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Circovirus Infection in Cattle

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LIST OF ABBREVIATIONS

aa	amino acid
AvGyV2	avian gyrovirus 2
BaCV	barbel circovirus
BNP	bovine neonatal pancytopenia
BTV	blue tongue virus
BVDV	bovine viral diarrhea virus
CaCV	canary circovirus
cap	capsid (gene)
Cap	capsid (protein)
CAV	chicken anemia virus
CfCV	cat fish circovirus
CsaCV	chimpanzee circovirus
CyCV	cyclovirus
DCVC	s-(1,2-Dichlorovinyl)-L-cysteine
DfCyV	dragonfly cyclovirus
DNA	deoxyribonucleic acid
DuCV	duck circovirus
ELISA	enzyme-linked immunosorbent assay
FiCV	finch circovirus
ICTV	international committee on taxonomy of viruses
GAGs	glycosaminoglycans
GoCV	goose circovirus
GuCV	gull circovirus
HGyV1	human gyrovirus 1
IFA	immunofluorescence assay

LIST OF ABBREVIATIONS

IgG	immunoglobulin G
IPMA	immunoperoxidase monolayer assay
ISH	*in situ* hybridization
kb	kilo base
MHC	major histocompatibility complex
nt	nucleotide
ORF	open reading frame
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PCV1	porcine circovirus type 1
PCV2	porcine circovirus type 2
PCVAD	porcine circovirus associated disease
PDNS	porcine dermatitis and nephropathy syndrome
PK15	porcine kidney 15
PiCV	pigeon circovirus
PMWS	post-weaning multisystemic wasting syndrome
PNP	proliferative and necrotizing pneumonia
PRDC	porcine respiratory disease complex
RaCV	raven circovirus
RCR	rolling circle replication
rep	replication (gene)
Rep	replication (protein)
ssDNA	single stranded circular deoxyribonucleic acid
StCV	starling circovirus
SwCV	swan circovirus

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1 INTRODUCTION

1 INTRODUCTION

1.1 Study objectives

Circoviruses are small, spherical viruses with a circular single-stranded DNA genome of 1.7 to 2.4 kb in size. The genome contains two major open reading frames (ORFs), encoding capsid (Cap) and replication (Rep) proteins (TODD et al. 2000). Porcine circovirus (PCV) was first identified as a cell culture contamination (TISCHER et al. 1974). Subsequently, research studies have shown that PCV was widespread in the pig population (TISCHER et al. 1986; ALLAN et al. 1995; TISCHER et al. 1995). The findings of JACOBSEN et al. (2009) showed that PCV2 has been present in the pig population in northern Germany since 1962. In 1991, a severe wasting syndrome in pigs was observed in Canada (HARDING and CLARK 1997), the United States of America (USA) and shortly thereafter in Europe (DAFT et al. 1996; LECANN et al. 1997). A PCV was found to be associated with the new syndrome (ELLIS et al. 1998a). Compared to the previously described circovirus in pigs the virus showed less than 80% sequence homology, indicating a new genotype (MEEHAN et al. 1998). Today, two distinct PCV genotypes are known: The non-pathogenic PK-15 cell line associated PCV1 and the pathogenic disease causing PCV2 (MEEHAN et al. 1998). PCV2-associated microscopic lesions were identified in archived tissues as early as 1985 in Spain (RODRÍGUEZ-ARRIOJA et al. 2003). Based on the sequence of capsid gene, the PCV2 can be further subdivided into PCV2a, PCV2b and PCV2c (OLVERA et al. 2007; GRAU-ROMA et al 2008; SEGALÉS et al. 2008), and is now present in all major pork producing countries.

The first detection of antibodies against PCV in sera of cattle, humans and mice was reported in 1995 (TISCHER et al. 1995). Studies performed in Canada also detected circovirus DNA in lung tissue samples of bovine respiratory disease and aborted foetuses (NAYAR et al. 1999). In two other studies, however, no antibodies to PCV2 were detected in

sera from cattle (n=405), sheep (n=120), horse (n=100) and humans (n=120) (ALLAN et al. 2000a; ELLIS et al. 2001).

Since the early 1990s, PCV2 has been associated with a number of disease manifestations including post-weaning multisystemic wasting syndrome (PMWS), respiratory disease, porcine dermatitis and nephropathy syndrome (PDNS), reproductive failure, enteritis, and neuropathy (CHAE 2005). Today, the combination of all the above described disease manifestations is summarized as porcine circovirus associated disease (PCVAD).

Bovine neonatal pancytopenia (BNP) is a syndrome affecting neonatal calves from different breeds and gender less than four weeks of age and was first observed in 2007. This syndrome is characterized by severe pancytopenia and bone marrow depletion leading to internal or external bleeding (FRIEDRICH et al. 2009), and it is associated with high mortality rates. It is known, that chicken infectious anemia virus, which is a member of the family *Circoviridae,* causes haemorrhages due to bone marrow depletion, severe anemia and atrophy of the thymus and bursa of Fabricius. To investigate a possible involvement of circoviruses in the etiology of BNP, we studied the presence of circoviruses in confirmed BNP-cases, and their relationship to this syndrome (see Chapter 4.1).

Moreover, PCV2 DNA has been found in 5/19 store bought beef samples from the USA by using consensus PCR screening (LI et al. 2011). The full-length genomes of PCV2 were also detected in three beef specimens from USA. These sequences shared 99% nucleotide identity with PCV2b (LI et al. 2011). The PCV1 and PCV2 were also detected in 5% (13/247) of human stool sample from the USA (LI et al. 2010). In order to study the prevalence of PCV2 in calves populations affected with BNP, 181 samples were tested using broad spectrum PCR (see Chapter 4.2).

Circovirus infections in cattle have not been convincingly described so far, and the serological investigations on circovirus-specific antibodies led to inconsistent results

(TISCHER et al. 1995; ALLAN et al. 2000a; ELLIS et al. 2001). It was important to study if PCV2 is pathogenic to calves. Accordingly, the susceptibility and immune response of calves to experimental PCV2 inoculation and the production of antibodies after vaccination with a commercial PCV2 vaccine were investigated (see Chapter 4.3).

1.2   Thesis organization

The present Thesis has been prepared in a cumulative format. The Thesis contains an introduction, a literature review, three separate scientific manuscripts, and a general discussion. References for the introduction, literature review, and the general discussion are cited at the end of the Thesis in the references section.

The first manuscript describes the etiological investigation regarding the BNP and showed the detection of PCV2 in affected calves. The second manuscript describes the prevalence of PCV2 in BNP in Germany and the whole-genome sequence of two PCV2 genomes derived from calves. The third manuscript investigates the susceptibility and immune response of calves to experimental PCV2 inoculation.

2 LITERATURE REVIEW

2 LITERATURE REVIEW

2.1 Circoviruses

2.1.1 Taxonomy

The family *Circoviridae* describes some of the smallest known viruses, with genomes ranging from 1.7 to 2.4 kb. The most recent taxonomy list from the International Committee on Taxonomy of Viruses (ICTV) (BIAGINI et al. 2012) classifies two genera of animal viruses, *Circovirus* and *Gyrovirus*, within in the family *Circoviridae*. Chicken anemia virus (CAV) is so far the only member of the genus *Gyrovirus* while members of the genus *Circovirus* include porcine circovirus-1 (PCV1), porcine circovirus-2 (PCV2), beak and feather disease virus (BFDV), pigeon circovirus (PiCV), goose circovirus (GoCV), canary circovirus (CaCV), duck circovirus (DuCV), finch circovirus (FiCV), gull circovirus (GuCV), raven circovirus (RaCV), starling Circovirus (StCV) and swan Circovirus (SwCV) (BIAGINI et al. 2012). Novel variants of circoviruses have been found in fish, in the faeces of a number of mammals including chimpanzees, bats, chickens, humans, and in the tissues of sheep, cattle, and camels (LI et al. 2010; LI et al. 2011; LORINCZ et al. 2011). Some of these novel viruses were classified in a new proposed genus *Cyclovirus* (LI et al. 2010). In 2011, circoviruses were found in invertebrates, specifically dragonflies, suggesting that members of the *Circoviridae* family may also infect insects (ROSARIO et al. 2011). The members of the family *Circoviridae* are shown in Table 1.

