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Bone marrow depletion with haemorrhagic diathesis in calves in Germany: Characterization of the disease and preliminary investigations on its aetiology

Panmyelophthise mit hämorrhagischer Diathese bei Kälbern in Deutschland: Charakterisierung des Erkrankungsbildes und bisherige Untersuchungsergebnisse zur Aufklärung der Ätiologie

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Summary

Since 2007 a new fatal haemorrhagic diathesis in calves has been observed in all areas of Germany. Analysis of 56 cases submitted for necropsy allowed its characterization. Calves fell ill within the first month of life independent of breed and sex. Only single or a few animals per herd were affected. Petechial and ecchymotic haemorrhages in many organs and tissues, particularly in skin, subcutis and gastrointestinal tract, were major findings in all animals. Microscopically a severe depletion of bone marrow cells was always observed. Lymphocytic depletion (43%) and inflammatory lesions (46%) were less frequently observed. Blood analysis of five animals indicated an aplastic pancytopenia. The resulting thrombocytopenia is regarded as major pathomechanism of this Haemorrhagic Disease Syndrome (HDS).
Pedigree analysis gave no indication of hereditary disease. Tests for specific toxins such as S-(1,2-Dichlorovinyl)-L-cysteine (DCVC), furazolidone, or mycotoxins resulting in bone marrow depletion were negative. Bacterial infections, Bovine Viral Diarrhoea Virus, and Bluetongue Virus were ruled out as cause of the disease.
HDS shares similarities with a circoviral infection in chickens (chicken infectious anaemia). A broad-spectrum PCR allowed detection of circoviral DNA in 5 of 25 HDS cases and in 1 of 8 non-HDS cases submitted for necropsy. Sequencing of the whole viral genome revealed a high similarity (up to 99%) with Porcine Circovirus type 2b. Single bone marrow cells stained weakly positive for PCV2 antigen by immunohistochemistry in 1of 8 tested HDS animals.
This is the first report of circovirus detection in cattle in Germany. The exact cause of HDS still remains unknown. A multifactorial aetiology involving infection, poisoning, immunopathy, or a genetic predisposition is conceivable. Additional research is necessary to clarify the pathogenesis and the potential role of PCV2 in HDS.

Keywords: calf, bone marrow depletion, haemorrhage, thrombocytopenia, circovirus

Zusammenfassung

Seit 2007 wird bei Kälbern in ganz Deutschland eine neue, meist tödlich verlaufende hämorrhagische Diathese beobachtet. Die Erkrankung wurde anhand von 56 Sektionsfällen charakterisiert. Die Kälber erkrankten durchweg im ersten Lebensmonat. Es handelte sich in der Regel um Einzeltiererkrankungen, wobei verschiedene Rassen und Tiere beiderlei Geschlechts betroffen waren. Hauptbefunde bei allen Tieren waren massive Blutungen, die vornehmlich in Haut, Unterhaut und im Gastrointestinaltrakt zu finden waren. Histologisch wurde immer eine hochgradige Hypo- bis Atrophie des Knochenmarks festgestellt. Zusätzlich konnten eine Lymphozytendepletion (43 %) und entzündliche Veränderungen (bei 46 % der Tiere) beobachtet werden. Blutanalysen von fünf Tieren zeigten eine aplastische Anämie. Damit wird dieses Hämorrhagische Diathese Syndrom (HDS) hauptsächlich durch eine Thrombozytopenie verursacht.
Abstammungsanalysen ergaben keinen Hinweis auf eine Erbkrankheit. Toxine wie z. B. S-(1,2-Dichlorovinyl)-L-cysteine (DCVC), Furazolidon oder Mykotoxine, die Knochenmarksschäden hervorrufen können, wurden nicht nachgewiesen. Auch Infektionen mit Bakterien oder mit dem Virus der Bovinen Virus Diarrhoe (BVDV) sowie der Blauzungenkrankheit (BTV) wurden als Ursache ausgeschlossen.
HDS hat gewisse Ähnlichkeit mit Circovirusinfektionen beim Geflügel (Infektiöse Anämie der Küken). Mit Hilfe einer Breitspektrum-PCR konnte Circovirus-DNS bei fünf von 25 HDS-Kälbern und einem von acht Nicht-HDS-Kälbern nachgewiesen werden. Die vollständige DNS-Sequenz zeigt eine starke Ähnlichkeit (bis zu 99 %) zum Porzinen Circovirus Typ 2b (PCV 2b). In einer PCV2-spezifischen Immunhistologie färbten sich einzelne Knochenmarkszellen bei einem von acht HDS-Kälbern schwach positiv.
Es konnte erstmals ein Circovirus bei Rindern in Deutschland nachgewiesen werden. Die Ursache des HDS bleibt jedoch unklar. Ein multifaktorielles Geschehen, an dem Infektionen, Intoxikationen, Immunopathien oder genetische Prädisposition beteiligt sein können, ist denkbar. Es sind weitere Untersuchungen notwendig, um die Pathogenese von HDS und eine möglichen Beteiligung von PCV2 aufzuklären.

Schlüsselwörter: Kalb, Panmyelophthise, Thrombozytopenie, Blutungsneigung, Circovirus

Introduction

In October 2007 the first cases of haemorrhagic diathesis in young calves were described in dairy cattle farms in Bavaria, Germany. In order to define the gross and histopathological lesions and to elucidate the aetiology, the calves were submitted for pathological examination. This report describes an apparently new form of haemorrhagic disease in calves and preliminary results of laboratory investigations to clarify its aetiology. In this regard, we detected a circovirus in some of the calves and analyzed its whole viral genome, which indicates a close relationship with PCV2.

Haemorrhagic diathesis in cattle has been associated with a variety of causes including viral infections, hereditary diseases, immune-mediated diseases, bacterial septicaemia, and poisoning. Bleeding tendency and thrombocytopenia are associated with non-cytopathic type 2 bovine viral diarrhoea virus (BVDV) infection (Ellis et al., 1998; Rebhun et al., 1989). Pathogenetically, altered bone marrow maturation, decreased numbers of circulating platelets, and altered platelet function contribute to haemorrhages (Ellis et al., 1998; Walz et al., 2001; Wood et al., 2004). An heritable haemorrhagic diathesis has been described in Simmental cattle. This Simmental hereditary thrombopathy is caused by dysfunction of platelets (Steficek et al., 1993). Immune-mediated thrombocytopenia is a known rare condition in cows. It may be classified as idiopathic thrombocytopenic purpura or as secondary entity (Yeruham et al., 2003). Examples of bacterial infections include *Pasteurella multocida*, a well known cause of haemorrhagic septicaemia in calves with petechial and ecchymotic haemorrhages, generalized hyperaemia, and pneumonia as clinical signs (Rhoades et al., 1967; Rimler, 1978).

