderate BT (hemorrhages on *A. pulmonalis* and/or *M. papillaris*, no or only 1–2 further alterations), score 3 = severe BT (hemorrhages on *A. pulmonalis* and *M. papillaris*, 3 and further alterations). For animals C11, D17, E27 no pathological score was assessed.

2.2.3. Histology

The following organs were fixed in 4% formalin, embedded in paraffin and stained with hematoxylin-eosin (HE) for histological examination: lung, myocard (left and right *M. papillaris*), *A. pulmonalis*, liver, spleen, kidney, adrenal gland, oral mucosa, lips, tongue, skin, hoove, skin from coronary band, intestine (small and large), pancreas, forestomach (when lesions present), lymph nodes (retropharyngeal, prescapular, mesenterial, iliacal, popliteal), brain and bone marrow.

2.3. Virological investigations

2.3.1. Tissue homogenization

To evaluate the presence and amount of BTV RNA, spleen, tonsils, lung and lymph nodes (prescapular,

mesenterial, iliacal, popliteal) were collected from all sheep. From three infected, randomly selected animals additional samples (liver, uterus, kidney, Peyer's patches, myocard, mammary gland, brain, adrenal gland, abomasum) were taken. For homogenization 1 g of tissue was removed with sterile instruments and minced in an Ultra-Turrax T25 homogenizer (Dispomix, IKA, Stauffen, Germany) after adding 10 mL of GMEM isolation medium containing antibiotics and fungicide (MEM with 25 mmol/L HEPES and penicillin/streptomycin; GIBCO, Invitrogen Corporation, Carlsbad CA 92008, USA). Iliacal and popliteal lymph nodes were pooled. The 10% (w/v) suspension of homogenized tissue was clarified by centrifugation and stored at -70°C prior to RNA extraction and cell culture isolation.

2.3.2. Extraction and detection of BTV-8 RNA

All EDTA blood samples were centrifuged at $1150 \times g$ for 10 min at 4°C . Plasma was removed and the sedimented erythrocytes were used in a 1:10 dilution in sterile phosphate buffered saline for RNA extraction. From erythrocytes and tissues RNA was extracted after addition of an internal positive control RNA (IPC) (Hoffmann et al., 2006) and analyzed for the presence of viral RNA by real-time reverse transcriptase PCR (rRT-PCR) according to Hofmann et al. (2008a). All samples were tested in triplicates. Any Ct value <50 was interpreted as positive.

2.3.3. Virus isolation in cell culture

Isolation of BTV in cell culture was performed using spleen and lung homogenates from all infected sheep. Briefly, confluent monolayers of KC cells (Mertens et al., 1996) were washed twice with serum-free medium and inoculated with a 10-fold dilution of sample. After an incubation of 1 h at 28°C growth medium was added and cell culture was incubated for 6 days at 28°C . Supernatant was removed and passaged in a 10-fold dilution on subconfluent, washed BHK-21 cells (baby hamster kidney cells, American Type Culture Collection) and incubated for 1 h at 37°C and 5% CO_2 . After 4 days of incubation at 37°C and 5% CO_2 , the cells were monitored for cytopathic effect (CPE). Entirely, inoculation on KC cells and two passages on BHK-21 cell culture were performed. Extraction of RNA and rRT-PCR was made from all supernatants.

2.4. Statistical analysis

Infected sheep only were included in statistical analysis. For evaluation of the statistical association between clinical signs and pathology, animals with scores 1 and 2 were grouped. Fever was defined as rectal body temperature $>40^{\circ}\text{C}$. To investigate breed-related differences the following three criteria for viremia were set up: (i) number of days until sheep first showed viremia detected by rRT-PCR, (ii) viremia peak defined as the lowest Ct value measured during the whole experiment, and (iii) the day after infection when viremia peak was recorded. For comparison between viremia and further parameters, daily determined Ct values were used. Continuous measurements such as viremia and pathological scores were compared between groups (clinical scores, fever, breeds) using the Kruskal Wallis ANOVA on

Ranks with Bonferroni Multiple Comparison Z-test. The overall level of statistical significance was set to 0.05.