PCV2 has been subdivided into three main subtypes, PCV2a, PCV2b and PCV2c (OLVERA et al. 2007; GRAU-ROMA et al 2008; SEGALÉS et al. 2008).

Retrospective studies have indicated that PCV2a was the most prevalent subtype in the pig population prior to 2003 (ALLAN et al. 2007), however recent data indicated that PCV2b was more common in PCVAD outbreaks from 2004 onwards (GAGNON et al. 2007). Today,

PCV2b is the most prevalent subtype in the pig population worldwide (GAGNON et al. 2007). Less prevalent PCV2 subtypes include PCV2c which has been found in archived samples from Danish pigs (DUPONT et al. 2008).

2.1.2 Viral structure and genome organization

Members of the family *Circoviridae* are non-enveloped, icosahedral viruses containing a single stranded circular DNA (ssDNA) genome. They represent the smallest known autonomously replicating viral genomes (BIAGINI et al. 2012). Figure 1 presents the structure of the members of genus *Circovirus* and genus *Gyrovirus.*
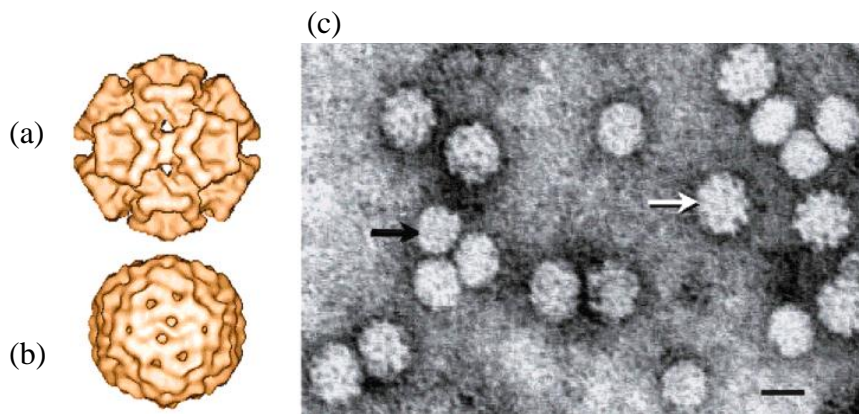

Figure 1: Circovirus particles. Left: Cryo-electron microscopy images of CAV (a) and PCV2 (b). Negative contrast electron microscopy of particles of CAV (black arrow, genus *Gyrovirus*) and BFDV (white arrow, genus *Circovirus*). Bar = 20 nm. Images taken from BIAGINI et al. (2012).

Table 1: The members of the family *Circoviridae*:

Genus	Species	Host	Genome (nt)	Reference
Circovirus	PCV1	Porcine	1759	TISCHER et al. 1974
	PCV2	Porcine	1768	MEEHAN et al. 1998
	DuCV	Duck	1991	HATTERMANN et al. 2003
	GoCV	Goose	1821	TODD et al. 2001a
	PiCV	Pigeon	2037	MANKERTZ et al. 2000
	RaCV	Raven	1898	STEWART et al. 2006
	SwCV	Swan	1785	HALAMI et al. 2008
	BFDV	Parrot	1993	RITCHIE et al. 1989
	GuCV	Gull	2035	TODD et al. 2007
	FiCV	Finch	1962	TODD et al. 2007
	StCV	Starling	2063	JOHNE et al. 2006
	CaCV	Canary	1952	TODD et al. 2001b
	CsaCV	Chimpanzee	1935	LI et al. 2011
	DogCV	Canine	2063	KAPOOR et al. 2012
	BaCV	Barbel fish	1957	LORINCZ et al. 2011
	CfCV	Cat fish	1966	LORINCZ et al. 2012
Gyrovirus	CAV	Chicken	2319	NOTEBORN et al. 1991
	AvGyV2	Chicken	2383	RIJSEWIJK et al. 2011
	HGyV1	Human	1886	SAUVAGE et al 2011
Cyclovirus	CyCV1-PK5006	Human	1732	LI et al. 2011
	CyCV-Chimp12	Chimpanzee	1747	LI et al. 2011
	CyCV-NG chicken 8	Chicken	1760	LI et al. 2011
	CyCV-PK goat11	Goat	1751	LI et al. 2011
	CyCV-PK beef23	Cow	1838	LI et al. 2011
	CyCV-TB	Bat	1703	LI et al. 2011
	DfCyV-A1	Dragonfly	1741	ROSARIO et al. 2011

Circoviruses have an ambisense genome organization containing two major inversely arranged open reading frames (ORFs). The largest ORF circovirus is located on the viral plus-strand (*rep* gene or ORF1, Figure 2). From ORF1 two proteins are expressed, which are both necessary for viral replication. Rep and Rep´ proteins are produced from differentially spliced transcripts. The Rep protein is translated from the full-length transcript (PCV1: 312 amino acid [aa]; PCV2: 314 aa); a spliced transcript encodes truncated and C-terminal by frame-shifted Rep´ (PCV1: 168 aa; PCV2: 178 aa) (FINSTERBUSCH and MANKERTZ 2009; BIAGINI et al. 2012). ORF2 (*cap* gene) encodes the major structural capsid protein (Cap) (PCV1: 232 aa; PCV2: 233 aa), which is also the main antigenic determinant of the virus. Cap displays a basic N-terminus rich in arginine residues, which is putatively involved in binding to viral DNA. A stem-loop structure with a conserved 9 bases motif in the loop, located between the 5-ends of the two main ORFs, is required to initiate the replication of the viral genome. The replication complex consists of Rep and a shorter Rep´ protein with a different carboxy terminus derived from a spliced transcript (FINSTERBUSCH and MANKERTZ 2009; BIAGINI et al. 2012).
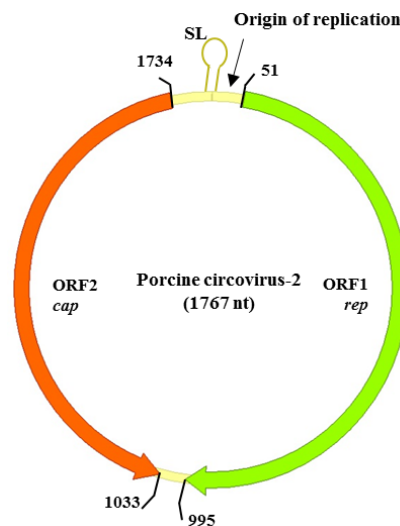

Figure 2: Genome organization of PCV2. ORF1 encoding the Rep protein, ORF2 encoding the Cap protein, the stem-loop structure (SL) and the origin of replication site (arrow) are shown. Modified from MEEHAN et al. (1998).

2.1.3 Entry and replication of circoviruses in pigs

The glycosaminoglycans (GAGs), heparan sulfate and chondroitin sulfate B serve as attachment receptors for PCV2 (MISINZO et al. 2006), but another molecule may be needed as an additional specific receptor (Figure 3). Viruses enter the host cell by several common pathways (VINCENT et al. 2005; MARSH and HELENIUS 2006) as e.g., clathrin-mediated endocytosis, macropinocytosis, caveolin-mediated endocytosis and clathrin and caveolin-independent pathways. After internalization, PCV2 is localized in endosomes. As the endosomes progress into the inner cell, the luminal pH of these vesicles drops to acidic values. Use of inhibitors made evident that a serine protease is essential for the release of PCV2 from the endosome (MISINZO et al. 2005), suggesting that this process may induce a proteolytic cleavage of the Cap protein.