Several toxins may be responsible for fatal haemorrhagic diathesis in cattle. Poisoning due to dichlorovinylcysteine (DCVC) in trichloroethylene-extracted soybean oil meal fed to calves (Lock et al., 1996) and also the antibiotic furazolidone (Hoffmann-Fezer et al., 1974; Hofmann et al., 1974) produce fatal aplastic anaemia, marked depletion of bone marrow and extensive haemorrhages. Ingestion of bracken fern (*Pteridium aquilinum*) causes acute poisoning in cattle with irreversible bone marrow hypoplasia as well (Maxie and Newman, 2007; Valli, 2007). In addition, poisoning with mycotoxins of *Stachybotrys chartarum* (atra) has been described in ruminants resulting in pancytopenic disease characterized by profuse haemorrhage and necrosis in many tissues (Harrach et al., 1983; Valli, 2007).

Focussing on haemorrhages due to bone marrow depletion and looking at other species, chicken infectious anaemia caused by chicken infectious anaemia virus (CIAV) is a well studied example. Severe anaemia, severe bone marrow depletion, atrophy of the thymus and Bursa of Fabricius, and haemorrhages are consistent findings in chicks infected with CIAV (Kuscu

and Gürel, 2008; Yuasa et al., 1979). One-day-old SPF chicks, experimentally inoculated with CIAV, showed decreased haematocrit values, became emaciated and depressed, with anaemia, particularly between days 12 and 20 post inoculation (Goryo et al., 1989). CIAV is classified into the family Circoviridae (Todd et al., 2005). It only infects chickens and is the sole member of the genus Gyrovirus. However, several members of a second genus, Circovirus, have been detected in mammalian and avian species including the porcine circoviruses PCV1 and PCV2.

TABLE 1: *Animals in the control group*

Case	Animal characteristics				Laboratory investigation	
No.	Age (days)	Sex	Breed	Weight (kg)	Circovirus	Diagnoses
1	0	F	SC	42	neg	abortion
2	n.n.	F	SC	46	neg	gastroenteritis
3	18	F	SC	42	neg	polyarthritis
4	n.n.	F	SC	38	neg	enteritis
5	9	F	SC	46	neg	enteritis, dehydration, pneumonia
6	n.n.	F	SC	30	neg	enteritis, dehydration
7	8	M	SC	49	pos	abomasoenteritis, dehydration
8	18	F	SC	34	neg	enteritis, dehydration, bronchitis

M: male; F: female; SC: Simmental cattle; neg: negative; pos: positive; n.n.: not named.

Members of the family Circoviridae are non-enveloped icosahedral particles with a circular single-stranded DNA (ssDNA) genome, 1759 to 2319 nucleotides (nt) in size (Todd et al., 2005). Viruses in the genus Circovirus possess an ambisense genome organization encoding the replication-associated (Rep) protein from the sense strand (open reading frame [ORF]-V1) and the capsid protein from the complementary sense strand (ORF-C1). Additional small ORFs have been recognized in some of the circoviruses, e. g., ORF3 encoding an apoptosis-inducing protein in PCV2-infected cells (Liu et al., 2005; Timmusk et al., 2008). In one of the non-coding regions, a stem-loop structure is present containing a conserved nonamer sequence which is involved in the initiation of the viral genome replication (Steinfeldt et al., 2001). The molecular biology of circoviruses has been reviewed recently (Mankertz, 2008).

With the exception of PCV1, all known circoviruses are pathogens, which cause immunosuppression and damage of the lymphoreticular tissues (Mankertz, 2008; Segales et al., 2005; Segales and Mateu, 2006; Todd, 2000). PCV2 is a virulent pathogen associated with a number of different syndromes and diseases in pigs such as the post-weaning multisystemic wasting syndrome (PMWS), the porcine respiratory disease complex (PRDC), reproductive failure associated to PCV2 and the porcine dermatitis and nephropathy syndrome (PDNS). However, only lesions typical of PMWS were demonstrated in both colostrum-deprived piglets and conventional pigs by PCV2 inoculation (Ellis et al., 1999; Kennedy et al., 2000), whereas the involvement of PCV2 in swine diseases other than PMWS has not been fully investigated (Allan et al., 2003; Chae, 2005).

Only limited data exist on circovirus infections in cattle. The presence of circoviruses was demonstrated by PCR in lung tissue samples from 6 of 100 cases of bovine respiratory disease and from 4 of 30 aborted fetuses (Nayar et al., 1999). The genome of this agent, tentatively named bovine circovirus (BCV), was nearly identical to that of PCV2, with 99% overall nt sequence identity. The presence of antibodies reacting with porcine circovirus in sera of humans, mice and cattle has been reported (Tischer et al., 1995). In another study, however, no antibodies to PCV2 were detected in sera from cattle, sheep, horse and humans (Allan et al., 2000; Ellis et al., 2001). Also, a seronegative neonatal calf and six seronegative 6-month-old beef calves that were experimentally infected with PCV2 failed to develop antibodies to the virus (Ellis et al., 2001).

Material and Methods

Case history

Between October 2007 and May 2009, 56 calves with haemorrhagic disease, originating from 45 dairy cattle farms in Bavaria, Germany, were presented for necropsy. Medical records were reviewed for age, sex, and breed. Owners were asked for previous disease history and previous medical treatment of calves, feeding of calves, contamination of forage with mould or bracken fern, and use of rodenticides.

Eight calves, sent for pathological examination for other reasons than haemorrhagic disease, were included as controls for the circovirus-specific PCR; they are listed in Table 1. Calf control No. 1 belonged to the same livestock as two calves with haemorrhagic disease (Nos. 11 and 15) and died shortly after birth for unknown reasons. No infectious agent was detectable in this case. Seven calves, included in the control group because of their age, suffered from severe polyarthritis or severe enteritis and died within the first month of life. None of the control animals showed any signs of bone marrow depletion.

Pathology and histopathology

Pathological examination was performed on all animals. A standard series of tissues including bone marrow from the femur and sternal bone, lung, liver, kidney, spleen, and lymph nodes were collected for histopathology. Additional samples were collected depending on further pathological findings, as required. Specimens of organ tissue were fixed in 10% buffered formalin. Specimens of sternal bone marrow were decalcified overnight in Ossa Fixona® (Waldeck, Münster, Germany). Following processing for paraffin embedding, 4-µm-thick sections were cut and stained with haematoxylin and eosin (HE).