3. Results

3.1. Virological findings

3.1.1. Viremia

The following results represent mean values. In all BTV-8 inoculated sheep viral genome was first detected by rRT-

PCR on 4.2 d.p.i. and all animals remained positive until the end of the trial (Fig. 1A–E). Peak of viremia was recorded on 7.7 d.p.i. with a Ct value of 27.3 (Fig. 1A–E). Fever was first measured on 6.9 d.p.i. and fever peak occurred on 8.3 d.p.i. with 41.1 °C. In sheep C11, D17 and E27 viremia peaked at 6 d.p.i. followed by an increase of 7.7 Ct values during the first month p.i. and thereafter an increase of 1.3 Ct values in second and third month p.i. (Fig. 2A). Viral RNA was no longer detectable in blood by 126, 140 and 133 d.p.i. in sheep C11, D17 and E27, respectively (Fig. 2A). All control

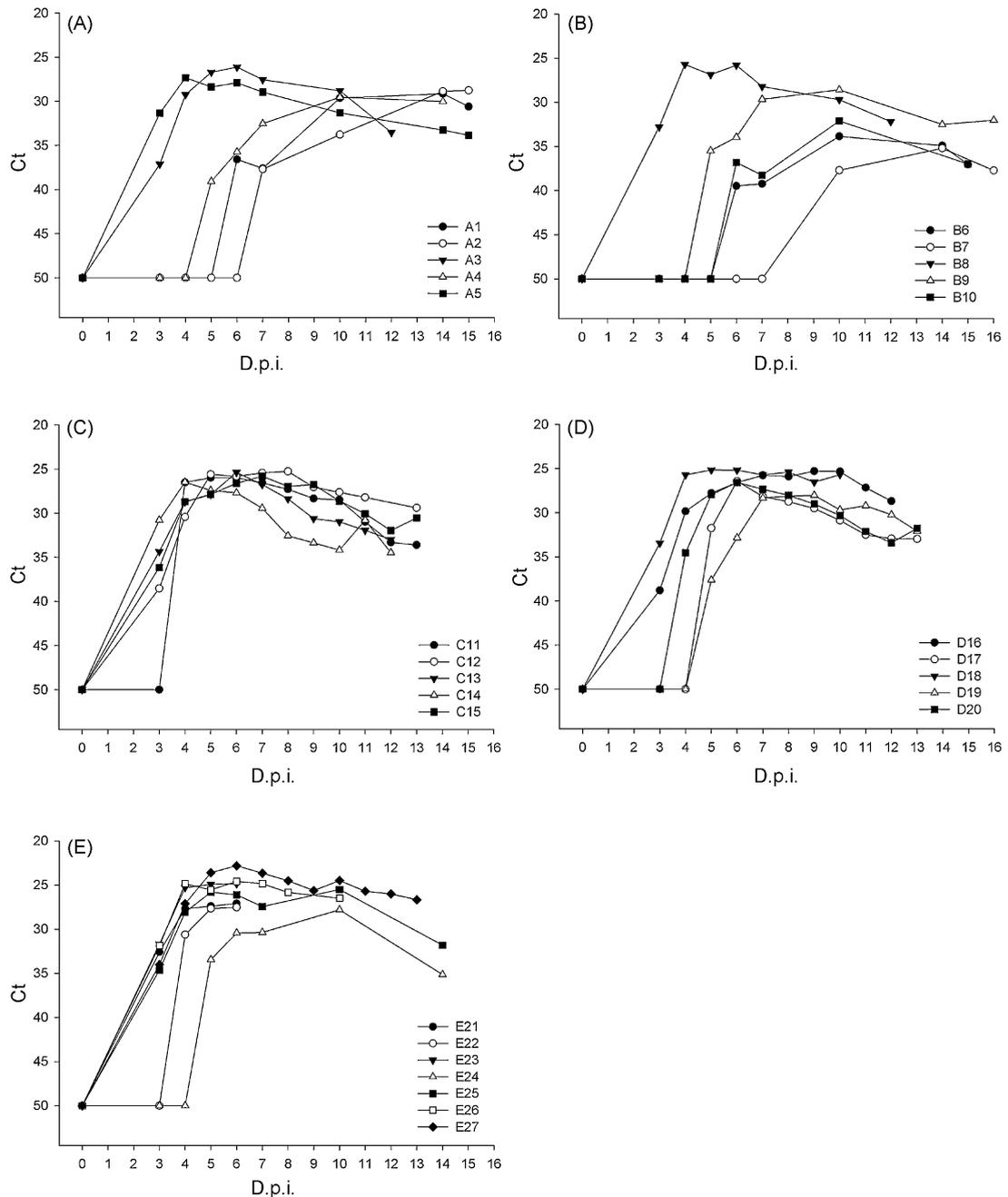


Fig. 1. Viremia of all 27 experimentally BTV-8 infected sheep belonging to breeds A–E between 0 and 16 d.p.i., determined by rRT-PCR (Ct), presented in figures (A)–(E), respectively.