Upon infection, the viral ssDNA genome is converted by host cell factors into a dsDNA replicative form that serves as template for viral DNA replication by cellular polymerase (Figure 3). The origin of replication is located within the non-coding region between the ORFs of *rep* and *cap* and overlaps with the promoter of the *rep* gene. A characteristic nonanucleotide sequence [5-(A/T) AGTATTAC-3] in PCV2 and PCV1, respectively, is conserved in all circoviruses and flanked by an inverted repeat (palindrome) of 11 nucleotides. Rep and Rep´ bind the stem loop, cutting a nick in the plus strand and a host-encoded DNA polymerase then extends the 3-hydroxyl group to copy the complementary circle using a rolling circle replication (RCR) mechanism (STEINFELDT et al. 2001; FAUREZ et al. 2009). The RCR strategy of PCV is similar to that of plant Geminivirus and Nanovirus and of bacterial plasmids within the pT181 family (TIMCHENKO et al. 1999; DEL SOLAR et al. 1998; GUTIERREZ 1999; DELWART and LI 2012). Recently, an alternative replication mechanism, the rolling-circle melting pot replication model was proposed for PCVs (CHEUNG 2004a; CHEUNG 2004b).
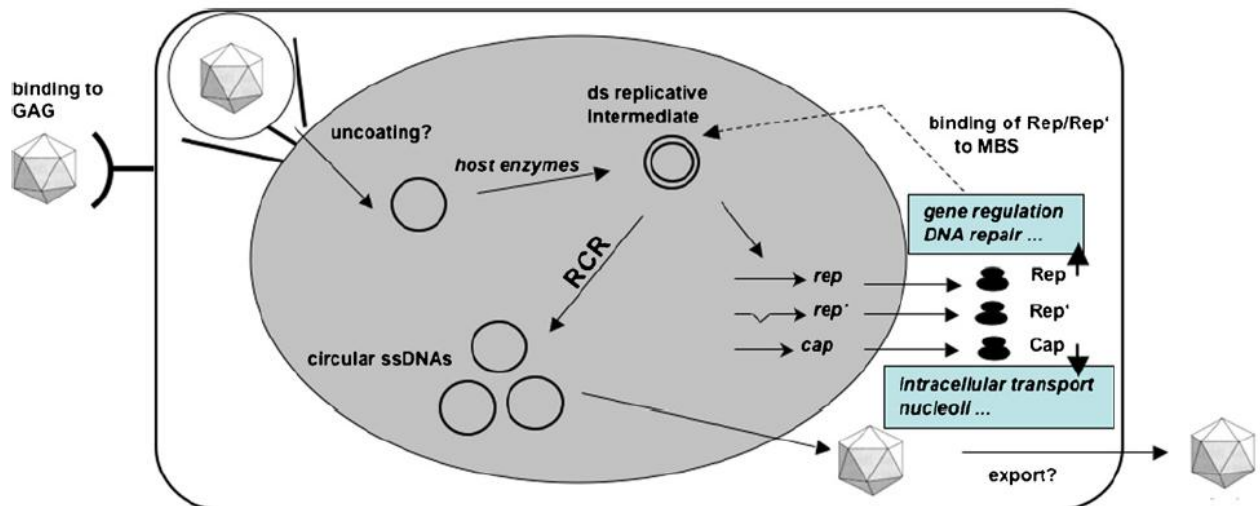
Figure 3: Life cycle of PCV. The virus uses GAGs as attachment receptors. The ssDNA genome is transported into the nucleus and converted by host enzymes into a dsDNA intermediate. The *rep* and *cap* mRNAs are transcribed, and the proteins are synthesized and imported from the cytoplasm. Rep/Rep´ bind to the dsDNA and initiate RCR by introduction of a nick that serves as primer. Events leading to assembly and release of virions have not yet been studied in detail. Figure and text taken from FINSTERBUSCH and MANKERTZ (2009).

In consequence, both minus- and plus-DNA strands are available as templates during initiation as well as termination of DNA replication.

2.1.4   Clinical manifestation of porcine circovirus-2 (PCV2) infection in pigs

Clinical signs and pathological features are still the corner-stones to suspect and diagnose overt disease associated with PCV2 infection. The clinical and pathological scope of this viral infection has been expanded over time. From the initial description of PMWS, some enteric, respiratory and reproductive disorders have been subsequently linked with PCV2. PDNS has also been associated with PCV2 infections. All together, the clinical signs have been grouped under the name PCVAD (SEGALÉS 2012).

PMWS was at first described in 1991 in Canada as a sporadic disease characterized by wasting and jaundice (HARDING and CLARK 1997). PMWS is defined by wasting, paleness

of the skin, respiratory distress, diarrhea and jaundice in late nursery and fattening pigs (SEGALÉS et al. 2005). Affected animals displayed characteristic lesions in multiple tissues (multisystemic), mainly in lymphoid organs (HARDING and CLARK 1997; ROSELL et al. 1999). PCV2 has been suggested to play a role in reproductive disorders, the so-called porcine respiratory disease complex (PRDC), enteritis, PDNS and proliferative and necrotizing pneumonia (PNP) (OPRIESSNIG et al. 2007; SEGALÉS et al. 2005). Table 2 summarizes PCV2 diseases from a clinical point of view using the proposed terminology (SEGALÉS 2012).

2.1.5 Pathogenesis of PCV2

Large parts of the pig populations (healthy or diseased pigs) at some point in their lives become infected with PCV2. The pathogenesis of PCV2 infection and the major cell types that support PCV2 replication are poorly understood. It has been suggested that PCV2 initially replicates in the tonsils. PCV2 antigen was first observed in minimal amounts in mesenteric lymph nodes at day 10 post infection (p.i.) with increasing density and distribution of PCV2 antigen at day 14, 17, 21, and 26 p.i. (ALLAN et al. 2000b). Large amounts of PCV2 antigen or nucleic acids are often detected in the cytoplasm of macrophages and dendritic cells by IHC or ISH (SORDEN et al. 1999; ALLAN AND ELLIS, 2000). It was found that antigen presenting cells in general stained positive by IHC for PCV2 antigen (CHIANINI et al. 2003). In contrast, PCV2 antigen was only sporadically detected in lymphocytes. In the thymus, PCV2 was only detected in few histiocytic cells in the medulla suggesting that thymocytes and T cells are not the primary target cells for PCV2 replication (CHIANINI et al. 2003). PCV2 antigen is also found in epithelial cells in lungs and kidneys, in smooth muscle cells, and in endothelial cells in several tissues in pigs experimentally-infected with PCV2 (KENNEDY et al. 2000) as well as in pigs with naturally occurring

PCV2-associated PMWS (MCNEILLY et al. 1999; ROSELL et al. 1999). KENNEDY et al. (2000) demonstrated PCV2 antigen in infiltrating macrophages in the tunica albuginea, in interstitial macrophages and in germinal epithelial cells in the testes, and in infiltrating macrophages in the epididymides of boars 24 to 29 days after they had been coinfected with PCV2 and porcine parvovirus (PPV) at 3 days of age. PCV2 was also found in the parenchyma of the secondary sex glands in a naturally infected boar (OPRIESSNIG et al. 2006). PCV2 targets mainly cardiomyocytes, hepatocytes, and macrophages during fetal life, and mainly monocytes in early post-natal life (SANCHEZ et al. 2003).

2.1.6 Diagnosis of PCV2

The first step in diagnosis always involves clinical sign assessment when overt disease is perceived. Table 2 summarizes dominating clinical signs in each of the PCVDs, even it is important to note that most of these symptoms are not specific of each of the PCVDs.

2.1.6.1 Polymerase chain reaction (PCR)

There are several PCR assays for the detection of PCV2-specific nucleic acids described in the literature (MOROZOV et al. 1998; CHOI et al. 2000; HAMEL et al. 2000; Shibata et al. 2003; GRIERSON et al. 2004). Variants of the regular PCR assay including multiplex PCR, nested PCR and quantitative real time PCR are also used for the detection of PCV2 DNA. Quantitative real time PCR assays have been developed, and these assays allow for determination of the amount of PCV2 genomic copy numbers in the serum or tissues. PCR reaction and detection are combined in one step which decreases the turn-around time (LIU et al. 2000; LADEKJAER-MIKKELSEN et al. 2002; ROVIRA et al. 2002; OPRIESSNIG et al. 2003; BRUNBORG et al. 2004; OLVERA et al. 2004; CHUNG et al. 2005).

Quantitative real time PCR, which can detect one genomic copy, is considered to be very sensitive for most applications. The amount of PCV2 nucleic acids in serum and tissues has been presented to be prognostic of the clinical outcome (BRUNBORG et al. 2004; OLVERA et al. 2004). Pigs with 10^7 or greater PCV2 genomic copies per milliliter of serum are likely to develop PCV2-associated lymphoid lesions and disease (BRUNBORG et al. 2004).

2.1.6.2 *In situ* hybridization (ISH)

ISH uses a labelled DNA probe that binds a specific sequence of the PCV2 genome (MCNEILLY et al. 1999; ROSELL et al. 1999; SIRINARUMITR et al. 2000). Hybridization is done overnight and followed by a color reaction. This method allows localization of PCV2 in histological investigations. The method in general is time consuming.