Immunohistochemistry

Immunohistochemistry (IHC) was performed on 4-µm sections mounted on Superfrost® Plus glass slides. A mouse monoclonal antibody, 36A9, directed against the VP2 protein (ORF2) of PCV2 (Ingenasa, Madrid, Spain) was applied to tissue sections of bone marrow, spleen, and lymph node of 8 affected calves. Reactivity of the antibody was assessed in each run on sections of lymph node and Peyers Patches collected from a pig with confirmed PCV2 infection based upon immunohistochemistry and PCR analysis. Pre-stain treatment included xylene washes to deparaffinise the sections and

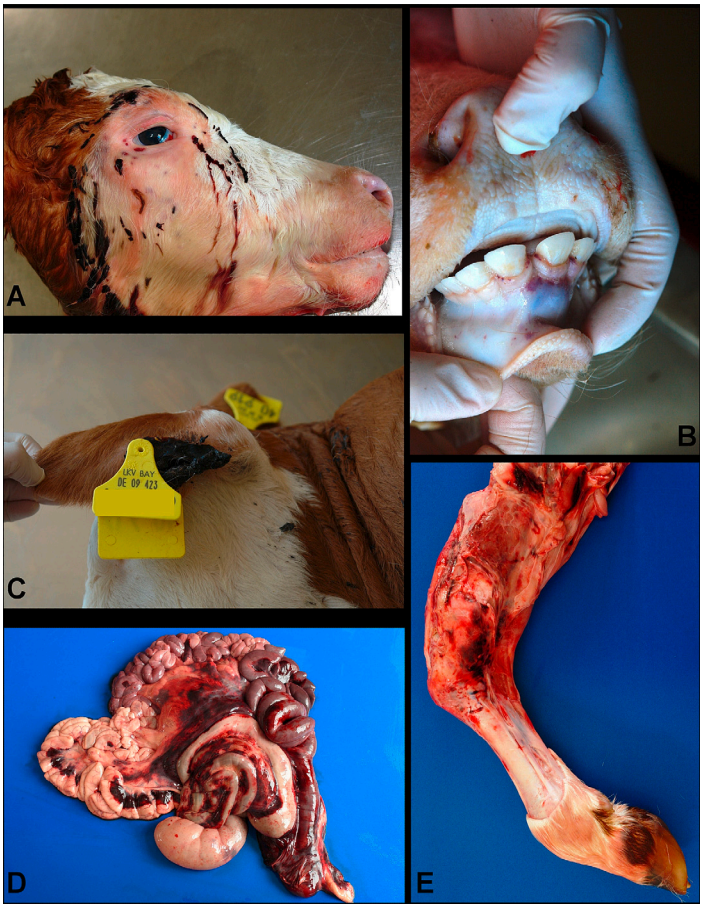

FIGURE 1: *Localization of haemorrhages in diseased calves. A: Focal acute haemorrhages in the skin of the head. Small tufts of hair stuck together by dried blood. B: Petechial and ecchymotic haemorrhages in the mucosa of the lower lip and gingiva. C: With the exception of bleeding associated with injection sites and ear tagging, there was no evidence of traumatic skin injury. D: Moderate focal haemorrhages in the mesenterium of small and large intestine. The segmental dark red discoloration of the small intestine is due to severe intraluminal bleeding. E: Subcutis of the carpus. Subcutaneous haemorrhages are most often seen over bone protrusions and mechanically strained parts of the body.*

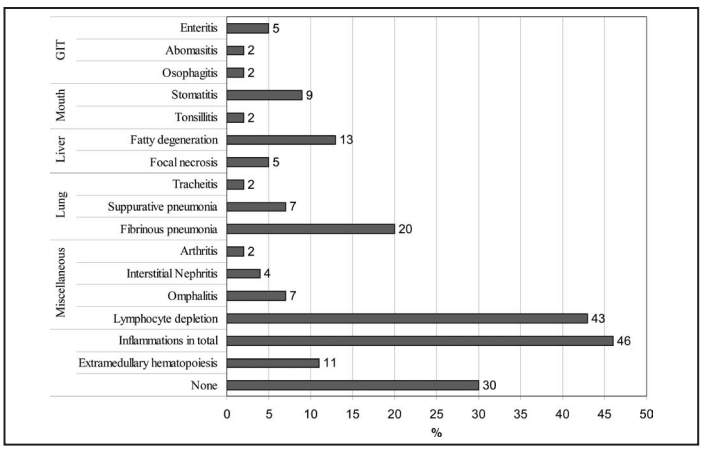

FIGURE 2: *Frequency of additional findings in calves with pancytopenia and haemorrhagic disease. Some animals showed several additional lesions. Inflammation of different organs were found frequently, but 30% of the animals investigated had no further lesions. GIT: gastrointestinal tract.*

serial graded ethanol washes for rehydration followed by treatment with 3% hydrogen peroxide to quench endogenous tissue peroxidase activity. Staining was performed using the Histostain®-Plus Bulk Kit and the chromogen reagent AEC Single Solution (InvitrogenTM, Camarillo, CA, USA) according to the manufacturer's instructions. Finally, sections were counterstained with Mayer's haematoxylin.

Slides classified as PCV2 positive showed an intracytoplasmic, bright red signal in a granular pattern. Eight