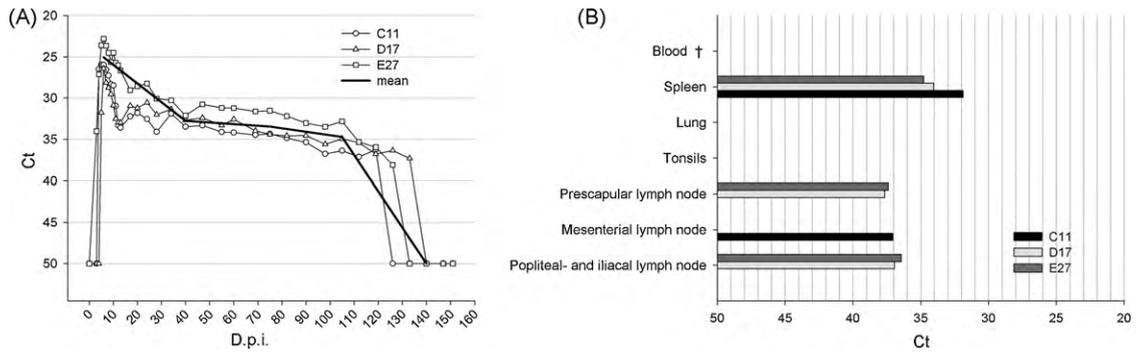


Fig. 2. Long-term follow-up in experimentally BTV-8 infected sheep C11, D17 and E27: (A) entire viremia period determined by rRT-PCR (Ct) of all sheep up to 151 d.p.i. and mean increase of Ct values per month. (B) Ct values of tissue homogenates and blood (†) collected at necropsy 151 d.p.i.

animals remained constantly negative for BTV-8 RNA and antibody (data not shown).

3.1.2. Viral RNA loads in tissues

Mean amounts of BTV-8 RNA in tissues (spleen, lung, tonsils; prescapular, mesenterial, popliteal and iliacal lymph node) and blood taken at necropsy from all infected animals except of C11, D17 and E27 are shown in Fig. 3. All additionally collected samples from three sheep namely liver, uterus, kidney, Peyer's patches, myocard, skeletal muscle, mammary gland, brain, adrenal gland and abomasum contained similar levels of viral RNA (Ct values 31.2–36.8), whereas liver showed slightly lower Ct values (27.0–31.5). Tissues from C11, D17 and E27 collected at necropsy at 151 d.p.i. yielded Ct values between 31.9 and 37.7 in spleen and lymph nodes, whereas no viral RNA could be detected in blood at this time (Fig. 2B). No obvious inhibition was observed in any of the investigated tissues as determined by IPC.

3.1.3. Virus isolation in cell culture

After inoculation on KC cells and two passages on BHK-21 cells, no CPE was observed and all supernatants were either negative in rRT-PCR or yielded considerably higher Ct values, interpreted as negative (input RNA, no viral

replication). In contrast, cells infected with cell culture-adapted BTV-8 used as positive control showed CPE and a distinct decrease of Ct values.

3.2. Pathology

3.2.1. Macroscopical lesions

Frequently observed macroscopical alterations are summarized in Fig. 4.

All control animals showed moderate enlargement of the mesenterial lymph nodes, two had a slight enlargement of the prescapular lymph nodes, in three animals the white pulp of the spleen was slightly visible and one animal presented a slight hemorrhage subserosal in the pyloric area.

Infected animals euthanized between 10 and 16 d.p.i.: the slaughtering criterion was the expression of clear BT-typical clinical signs and therefore resulted in the following schedule: 10 d.p.i.: 2/24, 11 d.p.i.: 1/24, 12 d.p.i.: 7/24, 13 d.p.i.: 4/24, 14 d.p.i.: 3/24, 15 d.p.i.: 6/24, 16 d.p.i.: 1/24. 83% of sheep of all breeds (20/24 animals) showed a hemorrhage in the *Tunica media* of the *A. pulmonalis* (Fig. 5A). The hemorrhages were arranged multifocally and pinpoint in size to confluent and large inducing bulging and thickening of the arterial wall. A