2.1.6.3 Enzyme-linked immunosorbent assay (ELISA)

There are several ELISA assays for PCV2 antibody detection commercially available (NAWAGITGUL et al. 2002; BLANCHARD et al. 2003; LIU et al. 2004). Recently, IgG and IgM PCV2-ELISA assays have been introduced in Europe (Ingezim PCV IgG® and Ingezim PCV IgM®, Ingenasa, Madrid, Spain). A variant of the regular ELISA assay is the competitive (blocking) ELISA (WALKER et al. 2000), which also has been developed for PCV2 (Synbiotics, Europe, Lyon, France). An antibody-detection blocking ELISA to detect PCV2-specific antibodies in faeces is also described and is commercially available in Europe (Synbiotics Europe, Lyon, France).

Table 2: Proposed terminology for porcine circovirus diseases (PCVDs) with case definition based on clinical findings, the most frequent gross and histopathological features and laboratorial findings of affected animals (modified from SEGALÉS 2012).

PCV2 subclinical infection (PCV2-SI)	None	Decreased weight daily gain without any evident clinical sign	None	None or slight lymphocyte depletion with granulomatous inflammation of lymphoid tissues	IHC/ISH: none or low amount in lymphoid tissues qPCR: $<10^5$ to 10^6 copies/g
PCV2 systemic disease (PCV2-SD)	PMWS, Porcine circovirosis PCV2-associated systemic infection	Wasting, decreased daily weight gain clinically evident, ill thrift or poor-doer	Long rough hair coat, prominent backbone and relatively oversized head Lymph node enlargement; White spots on kidney's cortices Atrophic and discolored liver, slightly rough hepatic surface; Catarrhal enteritis with or without mesenteric edema	Lymphocyte depletion with granulomatous inflammation of lymphoid tissues Lymphohistiocytic to granulomatous interstitial pneumonia Interstitial nephritis Granulomatous enteritis	IHC/ISH: moderate to high amount in lymphoid tissues; from none to high amount in non-lymphoid tissues, depending on lesional severity qPCR: $>10^6$ copies/g
PCV2 lung disease (PCV2-LD)	PCV2-associated respiratory disease, PNP	Respiratory distress, dyspnea	Lack of pulmonary collapse and tan-mottled lungs	Granulomatous bronchointerstitial pneumonia	IHC/ISH: moderate to high amount in lung and negative or low amount in lymphoid tissues qPCR: not determined
PCV2 enteric disease (PCV2-ED)	PCV2-associated enteritis	Diarrhea	Catarrhal enteritis Intestinal mucosa thickened Enlargement of mesenteric lymph nodes	Granulomatous enteritis Lymphocyte depletion with granulomatous inflammation in Peyer's patches	IHC/ISH: moderate to high amount in intestinal mucosa and Peyer's patches qPCR: not determined
PCV2 reproductive disease (PCV2-RD)	PCV2-associated reproductive failure	Abortions or mummifications Regular return-to-estrus	Fetal mummification Fetal hepatic enlargement and congestion Fetal cardiac hypertrophy Ascites, hydrothorax and hydropericardium in fetuses	Non-suppurative to necrotizing or fibrosing myocarditis of fetuses Chronic, passive, hepatic congestion in fetuses Mild pneumonia in fetuses	IHC/ISH: moderate to high amount in fetal myocardium qPCR: $>10^7$/500 ng DNA of myocardium, liver, and spleen
Porcine dermatitis and nephropathy syndrome (PDNS)	None	Dark red papules and macules on skin, mainly in hind limbs and perineal area	Irregular, red-to-purple macules and papules in the skin; subcutaneous haemorrhages and edema of affected areas; Enlarged lymph nodes Cutaneous scars in animals that recovered from the acute phase; Bilaterally enlarged kidneys, small cortical petechiae and edema of the renal pelvis	Systemic necrotizing vasculitis Fibrino-necrotizing glomerulitis with non-purulent interstitial nephritis Chronic, fibrous interstitial nephritis with glomerulosclerosis; from none to mild/moderate lymphocyte depletion	IHC/ISH: none or low amount in lymphoid tissues qPCR: $<10^6$ copies/g

2.1.7 PCV2 in non-porcine hosts

2.1.7.1 Cattle

Evidence for circovirus infection in mammals other than pigs is questionable, and studies have been restricted to PCV. The presence of antibodies reacting with PCV in sera of cattle has been reported (TISCHER et al. 1995). The first detection of circoviruses was demonstrated in 1999 by PCR in lung tissue samples from six out of 100 cases of bovine respiratory disease and from four out 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. In other studies, however, no antibodies to PCV2 were detected in sera from 185 bovine sera, randomly selected from serum banks in Northern Ireland. Moreover, 120 sera from cattle reared in farms in close proximity to pigs, having PMWS-like clinical disease, were also tested negative for PCV2 antibodies (ALLAN et al. 2000a). In this context, experimentally PCV2 infected dairy and beef calves did not develop virus-specific antibodies (ELLIS et al. 2001). Recently, a circovirus was also demonstrated in the bone marrow of five out of 25 BNP-affected calves in Germany (KAPPE et al. 2010). The whole genome analysis revealed a close relationship to PCV2. In addition, PCV2 sequences were also detected in five out of 19 beef samples from the USA, and the full-length genomes from three USA beef specimens shared 99% nt identity with PCV2 (LI et al. 2011).

2.1.7.2 Rodents

Reactivity to PCV antigen was tested in mice (NMR1, BALBc and black C57) sera using immunofluorescence assay (IFA). Percentage of sera reacted positive was between 12 to 69%. (TISCHER et al. 1995). Seventy-three mice were tested and found to be negative for PCV2-

specific antibodies by immunoperoxidase monolayer assay (IPMA) (RODRÍGUEZ-ARRIOJA et al. 2003) and 50 mice were found to be negative for PCV1-specific antibodies as determined by IFA (ALLAN et al. 1994a). Several studies have investigated the susceptibility of mice for PCV2-infection. One experimental study using BALB/c mice found detectable amounts of PCV2 antigen in the nuclei and cytoplasm of histiocytes and apoptotic cells in germinal centers of lymph follicles as well as in hepatocytes in the liver (KIUPEL et al. 2001). This group also showed mild lymphoid depletion and histiocytic infiltration of lymphoid follicles.

2.1.7.3 Humans

One study reported the presence of weakly cross-reactive anti-PCV antibodies in sera of humans (TISCHER et al. 1995). The DNA of PCV1 or PCV2 were detected in 12/247 (5%) human stool samples from the US (LI et al. 2010). Human exposure may have occurred by using a live attenuated oral human rotavirus vaccine (Rotarix®, GlaxoSmithKline, Rixenxart, Belgium), containing PCV1 (McCLENAHAN et al. 2011a). Rotarix® was found to contain full-length PCV1 genomes that are particle-associated, and cell culture assays in swine testis (ST) and PCV-free porcine kidney (PK-15) cells confirmed that PCV1 sequences in this vaccine represent infectious virus (VICTORIA et al. 2010; McCLENAHAN et al. 2011b). In other studies, no PCV DNA was found after screening more than 1000 samples from various tissues of both healthy and immunosuppressed humans (HATTERMANN et al. 2004), in plasma samples from 18 xenotransplantation recipients of pig islet cells (GARKAVENKO et al. 2004) or by consensus PCR in 200 human plasma samples (LI et al. 2010). PCV2 was reported in a colon biopsy from an ulcerative colitis patient, although contamination with PCV2 from stool is difficult to exclude (BERNSTEIN et al. 2003). A more recent study showed productive infection with PCV1 of a human hepatocellular carcinoma cell line

(BEACH et al. 2011). PCV detection in human stool likely reflects its transit through the gut without enteric viral replication (DELWART and LI 2012).