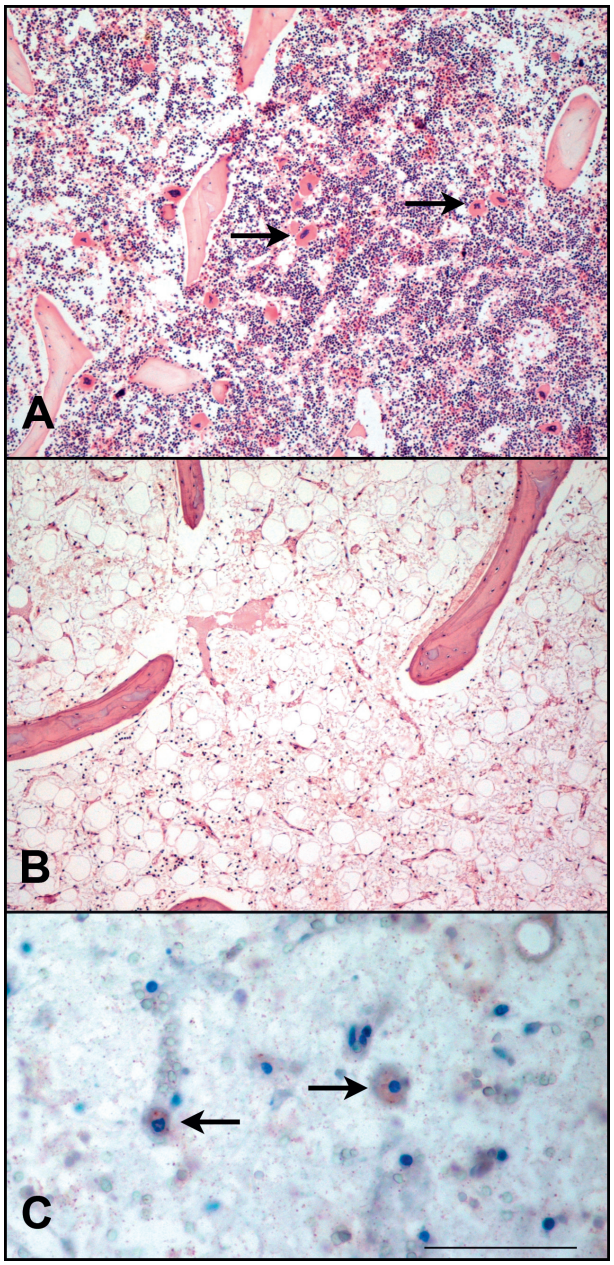

FIGURE 3: *A–B: Histological investigation of bone marrow (sternum) after decalcification, HE stain, 100x magnification. A: Normal bone marrow of a three-week-old calf with haematopoietic tissue including several megakaryocytes (arrows). B: Bone marrow of an affected calf with severe loss of haematopoietic tissue. Only stromal fibroblasts and fat cells remained. C: Immunohistochemistry for detection of PCV2-specific antigen, sternum, calf No. 1. Single bone marrow cells showed mild finely granular cytoplasmic staining. Bar 50µm.*

affected calves (Nos. 1–7 and 17), including the animals tested positive for PCV2 by PCR and all calves of the control group (n = 8), were tested. Immunohistochemistry was repeated if results were positive or questionable.

Haematology
EDTA blood samples were available from 5 calves (Nos. 2, 53–56). The samples were collected from diseased calves shortly before death. Blood analysis was performed within 48 hours after collection. Complete blood count was calculated including white blood cell count, haemoglobin level and parameters of red blood cells using the CELL-DYN® 3500 (Abbott, Wiesbaden, Germany) equipment. Platelets were counted microscopically using Thrombo Plus-tubes (Sarstedt, Nümbrecht, Germany).

Toxicology
The following samples were tested for specific toxins: Urine and blood samples of calves Nos. 21 and 22 were analyzed with specific methods to detect dichlorovinylcysteine (DCVC) and its metabolites. Gas chromatography-mass spectrometry (GC-MS) method was used for the detection of volatile organic compounds, coumarine derivatives and chemotherapeutics, such as sulphonamides, in urine samples of calf No. 25 and renal tissue of calf No. 8. Samples of urine and liver of three calves (Nos. 23, 34, and 36) were tested for pharmaceutical drugs using GC-MS method and high-performance liquid chromatography (HPLC) method.

Forage samples (silage, hay, soybean extract meal, and straw) were collected from a farm with two affected calves (calves Nos. 1 and 2). A sample of straw was suspicious due to greyish discoloration and mouldy smell. Mycotoxicological investigations as well as a cytotoxicity assay were performed with regard to Aflatoxin B1 and toxins of *Stachybotrys chartarum*. Attempts to demonstrate the presence of mycotoxins (Fumitremorgen C, Verrucologen, Aflatoxin B1, Fumagillin, Gliotoxin, Verrucarol NH4+, Deoxynivalenol, Nivalenol, Zearalenon, Satratoxin G, Satratoxin H, Verrucarin A, Roridin A, Roridin L, Satratoxin F, and Verrucarin J) were made using LC-MS/MS analysis as published recently (Gottschalk et al., 2008). Cytotoxicity (MTT) assays were performed according to the method of Reubel et al. (1987).

Microbiological culture
A standard set of organs (lung, liver, spleen, kidney, and small intestine) of all animals of the study and the control group as well as additional samples depending on pathological findings were examined for the presence of bacteria. Each sample was investigated by inoculating Columbia blood agar with 5% defibrinated sheep blood and Water-blue-metachrome-yellow lactose agar. Brain-heart-infusion-agar and chocolate-agar were used for detection of microaerophilic bacteria in lungs. For anaerobic examination, Zeissler agar was used. Salmonella were isolated in Rappaport-Vassilioadis medium after pre-enrichment in buffered peptone water and Xylose lysine desoxycholate agar.

Virology
Renal and thyroid tissues of all affected animals were tested for the presence of BVDV by direct immunofluorescence assay using a diagnostic kit (Bio-X Diagnostics, Jemelle, Belgium) according to the manufacturer's instructions. For isolation of BVDV, monolayers of bovine KOP-R cells (RIE 244, CCLV Federal Research Centre for Virus Diseases of Animals, Island of Riems, Germany) were inoculated with organ homogenates. The cells were screened daily for cytopathic changes. After a second cell culture passage, the cells were examined by direct immunofluorescence assay as described, and by an indirect ELISA for the detection of BVDV-specific antigens (SERELISA BVD p80 Ag Mono Indirect, Synbiotics, Lyon, France). For the demonstration of BVDV-specific nt sequences, RNA was isolated from tissue samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and a commercial real-time RT-PCR protocol (Virotype BVDV Kit; Labor Diagnostik Leipzig, Leipzig, Germany) was applied according to the manufacturer's instructions.

For detection of BTV-specific sequences, a real-time RT-PCR protocol covering all 24 BTV serotypes (Toussaint et al., 2007) was carried out with RNA isolated from spleen tissue of all affected calves.

Out of a total of 56 calves in the study group, 25 were randomly selected (Nos. 1, 2, 4–13, 15–22, 31, 34, 41, 42, 45) for the detection of mammalian and avian circoviruses including PCV2; all calves in the control group were also investigated. DNA was extracted from tissues including blood, bone marrow, spleen, thymus, kidney, and liver using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), and a nested broad-spectrum PCR protocol was applied as recently described (Halami et al., 2008). A further PCR protocol, routinely applied for specific detection of PCV2, was performed according to Bogner et al. (2005). Precautions were made to exclude laboratory DNA contamination during PCR analysis. DNA isolation, preparation of PCR mastermix and analysis of PCR products were performed in separate rooms with different sets of pipettes and single-use filter tips. Each set of reactions was screened for contamination using a negative reagent control and a negative DNA isolation control. The laboratory has never been used for routine PCR diagnostics for PCV2 infection prior to the commencement of this investigation.

Amplification of the whole circovirus genome
The complete genome of the detected circovirus was amplified by PCR using a pair of inverse primers (5'-AGC TCC ACA CTC GAT CAG TAAG-3' and 5'- CCT AGA TCT CAG GGA CAA CGG AG-3'), designed according to the sequence amplified by the nested broad-spectrum PCR. Amplification was performed using the High Fidelity PCR Enzyme Mix (Fermentas, St. Leon-Rot, Germany) with the following cycling conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec and 70°C for 4 min, and a final extension at 70°C of 10 min.