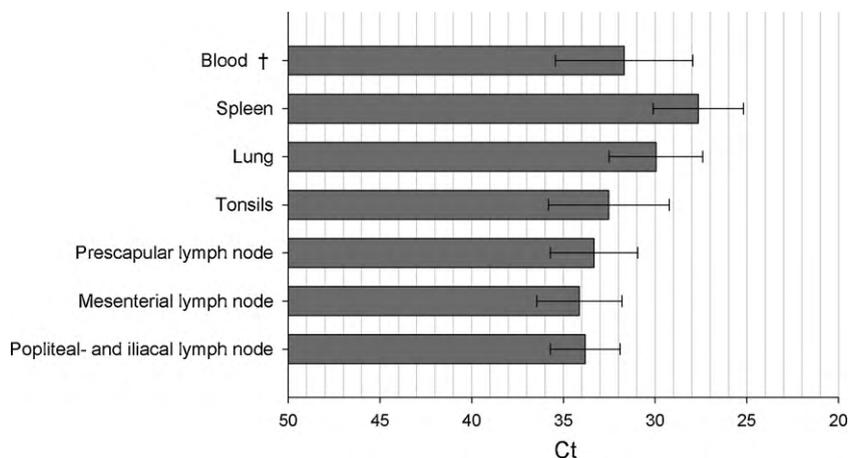


Fig. 3. Viral RNA detection by rRT-PCR (Ct) in tissue homogenates and blood samples (†) obtained at necropsy of 24 (C11, D17 and E27 excluded) experimentally BTV-8 infected sheep euthanized between 10 and 16 d.p.i.

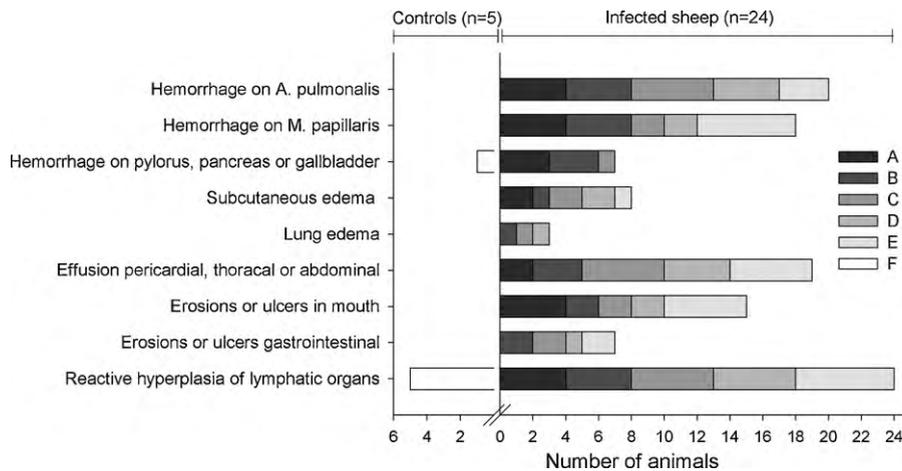


Fig. 4. Frequently observed macroscopical lesions in 24 (C11, D17 and E27 excluded) experimentally BTV-8 infected sheep of all breeds (A–E) including controls (F) euthanized between 10 and 16 d.p.i.

band-like hemorrhage on the left *M. papillaris* was present in 75% (18/24 animals) (Fig. 5B). An activation of lymphatic organs was observed in 100% (24/24 animals) either as lymphadenomegaly of different lymph nodes such as the mesenteric ones (Fig. 5C), or as prominently visible lymph follicles in the white pulp of the spleen. Erosions and ulcerations on the nose and mouth were present at different stages and distributions in 63% (15/24 animals). Thoracal, pericardial and/or abdominal effusion was found in 79% (19/24 animals) (Fig. 5D). Subcutaneous edema in the intermandibular space, around the ligamentum nuchae or in any other location was not prominent, 33% (8/24 animals) showed edema to some extent. In the gastrointestinal tract 25% (6/24 animals) of animals of breeds A, B, D and E presented erosions and ulcerations in the ruminal pillars (Fig. 5E) or in the area of the *Sulcus reticuli*. Hemorrhages in the pancreas, gallbladder or subserosal in the pyloric area (Fig. 5F) were visible in 33% (8/24 animals) of animals of breed A, C, D, and E. Lung edema was observed in one animal of each breed A, B and D, corresponding to 13% (3/24 animals). Reddening of the coronary band was present in 13% (3/24 animals) of sheep of breed C and E. No differences regarding the severity of the pathological lesions were observed between animals euthanized at 10 d.p.i. and those slaughtered at 16 d.p.i.

Infected animals euthanized at 151 d.p.i.: C11, D17 and E27 showed well visible white pulp of the spleen and in C11 and D17 slightly enlarged retropharyngeal lymph nodes were recorded.

3.2.2. Histology

The histological findings corresponded to the macroscopical lesions described above.

In control animals reactive hyperplasia in the mesenteric and prescapular lymph nodes and slight reactive hyperplasia of the white pulp of the spleen were recorded.