2.2 Bovine neonatal pancytopenia (BNP)

A mysterious haemorrhagic disease of calves emerged since 2007 in several European countries, affecting solely newborn calves under four week of age (FRIEDRICH et al. 2009; PARDON et al. 2010). An increasing number of calves during the last years were affected by this syndrome. The affected calves were reported for several breeds. Both genders were affected equally (FRIEDRICH et al. 2009). Investigations showed that affected animals were found in several countries including France, Germany, United Kingdom, Ireland, Netherlands, Belgium, Luxembourg, Italy, Spain and Canada (FRIEDRICH et al. 2009; PARDON et al. 2010; GOSSELIN et al. 2011). At the begin the syndrome was named "blood sweating", "haemorrhagic diathesis", "idiopathic haemorrhagic diathesis" and "bleeding calf syndrome". It was finally designated bovine neonatal pancytopenia (BNP) at the Satellite Symposium of the European Buiatric Congress in December 2009 in Marseille (France). BNP is characterized by high susceptibility to internal or external bleeding due to severe pancytopenia and bone marrow depletion leading to a high mortality rate (FRIEDRICH et al. 2009). In the initial phase, haematological investigations showed leukocytopenia and thrombocytopenia (FRIEDRICH et al. 2009; PARDON et al. 2010).

Clinical signs in affected calves may include severe external and internal cutaneous haemorrhages, bleeding after injections, petechiation of mucosae, melaena, pyrexia, and severe secondary infections can be explained by the pronounced thrombocytopenia and leukocytopenia (FRIEDRICH et al. 2009; PARDON et al. 2010). Also some affected calves are found dead without clinical signs being observed (LAMBTON et al. 2012). Mortality may reach 90% in affected calves. Mild to subclinical manifestations are rarely observed

(PARDON et al. 2010). In affected calves bone marrow is profoundly affected with reduction of megakaryocytes, lymphoid and myeloid precursor cells (FRIEDRICH et al. 2009; PARDON et al. 2010).

BNP is transferred by colostral alloantibodies binding to peripheral blood-derived leukocytes and platelet antigens of calves (PARDON et al. 2011). Remarkably, calves develop a severe thrombocytopenia and leukocytopenia within few hours after passive transfer of colostral antibodies and die within several days from bleeding disorder and bone marrow depletion (FRIEDRICH et al. 2011; PARDON et al. 2011). Alloantibodies responsible for BNP can develop in cows previously vaccinated with a specific bovine viral diarrhea (BVD) vaccine (PregSure BVD; Pfizer, Berlin, Germany; vaccine A) (PARDON et al. 2011). Colostra of these cows transfer BNP to healthy calves, indicating a commonly expressed target antigen in responding calves (FRIEDRICH et al. 2011). Alloantibodies are also detectable in blood of respective BNP dams (PARDON et al. 2011). Further immunological characterization of these antibodies revealed that they were of subclass IgG1 (ASSAD et al. 2012).

3 AIM OF THE WORK

3   AIM OF THE WORK

The detection of PCV (antigens and antibodies) in species other than pigs reflects the possibility of cross-species transmission of circoviruses. However, inconsistent data exist on PCV infections in cattle. It remains a question of value whether PCV is capable of replication in cattle causing clinicopathological condition

Therefore, the aim of the first part of this study was designed to investigate the possible involvement of circoviruses in the etiology of BNP in calves.

To deliver further knowledge about the possibility of cross-species transmission of PCV, in the second part of this study the prevalence of PCV2 in 181calves populations affected with BNP was investigated and the whole genome sequences of the PCV2-positive samples were analysed.

In the third part of the study, the susceptibility and immune response of calves to experimental PCV2 inoculation and the development of PCV2 specific antibodies after vaccination with PCV2 commercial vaccine were investigated.

4 RESULTS

Bone Marrow Aplasia with Haemorrhagic Disease in Calves in Germany: Characterization of the Disease and Preliminary Investigations on its Aetiology

4   RESULTS

4.1   PUBLICATION 1:   Bone Marrow Aplasia with Haemorrhagic Disease in Calves in Germany: Characterization of the Disease and Preliminary Investigations on its Aetiology

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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.

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).

TABLE 1: *Animals in the control group*

Case No.	Animal characteristics				Laboratory investigation	
	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.

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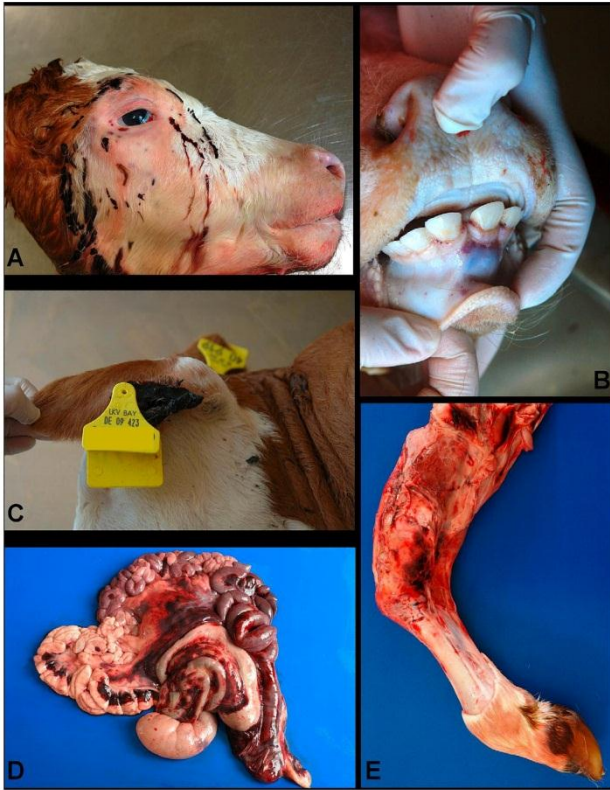
serial graded ethanol washes for rehydration followed by treatment with 3% hydrogen peroxide to quench endogenous tissue peroxidase activity. Staining was performed using the Histostain®-Plus Bulk Kit and the chromogen reagent AEC Single Solution (InvitrogenTM, Camarillo, CA, USA) according to the manufacturer's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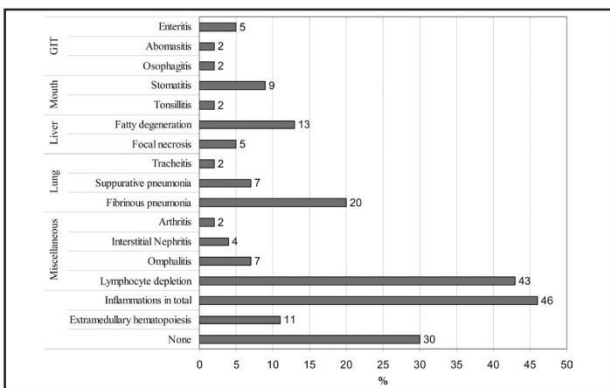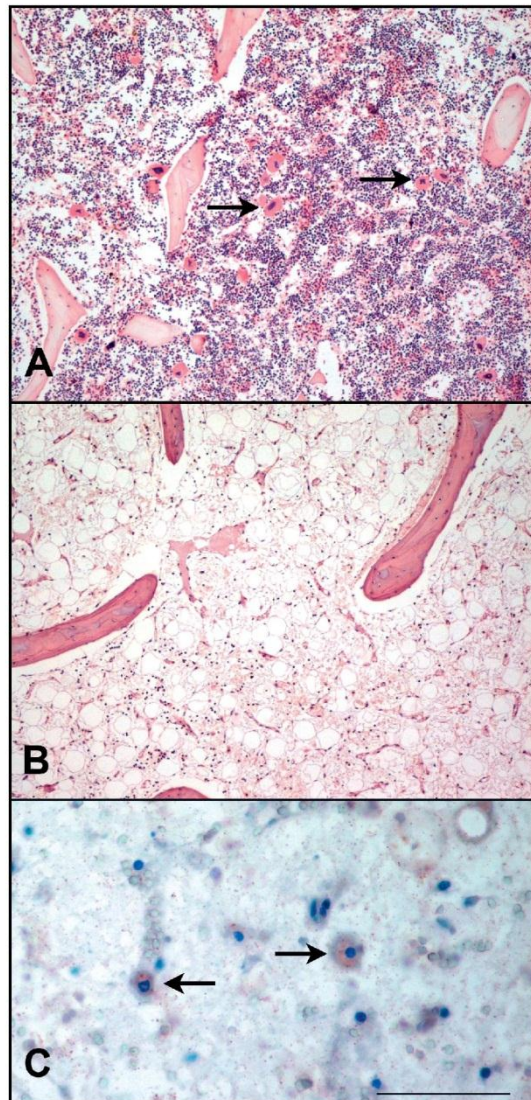