DNA sequencing and phylogenetic analysis
The PCR products were cloned using the GeneJET™ PCR Cloning Kit (Fermentas, St. Leon-Rot, Germany). The insert of the plasmids was sequenced using the primers pJet1 forward and pJet1 reverse (Fermentas, St. Leon-Roth, Germany) or specific primers in an ABI Prism device (Applied Biosystems). The complete genome sequence of the detected circovirus was reassembled from the sequence fragments using the EditSeq module of the Lasergene DNASTAR software package (DNASTAR, Inc., Madison, WI, USA) and subsequently deposited in the GenBank database with the accession no. FJ804417. Sequence similarity searches were per-

formed using the BLAST 2.2.14 search facility. Sequence alignments and construction of phylogenetic trees were carried out with the CLUSTAL W method (Thompson et al., 1994) using the MegAlign module of the above-mentioned software package. The strain designations and GenBank accession numbers are presented in Figure 5.

Pedigree analysis

All calves and their parents were identified and traced by their ear tags. The pedigree of all cases was constructed from the pedigree that is used for the joint breeding evaluation of Germany and Austria. The graphical presentation of the pedigree was performed with the Pedigraph TM software, and sires occurring more than once were identified.

Results

Evaluation of case histories

Simmental cattle were affected most frequently (86%, n = 48), followed by Holstein Friesian cattle (4%, n = 2) and calves of mixed or unknown breed (11%, n = 6). Age at time of death ranged from 7 to 32 days (17 days on average). 85% of the calves fell ill in the second to third week of life. Male and female calves were affected equally.

The calves were healthy at time of birth and during the first days post partum. Owners and attending veterinarians reported spontaneous transcutaneous bleeding without any obvious injury and haemorrhages in several mucosal surfaces as well as excessive bleeding associated with trauma or standard management procedures such as ear tagging or injections. Sometimes additional signs such as fever, diarrhoea, or dyspnoea were recorded. Single or few calves on a farm were affected at the same time. If several calves fell ill, the disease emerged at irregular intervals. Medical treatment was unsuccessful and most calves died within days (n = 50) or had to be euthanized (n = 6) as a consequence of blood loss.

Owners were asked for feeding history and previous medical treatment of calves, contamination of forage with mould or bracken fern, and use of rodenticides to provide information of possible poisoning. All calves had received colostrum in the first days of life. Thereafter, most farmers fed fresh whole milk. In general, calves remained untreated until first signs of haemorrhages emerged. Some calves received preventive medication, or because of acute diarrhoea, some were treated with halofuginone against Cryptosporidia. As bracken fern is rarely a component of pastures in Germany, any problems due to bracken fern contamination had not been reported. Rodenticides were used on the farms, but owners excluded the possible ingestion by cows or calves. Only one of the farmers mentioned having experienced health problems in cattle due to mouldy forage.

Pedigree analysis

The pedigree of all calves was constructed. The parentage of the calves was diverse and indicated no monogenic (recessive or dominant) genetic cause of disease. Even though some sires were represented several times, the number of calves was too small to obtain meaningful results from this analysis.

Gross pathology

At necropsy, the carcasses of the 56 calves in the study group were in good nutritional state with bodyweights between 38 and 72 kg depending on age (53 kg on average). In most of the animals, the abomasum contained coagulated milk, and some straw was found in the rumen. There was no indication of an uptake of toxic plants such as bracken fern. Predominant pathomorphological findings in all 56 calves were severe acute haemorrhages in various organs and tissues. Examples of haemorrhages are shown in Figure 1. Most animals (88%) showed multifocal petechial to ecchymotic haemorrhages in skin and subcutis. Haemorrhages in the serosal and mucosal surfaces of the gastrointestinal tract, in some cases with severe melena, occurred very frequently (98%). Furthermore, haemorrhages in the heart, the meninges, and skeletal muscle were common findings (up to 84%). The bone marrow of long bones and sternum was pale red. Depending on the duration and intensity of bleeding, most carcasses were pale.

Inflammatory lesions were additional occasional findings. Fibrinous or suppurative pneumonia (in total 27%) and focal ulcerative to necrotizing inflammation in the oral cavity (in total 11%) were observed most frequently. Additional pathological and histological findings are listed in Figure 2.

Histopathology

The major histopathological finding was a marked hypocellularity to acellularity of haemopoietic tissue in the bone marrow in each of the 56 animals (Fig. 3). All haematopoietic lineages of erythroid and myeloid cells were affected in the same way. In some cases, small islands of haematopoietic tissue remained. Occasionally, focal degeneration and apoptosis of precursor cells was present in these locations. Spaces between stromal cells were hyperaemic or filled with homogeneous eosinophilic material, or haematopoietic tissue was replaced by fat tissue. Only five of the 56 calves (9%) showed evidence of extramedullary haematopoiesis. Bleeding sites showed no further changes which would explain the bleeding tendency due to previous tissue damages such as vasculitis, inflammatory reactions, or tissue disruption. In 43% of the affected calves (n = 24), lesions in lymphatic tissues became evident as an increased number of apoptotic lymphocytes in lymphoid follicles or low cellularity of spleen and lymph nodes with small follicles. These changes were summarized as lymphocytic depletion. Apart from haemorrhages, thymic tissue was unremarkable. An occasional and infrequent finding was the presence of a few multinucleated giant cells in lymphatic tissue (n = 2). The cellular inflammatory reaction in some ulcerative lesions of the oral cavity was mainly composed of mononuclear cells with strikingly few neutrophils. Likewise, in some cases of fibrinous pneumonia, the inflammatory exudate consisted of large quantities of fibrin with very few neutrophils. Additional histological findings are also listed in Figure 2. There was no evidence of jaundice or haemolysis. No inclusion bodies were recognized in haematopoietic or lymphatic tissues.

Haematology

EDTA blood was available from 5 calves (Nos. 2, 53–56). Blood analysis revealed severe thrombocytopenia, moderate to severe leucopenia, and moderate relative lymphocytosis in all 5 calves. Additionally, 4 of theses calves showed a marked decrease of neutrophil granulocytes (granulocytopenia). 3 calves were anaemic. The haematocrit of 2 calves was still within physiological limits. Detailed haematological results are presented in Table 2.

TABLE 2: *Haematological results of five calves with haemorrhagic disease and bone marrow atrophy*

	Reference Range	Unit	No. 2	No. 53	No. 54	No. 55	No. 56
Haemoglobin	5.6–8.7	mmol/l	4.9	1.2	5.3	2.65	4.5
Haematocrit	0.22–0.44	l/l	0.23	0.06	0.27	0.13	0.22
Erythrocytes	5.0–9.7	T/l	6.1	1.32	6.3	2.94	5.6
White Blood Cell Count	4.0–12.0	G/l	0.85	1.47	0.285	0.9	1.0
MCV	36–50	fl	37.3	42.1	43.9	43.2	39.5
MCH	13–17	pg	12.9	14.6	13.7	14.5	13.0
MCHC	32–39	mmol/l	21.5	21.5	19.4	20.9	20.4
Neutrophil granulocytes	25–45	%	1	4	28	3	4
Eosinophil granulocytes	0–14	%	1	0	0	0	0
Basophil granulocytes	0–1	%	1	0	0	2	0
Monocytes	1–8	%	1	2	4	11	7
Lymphocytes	45–65	%	96	94	68	84	89
Platelet count	300–800	G/l	33.7	12.5	82	60	55

Toxicology

Toxicological screening of urine and renal tissue of calves Nos. 8 and 25 indicated no evidence for uptake of substances such as trichloroethylene, anticoagulants or sulfonamides. The antibiotic furazolidone was not detectable in samples of urine and liver of calves Nos. 23, 34, and 36 using HPLC method. However, metamizol was found in calves Nos. 23 and 34 and a combination of sulfamethazin and trimethoprim was found in calf No. 36. These results were interpreted to be the result of therapeutic administration shortly before death.