Infected animals euthanized between 10 and 16 d.p.i.: almost all sheep presented multifocal to focal-extensive acute hemorrhages in the *T. media* of the *A. pulmonalis* (Fig. 6A–C). Sometimes activated endothelial cells in

small vessels (Fig. 6B) and a slight lymphocytic arteritis (Fig. 6A) could be identified and fibrinoid vessel wall degeneration was sporadically visible. In one case a focal erythrophagocytosis and pyknotic cells were assessed in the *T. media* of the *A. pulmonalis*. Hyperemia and hemorrhages in the left *M. papillaris* of the heart were a common finding (Fig. 6D) and in few cases muscle degenerations in the apex were observed. All sheep showed strong reactive hyperplasia in Peyer's patches, all investigated lymph nodes and in the lymph follicles of the white pulp of the spleen. Sporadically activated lymph nodes were accompanied by hemorrhagic lymphadenitis. Some animals showed a hemorrhage in the region of the omasal folds with acute muscle degeneration or hyperemia in the esophageal groove. Erosive and ulcerative glossitis (Fig. 6E), gingivitis and ulcerative necrosuppurative dermatitis and/or cheilitis were often recorded. Multifocal erosive and necro-ulcerative rumenitis (Fig. 6F), sometimes accompanied by thrombi formation, and acute hyperemia in the *S. reticuli* were found in some animals. Acute focal-extensive hemorrhages serosal, subserosal and in the muscularis of the pylorus were sporadically accompanied by acute muscle fiber degeneration and endothelial activation in single vessels in the pyloric wall. Some animals presented acute multifocal hemorrhages in the pancreas and in the gallbladder wall. The macroscopically determined reddening of the coronary band could histologically not be confirmed as coronitis.

Infected animals euthanized at 151 d.p.i.: in sheep C11 and D17 a slight lymphocytic arteritis was visible in the *T. media* of the *A. pulmonalis*. All animals showed a reactive hyperplasia of the white pulp of the spleen, tonsils, the prescapular, retropharyngeal and mesenteric lymph nodes and the Peyer's patches.

3.3. Statistical comparisons

3.3.1. Breed differences with respect to viremia

Breeds A and B showed by trend a slightly lower viremia compared to breeds C, D and E as (i) viral RNA was first