FIGURE 2: *Frequency of additional findings in calves with pancytopenia and haemorrhagic disease. Some animals showed several additional lesions. Inflammation of different organs were found frequently, but 30% of the animals investigated had no further lesions. GIT: gastrointestinal tract.*

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 dichlorovinyl-cysteine (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 PedigraphTM 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
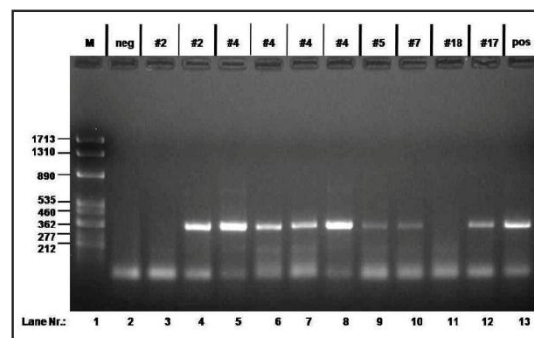

FIGURE 4: *Detection of circovirus DNA in samples from calves with haemorrhagic disease. A nested broad-spectrum PCR was performed using DNA extracted from bone marrow (lanes 3, 5, 9–12), blood (lanes 4 and 8), liver (lane 6) or kidney (lane 7) of calves with numbers indicated above the lanes. Neg: negative 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.*

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).

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-
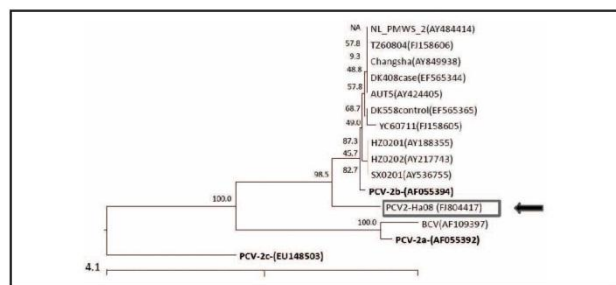

FIGURE 5: *Phylogenetic relationship of the circovirus PCV2-Ha08 detected in a German calf with porcine circovirus type 2 strains. The phylogenetic tree was established on the basis of the complete nucleotide sequences of the reference strains PCV2a, PCV2b and PCV2c (bold font), the Canadian bovine circovirus (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 immuno-histochemistry 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.

Acknowledgement

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4.2 PUBLICATION 2:

Whole Genome Sequence of Two Strains of Porcine Circovirus 2 Isolated from Calves in Germany

4.2 PUBLICATION 2: Whole Genome Sequence of Two Strains of Porcine Circovirus 2 Isolated from Calves in Germany

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Abstract:

Two new strains of porcine Circovirus type 2 virus (PCV2), strain Ha09 and Ha10, have been detected in calves in Germany, and the complete genomic sequence of each virus has been sequenced and analysed. Phylogenetic analysis suggested that these strains belong to the PCV2b genotype cluster, the worldwide highly prevalent genotype.

Porcine circovirus type 2 (PCV2), which belongs to the family *Circoviridae*, is a non-enveloped, icosahedral, single-stranded circular DNA virus with a genome of about 1768 nucleotides (nt). PCV1 and PCV2 are the smallest viruses which replicate autonomously in mammalian cells (1). The PCV2 genome contains two major open reading frames (ORF1 and ORF2), encoding for the replication (Rep) and capsid (Cap) proteins (2), respectively. As well as a third gene (ORF3) encoding a virus-induced apoptotic protein. (3). PCV2, identified as the etiologic agent of porcine circovirus-associated diseases (PCVAD) causing severe economic losses (6), was also detected in rodents (7), cattle (8), and in calves affected with bovine neonatal pancytopenia (BNP) (9). There are three different recognized PCV2 genotypes, PCV2a, PCV2b, and PCV2c (4, 5). In this study, two PCV2 strains were detected and identified in calves affected with bovine neonatal pancytopenia (BNP) in Germany. During the screening of the prevalence of PCV2 in calves we investigated 181 EDTA-blood and tissue samples. Two out of 181 samples were found positive for PCV2, both from calves with signs typical for BNP. However, the first strain, designated Ha09, originated from the blood of a calf in Bavaria, whereas the second strain Ha10 was isolated from lung and brain of a calf in Saxony. The DNA was extracted from the samples using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany), and a nested broad-spectrum PCR protocol was applied as recently described (10). The complete genome of the detected

circovirus was amplified by PCR using a pair of inverse primers which previously described (9). The whole genome was amplified and subsequently cloned and sequenced.

The genome of the strain Ha09, which originated from Bavaria in the south of Germany, is comprised of 1768 nt, however, the strain Ha10, which was collected in Saxony in the east of Germany, contains 1767 nt. Both strains consist of 3 ORFs (ORF1, ORF2, and ORF3). The level of identity between the complete sequence of the Ha09 and Ha10 strains comprises 98.8%. These strains are more closely related to the PCV2b than other genotypes, PCV2a and PCV2c. The identity scores to the PCV2b based on the complete sequence of the Ha09 and Ha10 are 98.9% and 99.6%, respectively. A phylogenetic tree based on capsid nucleotide sequences revealed that the isolate from Saxony (Ha10), was more closely related to PCV2b than the isolate from Bavaria (Ha09). As compared to the published PCV2b sequences, a detailed analysis of the capsid amino acid sequences revealed four identical amino acid changes in the isolate from Bavaria, and 2 others in the isolate from Saxony. According to the type of these amino acids, it may be expected that these changes may not have any significant influence on the biological or antigenic characteristics of these isolates. This finding was further supported by phylogenetic analysis of the replication protein sequences. The obtained data will be helpful for analyses of the evolutionary characteristics and molecular pathogenesis of PCV2 and its prevalence in non-porcine hosts.

Nucleotide sequence accession numbers. The genome sequence of the Ha09 and H10 strains have been submitted to GenBank under the accession no. HQ231329 and HQ231328, respectively. The GenBank numbers of the reference strains are: PCV2b (accession no. AF055394), PCV2a (accession no.AF055392) and PCV2c (accession no. EU148503).

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4.3 PUBLICATION 3:

Susceptibility of Calves to Porcine Circovirus-2 (PCV2)

4.3 PUBLICATION 3: Susceptibility of Calves to Porcine Circovirus-2 (PCV2)

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Abstract

Circoviruses are known to infect pigs and birds and cause severe diseases with various clinical signs. Porcine circovirus-2 (PCV2), associated with severe economic losses, was detected in rodents, mosquitoes, cattle, and in calves affected with bovine neonatal pancytopenia (BNP). However, molecular and serological investigations on circovirus infections in cattle revealed inconsistent results. The aim of the study was to investigate the susceptibility and immune response of calves to experimental PCV2 inoculation. Animals were either intravenously inoculated with tissue-culture grown PCV2, with bone marrow from PCV2 positive and negative calves or immunized with a commercial inactivated PCV2 vaccine. The results showed that the animals inoculated with tissue-culture grown PCV2 and with PCV2 positive bone marrow displayed clinical signs including lymph node swelling, reddening of oral and ocular mucosa, and diarrhea 7-18 days post inoculation (p.i.). PCV2-specific antibodies were detected in the tissue-culture grown PCV2-infected animals and in the PCV2-immunized animals from day 11 and 7 p.i. onwards, respectively, but were absent in both bone marrow inoculated groups. PCV2 was detected by real-time quantitative PCR only in blood samples of the tissue-culture grown PCV2-infected animals and in various tissues (e.g., spleen, lymph nodes, thymus), with high copy numbers in blood between day 4 (5.16 $\log_{10}$ genomic copy number/ml) and 46 (5.33 $\log_{10}$ genomic copy number/ml) p.i. In conclusion, the seroconversion and the detection of PCV2 in lymphoid tissues for more than five weeks p.i. revealed that host susceptibility of PCV2 is not solely restricted to pigs.

Keywords: Porcine circovirus-2 (PCV2); Calves; Bovine neonatal pancytopenia (BNP)

1 Introduction

Circoviruses, the smallest viral pathogens (17-20 nm) known to infect eukaryotic organisms, belong to the family *Circoviridae* which contains three genera, *Gyrovirus, Circovirus* and the recently proposed genus „*Cyclovirus*". In addition to different avian circoviruses, the genus *Circovirus* contains porcine circovirus-1 (PCV1) and porcine circovirus-2 (PCV2) (Biagini et al., 2012). The PCV1 was first identified as a contaminant of a continuous porcine kidney cell line and found to be non-pathogenic in pigs (Allan and Ellis, 2000). However, PCV2 is associated with a group of diseases collectively called porcine circovirus associated diseases (PCVAD). These syndromes are 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) (Ellis et al. 1999).