In addition, analysis of urine and blood samples collected from 2 affected calves using specific methods for detection of DCVC and its metabolite N-acetyl-DCVC yielded negative results.

The condition of straw collected from one farm suggested a possible contamination with mould; however, no mycotoxins were detected. The cytotoxicity assay also showed negative results.

Microbiological culture

All calves with haemorrhagic disease were tested for the presence of potentially pathogenic bacteria. In some calves, more than one agent was detected. *E. coli* (n = 29) was detected most often, followed by *C. perfringens* (n = 14) in intestine and other organs. *P. multocida* (n = 3) and *P. aeruginosa* (n = 3) were found in few cases. *M. haemolytica*, *Pseudomonas* spp., *Staphylococci*, *Nocardia* spp. and *Salmonella enterica* were found only in single animals (n = 1 each). In 16 calves no bacterial pathogens were detected.

26 calves with haemorrhagic disease showed additional inflammatory lesions (Fig. 2). Pneumonia was observed most frequently (n = 15). Here, *E. coli* was isolated in lung tissue of 9 calves. *Nocardia* spp. (n = 1) or combinations of *P. multocida* and *P. aeruginosa* (n = 1), or *S. aureus* and *S. uberis* (n = 1), were detected in lung tissue of three calves. In lung tissue of three further calves with pneumonia, no pathogen was isolated. Enteritis due to infections with *E. coli* (n = 2) and *C. perfringens* (n = 1) were diagnosed in 3 calves.

Virology

All animals with haemorrhagic disease were tested for BVDV and BTV. Neither viral antigens nor the presence of the viral genomes could be demonstrated for either of these viral agents (data not shown).

Organ tissues collected from 25 affected calves (Nos. 1, 2, 4–13, 15–22, 31, 34, 41, 42, 45), were investigated for the presence of circoviral DNA by nested broad-spectrum PCR, using primers with binding sites in the ORF-V1 of the circovirus genome. In samples tested positive, agarose gel electrophoresis revealed bands with the expected length of approximately 350 bp. Figure 4 shows a negative bone marrow sample of calf No. 2 (lane 3), whereas a strong band with the expected size had formed when blood was analyzed (lane 4). In calf No. 4, bone marrow, liver, kidney and blood were positive (lanes 5–8). Weaker bands were detected when samples collected from other calves were investigated (lanes 9, 10 and 12); others remained negative (line 11). In total, 5 out of 25 calves of the study group (Nos. 2, 4, 5, 7, 17) and 1 out of 8 calves of the control group (control No. 7) tested positive in the circovirus PCR. The PCR products of three samples (calves Nos. 2, 4 and 17) were sequenced, and identities of 99% were obtained when compared with nucleotide sequences of PCV2 present in the GenBank database. Out of the 25 samples under investigation, the five samples tested positive in circovirus-specific PCR plus four randomly selected out of the samples tested negative were sent to another laboratory; a routinely used PCV2-specific PCR protocol revealed negative results in all cases (data not shown).

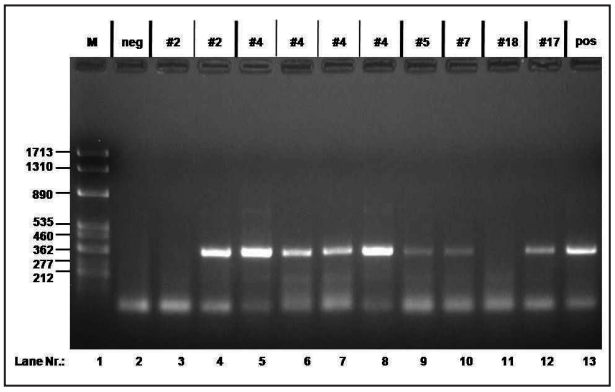

FIGURE 4: *Detection of circovirus DNA in samples from calves with haemorrhagic disease. A nested broad-spectrum PCR was performed using DNA extracted from bone marrow (lanes 3, 5, 9–12), blood (lanes 4 and 8), liver (lane 6) or kidney (lane 7) of calves with numbers indicated above the lanes. Neg: negative isolation control; pos: positive PCR control; M: molecular mass markers, with sizes indicated left in bp. The secondary PCR products with a size of approximately 350 bp had been separated on an ethidium-bromide stained agarose gel.*

Whole genome sequence analysis of strain PCV2-Ha08

Based on the sequence of the PCR products, inverse primers were created which were capable of amplifying the

TABLE 3: *Comparison of sequence similarities between PCV2-Ha08 and other circoviruses*

	PCV2-Ha08/ PCV1	PCV2-Ha08/ PCV2a	PCV2-Ha08/ PCV2b	PCV2-Ha08/ PCV2c	PCV2-Ha08/ BCV	PCV2-Ha08/ PCV2-DK558control
Genome[1]	78%	96.3%	98.9%	94.7%	96.1%	99%
Rep[2]	86.5%	99.4%	99.4%	99.4%	99.4%	99.7%
Cap[2]	68.5%	94%	97%	87.2%	91.9%	97.4%
ORF-3[2]	-	96.2%	99%	97.1%	96.2%	100%
NCR1[1]	84.8%	98.8%	100%	98.8%	98.8%	100%
NCR2[1]	78.9%	97.4%	97.3%	97.1%	97.4%	97.3%

The accession numbers of the sequences used are the same as in Figure 5.

[1] nucleotide sequence.

[2] amino acid sequence.

complete circovirus genome present in the sample of calf No. 4 tested positive with bone marrow, liver, kidney and blood. The strain was designated as PCV2-Ha08 and completely sequenced. The PCV2-Ha08 genome has a length of 1768 nucleotides. Sequence analysis revealed three ORFs with similarities to the PCV2 Rep and capsid protein and to the product of ORF3. The stem-loop structure, 11 bp in length and containing the conserved nonamer sequence, is evident in the non-coding region 1 (NCR1).