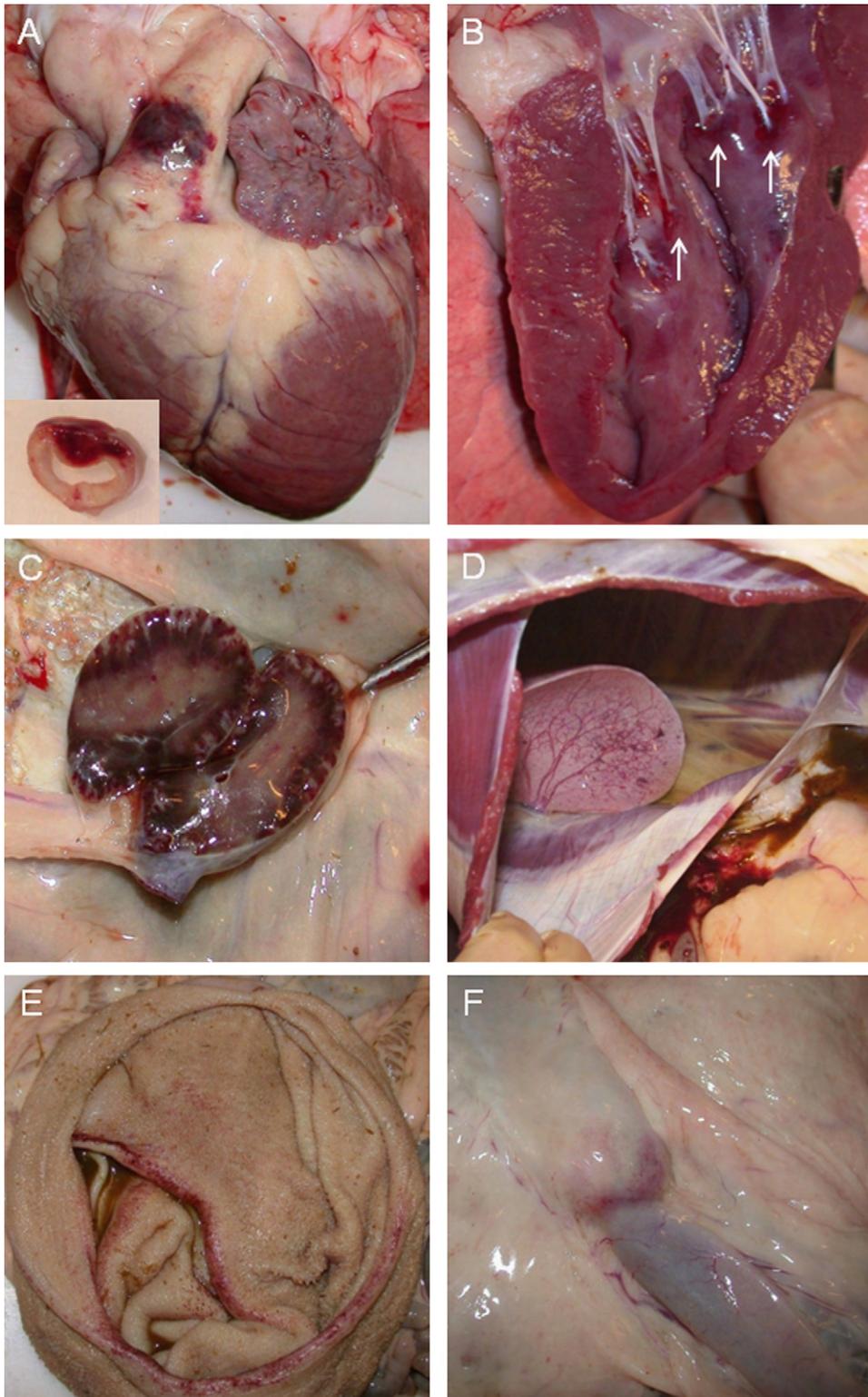


Fig. 5. Frequently observed macroscopical findings in 24 (C11, D17 and E27 excluded) experimentally BTV-8 infected sheep euthanized between 10 and 16 d.p.i.: **(A)** heart: hemorrhage at the base of the *A. pulmonalis* and cross-section of the hemorrhage located in the *Tunica media*. **(B)** Heart: multiple hemorrhages on the left *M. papillaris* (marked by arrows). **(C)** Mesenteric lymph node: enlargement and hemorrhage. **(D)** Thoracic cavity: effusion. **(E)** Rumen: erosions on ruminal pillars. **(F)** Pylorus: subserosal hemorrhage in the pyloric area.