Only limited data exist on circovirus infections in cattle. PCV2 was first detected by PCR in lung tissue samples from cases of bovine respiratory disease and aborted foetuses and tentatively named bovine circovirus (BCV). It was nearly identical to that of PCV2 (Nayar et al., 1999). PCV2 was also detected by PCR in the bone marrow of bovine neonatal pancytopenia (BNP) - affected calves (Kappe et al., 2010). The PCV2 sequences were also detected in beef samples from USA (Li et al., 2011). PCV has been reported in addition in sera from cattle, humans and mice has been reported (Tischer et al., 1995). In another studies, however, no antibodies to PCV2 were detected in sera from cattle, sheep, horse and humans (Allan et al., 2000; Ellis et al., 2001) and experimentally PCV2 infected dairy and beef calves did not produce virus-specific antibodies (Ellis et al., 2001). Due to these inconsistent results of molecular (Nayar et al., 1999; Ellis et al., 2001; Kappe et al., 2010; Li et al., 2011) and serological investigations (Tischer et al., 1995; Allan et al., 2000; Ellis et al., 2001), this study was initiated to investigate the susceptibility of calves to experimental PCV2 inoculation.

2 Materials and methods

2.1 Animals

Eight male German Holstein calves were obtained from a large commercial dairy farm in Saxony/Germany at an age of four weeks and kept in the premises of the Faculty of Veterinary Medicine, University of Leipzig. The animals were fed with milk substitute and hay *ad libitum*.

2.2 Virus, inoculum and vaccine

The PCV2b strain AFSSA P3 CS (AY321984), $10^{4.5}$ tissue culture infective doses ($TCID_{50}$) per ml, kindly provided by Pfizer, was used for inoculation. Preparations of bone marrow from a BNP-affected, PCV2-positive calf (5.86 $\log_{10}$ genomic copy number of PCV2/ml), and bone marrow from a healthy, and from PCV2-negative calf were used for inoculation. The commercial PCV2 based sub-unit vaccine containing the Cap protein expressed in a baculovirus system (Ingelvac® CircoFLEX^{TM}) was obtained from Boehringer Ingelheim GmbH, Ingelheim am Rhein, Germany.

2.3 Experimental design

The animals were randomly divided into four groups (designated G1-G4) of two animals each (Table 1). All animals were tested negative for bovine herpes virus-1 (BoHV1) and bovine virus diarrhea virus (BVDV) antigen by ELISA at the Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen, Leipzig (Germany). Calves in group1, 2 and 3 were inoculated intravenously (i.v.) with 10 ml (5.86 $\log_{10}$ genomic copy number/ml) bone marrow of BNP-affected PCV2-positive calves or 10 ml (10.07 $\log_{10}$ genomic copy number/ml) tissue-culture growth PCV2 or intramuscularly (i.m.) with the PCV2 vaccine, respectively. Animals of the group G4 served as controls and were inoculated i.v. with preparations of 10 ml bone marrow from healthy PCV2-negative calves.

2.4 Clinical investigations and sample collections

The animals were daily investigated for clinical signs according to common practice. EDTA-blood, serum, and nasal swabs were collected on day 0, 2, 4, 7, 9, 11 and 18 p.i. Samples were collected weekly until the termination of the study at day 65 p.i. (day 46 p.i. in the case of group 2). Anticoagulant blood was collected in 1.5 ml Eppendorf tubes. Serum was collected in 5 ml tubes, centrifuged at 2000 x g for 10 min at 4°C, and stored at -20 °C until use. Nasal swabs were collected using sterile swabs which were stored in 1.5 ml tubes containing 1 ml of sterile saline solution. All EDTA-blood samples were subjected to haematological analysis at the Department of Large Animal Medicine, Faculty of Veterinary Medicine, University of Leipzig.

2.5 PCV2 DNA detection and sequencing

Organ tissues were homogenized using TissueLyser II (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. PCV2 DNA extraction was automatically performed (QIAcube, Qiagen, Hilden, Germany). To verify the presence of PCV2 in all EDTA-blood, nasal swabs and tissue samples PCV2 DNA detection and quantification was done by a real-time quantitative PCR using the primers and probe as described by Brunborg et al. (2004) with some modifications. In brief, forward primer PCV2-84-1256U21 (5´-GTA GCG GGA GTG GTA GGA GAA-3´) and reverse primer PCV2-84-1319L21 (5´-GCC ACA GCC CTC ACC TAT GAC-3´) were used in combination with TaqMan-1286-1314 (5´-6-Fam-ATG TTA ACT ACT CCT CCC GCC ATA CCA T-Tamra-3´) as probe. The nucleotides modified in the present study are indicated with black boxes. For each sample, a volume of 2.5µl of the eluate was run in a 25µl reaction on a MxPro 3005 real-time PCR cycler (Stratagene, Agilent Technologies, Inc., Santa Clara, CA, USA). Results are given as the numbers of DNA copies per ml blood or per gram of tissue. Using the real-time qPCR assay Maxima[®] Probe qPCR Master Mix (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot, Germany), the thermocycler conditions were 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and

60°C for 1 min. Samples were considered negative if no signal was observed during the 40 amplification cycles. Three concentrations (10^7, 10^4, and 10^2 copies per sample) of a plasmid containing the complete genome of PCV2-Ha08 (GenBank Nr. FJ804417) were included in each run, serving as positive controls as well as to derive the standard curve used for quantitation of PCV2 DNA in tissue and blood samples. Each run included two negative controls (no template).

2.6   Amplification of the whole circovirus genome

The complete genome of PCV2 from a lymph node of calf in group 2 was amplified using a pair of inverse primers as previously described (Kappe et al., 2010). Amplification was performed using the Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific) with the following cycling conditions: iInitial denaturation 98°C for 5 min followed by 35 cycles of 98°C for 30 s, 60°C for 30 s and 72°C for 2 min, and a final extension at 72°C of 7 min. The product was purified and cloned using CloneJET PCR Cloning Kit (Thermo Fisher Scientific). The plasmid contains the complete genome was isolated and sequenced in an ABI Prism device (Applied Biosystems). Sequence alignments were carried out with the CLUSTAL W method using the MegAlign module of the Lasergene DNASTAR software package (DNASTAR, Inc., Madison, WI, USA). Sequence similarities were performed using the BLAST 2.2.14 search facility.

2.7   PCV2 antibody detection

All serum samples were tested using the SERELISA® PCV2Ab Mono Blocking kit (Synbiotics Europe, Lyon, France) according to the manufacturer's instructions. Results were expressed as the 'sample to negative corrected ratio' (SNc). Samples were considered negative for the presence of anti-PCV2 antibodies if the SNc ratio was greater than 0.40. They were considered positive with values less than or equal 0.40.

2.8   Necropsy and histopathology

At day 65 p.i. (day 46 p.i. for G2), calves were euthanized humanely using an i.v. administered overdose of pentobarbital. Necropsy was performed at the Landesuntersuchungsanstalt für das Gesundheits- und Veterinärwesen Sachsen, Leipzig, Germany. Sections of organs including bone marrow, tonsil, spleen, thymus, lymph nodes, liver, lung, kidney and ileum were collected and stored at -80°C for real-time quantitative PCR. Sections were also mixed with 10% neutral buffered formalin and routinely processed for histological examination.

2.9   *In situ* hybridization

In situ hybridisation (ISH) was used to identify the presence of viral nucleic acid of PCV2 in paraffin-embedded tissue samples as previously described (Bukovsky et al., 2007). Sections of inguinal lymph nodes, ileum, spleen, thymus and bone marrow samples were investigated. Samples of inguinal lymph node from naturally infected pigs served as positive controls for PCV2.