A sequence similarity search of the PCV2-Ha08 genome sequence with sequences of the GenBank database revealed the highest degree of identity (99%) with PCV2 isolate DK558control (EF565365), originating from a pig in Denmark. Comparison of the deduced amino acid sequences of the Rep, Cap and ORF3 product with that of selected porcine and bovine circoviruses revealed identities between 68.5% and 100% (Tab. 3). In all cases, PCV2-Ha08 was closely related to PCV2b-strains and showed the highest percentage of identity with isolate DK558control (EF565365).

A phylogenetic analysis was performed using the whole genome sequences of PCV2-Ha08, the bovine circovirus (AF109397), ten circoviruses sharing highest sequence similarity (determined by BLAST search), and three reference strains defining subtypes PCV2a, PCV2b and PCV2c. (Segales et al., 2008) As shown in the phylogenetic tree (Fig. 5), PCV2-Ha08 clearly clusters within the PCV2b subtype, however, it forms a separate branch within this group. In contrast, the bovine circovirus (AF109397), which had been previously described to infect cattle in Canada, clusters together with PCV2a.

Immunohistochemistry

In order to demonstrate the presence of PCV2-specific antigen, immunohistochemistry was performed on tissue sections of bone marrow, spleen, and lymph node of 8 affected calves including the five calves tested positive in the circovirus PCR (Nos. 1–7 and 17) and all calves of the control group (n = 8). Out of these calves, only single bone marrow cells of calf No. 1 showed mild immunoreactivity (Fig. 3C). All tissues of calves Nos. 2–7, and 17 and all tissues of the control calves were negative for PCV2-specific antigen.

Discussion

Here we describe a haemorrhagic disease syndrome (tentatively abbreviated to HDS) of calves, which could be distinguished from other haemorrhagic diseases by following clinical, pathological and histological criteria: The most prominent clinical signs were spontaneous bleeding without obvious injury, haemorrhage of mucosal surfaces and excessive bleeding associated with standard management procedures. Consistently, the haemorrhagic disease became evident in young calves within their first month of life. Severe hypocellularity of the bone marrow was found in all cases. The haematological results indicating aplastic pancytopenia in five of these animals supported this finding. It is safe to assume that the resulting thrombocytopenia causes the haemorrhagic disease. Furthermore, the haematological results revealed moderate to severe leucopenia and granulocytopenia. This finding is consistent with the severe bone marrow depletion observed in all animals and depletion of lymphatic tissues in 43% of the animals. The lack of proliferating lymphatic cells is likely to cause immunosuppression. This may explain the frequent occurrence of lesions such as pneumonia and ulcerative stomatitis as well as the lack of inflammatory cells in some of these lesions.

The calves were healthy at birth without any haemorrhage, but fell ill over the following days and weeks. Considering this, we hypothesize that the destructive insult may occur in the neonatal calf. Following bone marrow destruction, the onset of clinical signs will largely depend on the half-life of blood cells in the circulation, especially of platelets. Platelets' life span is merely 9 days (Paape et al., 2003; Valli, 2007). Anaemia is less significant, unless complicated by bleeding, due to the long life span of bovine erythrocytes of 120 days (Loesch et al., 2000; Valli, 2007).

To assess the aetiology of HDS, several causes of haemorrhage in cattle due to thrombocytopenia or thrombopa-

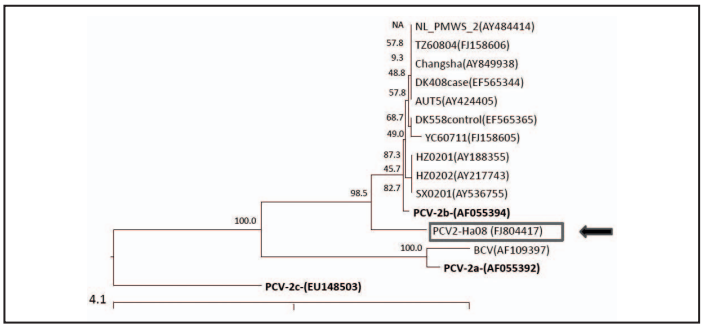

FIGURE 5: *Phylogenetic relationship of the circovirus PCV2-Ha08 detected in a German calf with porcine circovirus type 2 strains. The phylogenetic tree was established on the basis of the complete nucleotide sequences of the reference strains PCV2a, PCV2b and PCV2c (bold font), the Canadian bovine circovirus (BCV) and ten circoviruses which turned out to be most closely related to PCV2-Ha08 by a BLAST search. PCV2-Ha08 is marked with an arrow. The GenBank accession numbers of the sequences are shown in brackets. The tree is scaled in nucleotide substitution units.*

thy were investigated. Hereditary haemorrhagic diathesis is described in Simmental cattle and is known as Simmental hereditary thrombopathy (Steficek et al., 1993). Here, Simmental cattle were affected in the majority of cases, but two Holstein Friesian calves showed equivalent lesions. In southern Germany, Simmental cattle are the most common breed and may, therefore, be overrepresented in this study. The same clinical picture of HDS in different breeds and the results of pedigree analysis indicate no autosomal dominant or recessive hereditary disease. However, the number of animals in the study was not sufficient to make a definitive statement at present.

Several toxins and mycotoxins are known to cause haemorrhage in cattle. The medical history of the diseased calves and information about animal husbandry gave some indication for possible poisoning in individual cases, for example with mycotoxins or drugs. However, there was no consistency in the information provided which would apply to all affected farms and which would give reason to suspect a specific toxin. Nevertheless, some specific tests for toxins were carried out.

Particularly, poisoning with S-(1,2-Dichlorovinyl)-L-cysteine (DCVC) or furazolidone, both causing bone marrow depletion and haemorrhages, are consistent with the observed lesions (Lock et al., 1996; Hoffmann-Fezer et al., 1974; Hofmann et al., 1974). Trichloroethylene-extracted soybeanoil meal fed to calves produces fatal aplastic anaemia and, at higher doses, renal injury. DCVC, a metabolite of trichloroethylene, is the toxic factor in this entity. Currently, hexane, instead of trichloroethylene, is used for extraction of soybean oil. Testing of blood, renal tissue and urine of a total of 4 calves for trichloroethylene, DCVC, and its metabolite N-acetyl-DCVC yielded negative results. The antibiotic furazolidone is used for treatment or prophylaxis of bacterial and protozoan infections in human and animals. According to a national council regulation, the administration of furazolidone to food-producing animals is prohibited. Anyhow, 3 affected calves were investigated and proved to be negative with regard to furazolidone.

Ingestion of bracken fern (*Pteridium aquilinum*) causes symptoms of poisoning in grazing animals. Acute bracken fern poisoning in cattle produces irreversible bone marrow hypoplasia resulting in aplastic pancytopenia (Maxie and Newman, 2007; Valli, 2007). Also, poisoning with mycotoxins of *Stachybotrys chartarum* are described in ruminants and horses as pancytopenic disease (Harrach et al., 1983; Valli, 2007). Bracken fern poisoning and stachybotryotoxicosis seem to be unlikely in these cases because symptoms should emerge in animals of all ages and especially in those fed with a diet containing roughage. In this study, forage samples tested negative for mycotoxins and there was no indication of intake of bracken fern by calves or cows.