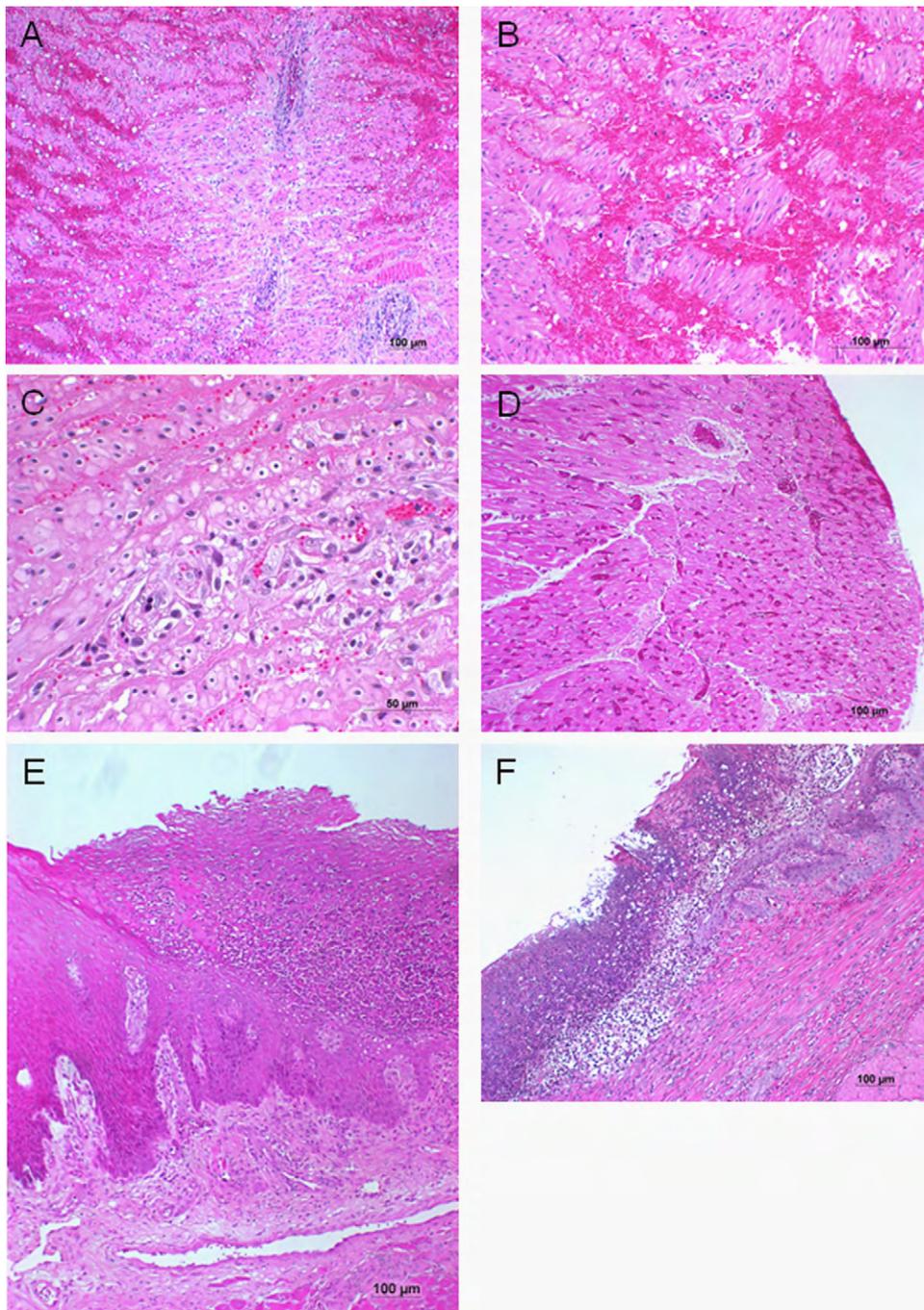


Fig. 6. Sections stained with HE showing frequently observed histological findings in 24 (C11, D17 and E27 excluded) experimentally BTV-8 infected sheep euthanized between 10 and 16 d.p.i.: **(A)** *A. pulmonalis*: severe diffuse acute hemorrhage in the *Tunica media* with activated endothelial cells of the *vasa vasorum* and few perivascular arranged lymphocytes. **(B)** *A. pulmonalis*: acute hemorrhage in the *Tunica media* with activated endothelial cells of the *vasa vasorum* and few perivascular arranged lymphocytes. **(C)** *A. pulmonalis*: acute hemorrhage in the *Tunica media* with activated endothelial cells of the *vasa vasorum*. Some endothelial cells are apoptotic. **(D)** *M. papillaris*: severe hyperemia and subendocardial acute hemorrhage. **(E)** Tongue: focally extensive necrosuppurative and ulcerative glossitis. **(F)** Rumen: severe diffuse necrosuppurative and ulcerative rumenitis.

detected in blood 3–4 days later, (ii) viremia peak were lower up to 10 Ct values, and (iii) viremia peak occurred 3 days later in these two breeds (Fig. 1A–E). These differences were not significant ($p > 0.05$).

3.3.2. Comparison between fever and viremia

Significant association ($p < 0.0001$) between daily recorded viremia and corresponding body temperature indicated that on days, on which sheep exhibited fever, a

higher viremia (lower Ct value) was recorded when compared to days when no fever was present. No fever was recorded on days with Ct values in blood >34.

3.3.3. Comparison between clinical scores and viremia

All infected sheep developed clinical signs of BTV infection (Worwa et al., 2008). A highly significant association ($p < 0.0001$) between clinical manifestation of BTV infection and Ct value was found. Healthy sheep (score 0) had significantly higher Ct values than sheep ranked with score 1, 2, and 3.

3.3.4. Comparison between clinical and pathological scores

Statistical correlation between pathological score and clinical score obtained before necropsy was shown to be significant ($p < 0.0001$). Pathological lesions of sheep evaluated with a clinical score of 0 and 1 were fully consistent, whereas sheep with a score 3 in pathology had shown varying clinical disease ranging from score 1 to 3.

4. Discussion

All inoculated sheep were clearly susceptible to infection with the northern European strain of BTV-8 (Fig. 1A–E). A higher or different susceptibility of the English Poll Dorset sheep (breed E) as reported in previous studies (Gibbs and Greiner, 1994; Hamblin et al., 1998; Darpel et al., 2007) could not be reproduced in these experiments as disease manifestation (Worwa et al., 2008), pathology and viral RNA loads were similar in all breeds (Fig. 1A–E). Infected sheep exhibiting fever or any clinical signs of acute BTV infection showed concurrently peak of viremia (lowest Ct values). Based on this observation, disease manifestation is an important indicator for the concurrent presence of high amounts of BTV in blood, which therefore represents a relevant determinant in BTV epidemiology and diagnostics.

As BTV is exclusively associated with erythrocytes in later stages of viremia (Brewer and MacLachlan, 1992; Barratt-Boyes and MacLachlan, 1994), the decrease of viral RNA observed in C11, D17 and E27 likely reflects the degradation of old erythrocytes in the spleen. However, the abrupt disappearance of viral RNA (Fig. 2A) does not reflect the natural clearance, which occurs gradually, but rather depends on the detection limit of the used rRT-PCR (approximately at Ct value ~40).

Interestingly, viral RNA was detected in spleen and lymph nodes of sheep C11, D17, E27 at 151 d.p.i., although their blood was already tested rRT-PCR-negative on 126, 140 and 133 d.p.i., respectively (Fig. 2A and B). Persistence of viral RNA in spleen could be explained by the accumulation of viral remnants due to the clearance of old, virus-carrying erythrocytes. Furthermore, the BTV genome consists of double-stranded RNA, which eventually contributes to higher resistance against RNAses and therefore increased stability compared to single-stranded RNA viruses.