Idiopathic thrombocytopenic purpura is described as a rare condition in cows (Yeruham et al., 2003). The cause of this autoimmune disease may be immune-mediated destruction of platelets (Lunn and Butler, 1991). Reported cases of thrombocytopenic purpura were associated with a recent multivalent botulism toxoid vaccination or inactivated vaccines against papilloma virus and clostridia, respectively (Lunn and Butler, 1991; Yeruham et al., 2003). Calves in this study were not vaccinated. Furthermore, the occurrence of bone marrow destruction is inconsistent with the immune-mediated thrombocytopenia described in cows.

Infections with *P. multocida* types B or E are known to cause haemorrhagic septicaemia in calves (Rimler, 1978). Endotoxins play a major role in the pathogenesis of this infection (Horadagoda et al., 2001). In this study, P. multocida was present in only three calves. A typing was not carried out. In our study, microbiological investigation of organ samples revealed a wide spectrum of potentially pathogenic bacteria in diseased calves. However, evidence of a specific pathogenic bacteria associated with the haemorrhagic disease could not be found. In 29% (n = 16) of all cases, no pathogenic bacteria were detected. In 17 calves isolated bacteria were associated with additional inflammatory lesions such as pneumonia or enteritis. It may be assumed that depletion of lymphatic tissue and bone marrow in these calves resulted in severe leucopenia and granulocytopenia associated with immunosuppression and secondary infections.

Infection with non-cytopathic BVDV type 2 may result in a severe bleeding tendency due to thrombocytopenia (Ellis et al., 1998; Rebhun et al., 1989). BVDV was detected in none of the calves under investigation. Furthermore, the bone marrow cellularity does not decrease in BVDV infections (Wood et al., 2004). In contrast, severe bone marrow depletion was a consistent finding in the cases reported in this study. On this account, it seems reasonable to exclude a BVDV infection.

Likewise, infection with BTV was excluded by laboratory examination. Several farmers suspected the BTV-8 vaccination to be correlated to HDS. Due to the fact that the first cases of HDS were reported in 2007 and nationwide vaccination against BTV-8 did not start until 2008, this speculation is unfounded.

Because HDS could not be explained by known causes of haemorrhagic disease, similar diseases in other species were considered. Chicken infectious anaemia is a disease strongly resembling the haemorrhagic disease in calves reported here. Therefore, a broad-spectrum PCR was performed for amplification of circoviral genomic sequences. A circovirus was detected in five of the affected calves. The analysis of the whole genome sequence of the circovirus PCV2-Ha08 revealed a close relationship to PCV2b. Circovirus infection in cattle has not been convincingly described so far. Serological investigations on circovirus-specific antibodies led to contradictory results (Allan et al., 2000; Ellis et al., 2001; Tischer et al., 1995). Only one study was able to detect a circovirus closely related to PCV2 in lung tissues and foetuses of cattle (Nayar et al., 1999). The only circovirus sequence originating from bovine tissue (Nayar et al., 1999) and available at the GenBank database is also closely related to PCV2. However, both strains cluster into different subtypes thus excluding the existence of a distinct PCV2 strain which is able to infect cattle. Circoviruses are generally thought to have narrow host ranges and detailed phylogenetic analyses revealed a strict co-evolution of circoviruses with their hosts (Johne et al., 2006). For PCV2, however, a slightly different evolutionary and epidemiological pattern has been described, which is consistent with a prolonged period of limited transmission in the past followed by a recent worldwide spread of this virus (Hughes and Piontkivska, 2008). It may be speculated that PCV2 has acquired specific properties allowing rapid spread and – in rare cases – transmission across the species barrier.

Interpretation of PCR results is sometimes difficult, especially with respect to DNA contamination. In our study, however, we implemented a strict regime to exclude laboratory DNA contamination. The successful amplification of the whole PCV2 genome from one sample argues against contamination with short PCR products. The negative

results of the PCV2-specific PCR protocol used routinely may be explained by a lower sensitivity of this protocol as compared to the nested protocol of the broad-spectrum PCR. The detection of PCV2-specific antigen by immunohistochemistry in individual bone marrow cells of one of the affected calves may support the PCR results but does not confirm a viral aetiology of HDS, because viral genome and viral antigen were not detected consistently in HDS-calves. Moreover, viral genome was even found in one calf of the control group. Immunohistochemistry was performed on calves with an end stage disease of HDS and the results are questionable. Efficient detection of PCV2-specific antigen might be restricted by the severe loss of bone marrow cells. In addition to bone marrow tissue, lymphatic tissues were investigated but tested negative for PCV2-specific antigen. The detection of PCV2 DNA in different organs might be explained by the presence of differentiated blood cells containing viral DNA.

Regarding PCV2-detection in one calf of the control group it has to be considered that control animals had been sent for pathological examination for reasons other than haemorrhagic diseases. In pigs PCV2 is associated with different syndromes and can even be found in healthy animals. According to this, it might be speculated that circoviruses contribute to several diseases in calves, too. On the other hand, it is also conceivable that immunosuppression in calves with HDS and also in calves of the control group enhances susceptibility to other infections. In this case, detection of PCV2 in calves may reflect an opportunistic or clinically unapparent infection.

Clearly, until now, results of our attempts to identify the causative agent of HDS have to be considered preliminary. An infection with a circovirus would be consistent with many of the observed clinical signs as most of the circoviruses cause lymphocyte depletion and the related CIAV also causes aplastic anaemia and haemorrhages in infected chickens. In our study, however, PCV2 was not detected in all clinical cases using the available diagnostic methods. Also, detection by PCR does not necessarily mean infection with a replicating virus. Although detection of PCV2 antigen by immunohistochemistry in some individual bone marrow cells is indicative of viral genome expression and replication, further investigations such as in situ hybridization and demonstration of viral antigens in affected tissues in early stages of the disease are necessary to assess the significance of PCV2 infections in cattle. Until then, the contribution of PCV2 to the aetiology of HDS remains hypothetical.

Most probably the underlying pathogenesis is even more complex and may include various causes such as infection, hereditary disposition, and immune-mediated destruction of blood cell precursors. Further possible causes of the disease and a broader spectrum of infectious agents should be screened in future studies in order to determine the cause of the haemorrhagic disease described here.

Added note: Currently, the number of calves affected by HDS in our institute increased to 220 (September 2009, data not shown). Furthermore, reports of HDS cases in other European countries like Great Britain (SAC, 2009) support the spread and relevance of this new disease entity in young calves.

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