Infectious virus could not be isolated in KC cells and subsequent passages in BHK-21 cells from any spleen and lung homogenates obtained between 10 and 16 d.p.i. This observation is supported by the fact that BTV infection of

endothelial cells is transient in affected tissues as labelling is markedly diminished by approximately 10 d.p.i. using immunohistochemistry (MacLachlan et al., 2009). Also, Hamblin et al. (1998) reported that titres of virus from tissues of sheep killed 12 d.p.i. were consistently lower compared to animals killed 7 d.p.i. The presented results highlight the low sensitivity of virus isolation for BTV diagnostics in spleen and lung homogenates obtained between 10 and 16 d.p.i., in contrast to the highly sensitive rRT-PCR, which detected viral RNA in tissues from all infected animals. Nevertheless, the discrepancy of results obtained by rRT-PCR compared to isolation in cell culture requires care in interpreting the significance of rRT-PCR results, as rRT-PCR cannot be used to assess viral infectivity. In addition, the isolation of a northern European BTV-8 field strain might present difficulties, as field strains may require previous passages in embryonated chicken eggs before growing efficiently in cell culture. In contrast, cell culture-adapted viruses use to retain their high replication capacity in cultured cells even after several passages in the natural host.

Subcutaneous edema and pulmonary edema was not a common finding in this study. However, three animals presented lung edema at necropsy, which in two animals also was clinically apparent by severe respiratory distress and foamy nasal discharge at 11 and 12 d.p.i. (Worwa et al., 2008). Hence, this demonstrates that BTV-8 is able to induce fatal BTV infection in indigenous breeds, since lung edema represents the cardinal symptom of fulminant BT (MacLachlan et al., 2008). Pericardial, pleural and abdominal effusions were prominent in the cases described here (Fig. 5D) and point to widespread endothelial injury leading to vascular leakage (MacLachlan et al., 2008). The acute hemorrhage in the wall of the *A. pulmonalis* (Figs. 5A and 6A–C) and in the left *M. papillaris* (Figs. 5B and 6D) was prominent and long-lasting as it was present histologically even in sheep C11, D17, E27 at 151 d.p.i. The hemorrhage in the *T. media* of the *A. pulmonalis* has been regarded as pathognomonic for BTV infection (Erasmus, 1975). However, septicemic pasteurellosis in sheep can cause similar hemorrhages and therefore has to be considered as differential diagnosis (Luján et al., 2005). Histologically, hemorrhages were mainly accompanied by an activation of endothelial cells and vasculitis was not frequently present. Activation of lymphatic tissues needs to be interpreted carefully as it was also observed in most of the control animals. However, hemorrhagic lymph nodes were exclusively present in BTV-8 infected sheep (Fig. 5C). Concerning the severity of the pathological lesions, no differences were observed between animals euthanized at 10 d.p.i. and those killed at 16 d.p.i. However, a correlation between pathological and clinical scores obtained prior to necropsy was shown to be significant. Therefore, it can be reasoned that sheep with clinical manifestation will also show pathological lesions at necropsy that are similarly corresponding to each other in their severities.

In summary, the present study describes a novel clinical score system which can be used for the evaluation of clinical signs of BT on a daily basis and which allows a comparison with further parameters such as viremia and BTV-induced pathology. Furthermore, it was demonstrated that residual

viral RNA is present in spleen and lymph nodes even when virus is not detected in blood using highly sensitive rRT-PCR. In contrast to rRT-PCR, virus isolation in cell culture proved not to be suitable for sensitive BTV detection in this study. Although not considered to be pathognomonic for BT, it is likely that the hemorrhage in the *A. pulmonalis* together with the hemorrhage in the *M. papillaris* represent a cardinal pathological finding in the post-mortem examination of BTV-infected animals.

In conclusion, this report demonstrates that the four most common Swiss sheep breeds are fully susceptible hosts of BTV-8, similarly to the highly susceptible Poll Dorset sheep. Moreover, the reproduction of fulminant BT in this study emphasizes a remarkably high virulence of BTV-8 for indigenous sheep breeds.

Conflict of interest statement

None of the authors of the publication “Virological and pathological findings in Bluetongue virus serotype 8 infected sheep” (Gabriella Worwa, Monika Hilbe, Valérie Chaignat, Martin A. Hofmann, Christian Griot, Felix Ehrensperger, Marcus G. Doherr, Barbara Thür) has any financial or personal relationships with other people or organizations that could inappropriately influence this work.

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