3   Results

3.1   Clinical investigations and macroscopic lesions

The animals infected with bone marrow of BNP-affected PCV2-positive calves (G1) and animals infected with tissue culture grown PCV2 (G2) developed mild dyspnoea and dry coughing during the first two weeks p.i. The rectal temperature was within normal limits (mean 39.4°C) and the joints appeared normal. Diarrhea was observed in calves in group 2 on day 10-12 p.i. Wasting was not observed in any of the calves. Gain of body weight was normal in all animals (data not shown) except for one calf in group 2. This animal started with the highest body weight in the experimental trial. The calves of group 1 developed swelling of superficial lymph nodes, reddening of oral and ocular mucosa, abnormal behaviour at day 7-18 p.i., however, calves of group 2 showed swelling of superficial lymph nodes, reddening of

oral and ocular mucosa at day 11-18 p.i. Necropsy of calves in group 1 and 2 revealed haemorrhages and enlargement of one or more internal and superficial lymph nodes (Fig. 1a, 1b, 1c). PCV2-vaccinated calves (G3) and control calves (G4) did not develop any clinical signs or pathological lesions (Fig. 1d).

3.2   Haematological investigations

Total leukocyte counts were markedly decreased on day 18 and 11 p.i. in the calves inoculated with bone marrow of BNP-affected PCV2-positive calves (G1) and the tissue culture grown PCV2-infected calves (G2), respectively (data not shown). No significant differences were observed in leukocyte differentiation, haemoglobin, haematocrit value, mean cell volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) among the animals of groups (G1-G4).

3.3   Histopathological findings

Microscopic lesions characteristic of BNP were present in none of the calves in the trial. Acute catarrhal enteritis was observed in calves from group 1 and 2. Calves in group 2 showed a moderate follicular and pulpy lymphatic hyperplasia with low lymphadenitis in the spleen as well as hyperaemia in the inguinal and mesenteric lymph nodes. The lymph nodes in the calves of group 3 and 4 remained normal throughout the observation period.

3.4   PCV2 DNA detection in blood and swab samples

Blood and swabs of all calves were tested negative for PCV2 DNA at the beginning of the experiment. Calves in group 1 and 3 as well as those in the control group 4 were tested negative for PCV2 throughout the study (until day 65 p.i.). However, PCV2 DNA was detected in blood samples collected from tissue culture grown PCV2-infected calves (G2) at day 4, 11, 18, 32, 39 and 46 p.i. using real-time quantitative PCR with mean concentrations of 5.2, 5.9, 5.4, 5.1, 5.3 and 5.3 $\log_{10}$ genomic copy number/ml, respectively.

3.5   Presence of PCV2 DNA in organs and tissues

Organs and tissues, namely bone marrow, tonsils, spleen, thymus, lymph nodes, liver, lung, kidney and ileum, collected from the calves in group 1, 3 and 4 were tested negative for PCV2 DNA at the end of the study (day 65 p.i.). Bone marrow, tonsils, spleen, thymus, lymph nodes, liver, lung, kidney and ileum of tissue culture grown PCV2-infected calves (G2), however, were positive for PCV2 DNA using real time quantitative PCR (Table 2). The highest mean load of viral DNA was found in the spleen and thymus (mean = 7.88 $\log_{10}$ PCV2 genomic copies/g tissue). A high amount of viral DNA was also detected in the lung (mean = 7.83 $\log_{10}$ genomic copy number/g). The lowest amount was detected in kidney (mean = 5.64 $\log_{10}$ PCV2 genomic copies /g).

3.6   Sequence analysis

The sequence of the amplified PCR product obtained from lymph node of calf in group 2 using the inverse primers has a length of 1767 nucleotides (nt). The strain was designated as PCV2-Ha11 and submitted to the GenBank with the accession number KJ920205. A sequence similarity comparison of the PCV2-Ha11 genome sequence to the sequence of the inoculum (AY321984) revealed that the degree of identity is 99.9%. There are differences in 2 nt different between the two genomes. One mutation (C1708T) is located in the capsid coding region without any changing the amino acid sequence. The second mutation (G365C) is located in the replicase coding region, which lead to a change in the amino acid sequence (Met105Ile).

3.7   Presence of PCV2 IgG antibodies

At the beginning of the experiment, all calves were tested negative for PCV2-specific antibodies using the competitive ELISA. Calves in group 1 and the negative control group (G4) remained seronegative for PCV2 throughout the study. However, all animals of group 2 and 3 developed specific antibodies from day 11 and 7 p.i., onwards (Fig. 2).

3.8   *In situ* hybridization

PCV2-specific DNA was not detected in the tested tissue samples (lymph nodes, ileum, spleen, thymus or bone marrow) from calves in all groups at necropsy (Fig. 3). A section of a lymph node taken from a naturally PCV2-infected pig served as positive control.

4   Discussion

Circovirus infections in pigs and birds are frequently reported, and cause a broad range of clinical diseases as associated with significant economic losses (Todd, 2000). Only limited information exists on circovirus infections in cattle. PCV2 uses glycosaminoglycans (GAGs), heparansulfate and chondroitin sulfate B as receptors for its attachment to host cells (Finsterbusch and Mankertz, 2009). The GAG receptors are present on a large number of bovine cells (Ruiz-Saenz et al., 2009), suggesting that PCV2 could infect cattle. The presence of circovirus genomes with similarities to PCV2 was demonstrated in calves (Halami et al., 2014) and beef (Li et al., 2011). However limited data exist with regard to the pathogenicity of PCV2 in cattle. Therefore, this work aimed to investigate the susceptibility and immune response of calves to experimental PCV2 inoculation.

A number of clinical signs such as respiratory dysfunction, enlargement of superficial inguinal and intestinal lymph nodes and diarrhea were observed in PCV2-infected calves (G2). These clinical findings resemble signs of PMWS in PCV2 infected pigs (Allan and Ellis, 2000). Although PCV2-associated systemic disease in pigs is clinically characterized by wasting or weight loss (Krakowka et al., 2004), only one calf in the PCV2-infected group lost body weight. Histopathological lesions revealed the presence of follicular and pulpy lymphatic hyperplasia with low lymphadenitis in the spleen, and hyperaemia in several lymph nodes in the PCV2-infected calves. Similar results were described by Galindo-Cardiel et al. (2011) in pigs affected by PCV2. The results also resemble histopathological findings in PCV2 infected pigs described by Ellis et al. (1999). The total leukocyte count was decreased

in calves of group 1 and 2 at day 18 and 11 p.i., respectively, could be attributed to the susceptibility of peripheral blood mononuclear cells (PBMC) from cattle and pigs to PCV1 (Allan et al., 1994). Although calves in group 1 developed mild dyspnoea, dry coughing, swelling of superficial lymph nodes and reddening of oral and ocular mucosa during the first two weeks p.i. as well as leukocytopenia, they remained sero-negative for PCV2. It is possible that the amount of PCV2 (5.86 $\log_{10}$ PCV2 genomic copies/ml) in the inocula used was not high enough to establish virus infection and to produce specific antibodies. Possibly that the virus present in the bone marrow of the BNP calf had lost its infectivity. It is also possible that agents other than PCV2 virus are responsible for these signs and leukocytopenia (Demasius et al., 2014). PCV2-immunized (G3) and control (G4) calves did not show any clinical signs or post mortem lesions. Seroconversion was observed using competitive ELISA in PCV2-immunized and PCV2-infected calves at day 7 and 11 p.i., respectively. These calves displayed high antibody titres throughout the experiment. Allan et al. (2000) did not find any lesions or seroconversion in lambs infected by a combined oral and nasal route with $10^{5.0}$ TCID$_{50}$ PCV2. It was reported that the cut off for the induction of histopathological lesions of PMWS in pigs was at 7 $\log_{10}$ PCV2 genomes per ml serum (Olvera et al., 2004) which might point towards a dose response effect *in vivo*. PCV2 DNA was detected and quantified in blood and tissue samples by real-time quantitative PCR (detection limit 2.5 copies) on day 4 p.i. in PCV2-infected calves (G2), and the calves remained positive for PCV2 throughout the study. Prolonged viraemia with detectable viral DNA up to day 125 p.i. was reported from PCV2-infected pigs (Shi et al., 2008; Gillespie et al., 2009; Patterson et al., 2011). Also high amounts of DNA can be detected in the lymphatic tissues including spleen, thymus and lymph nodes of PCV2 inoculated and diseased (PMWS) pigs (Brunborg et al., 2004). The nasal swab samples from PCV2-infected calves were negative indicating that no shedding of the virus via nasal discharge in cattle occurred. However, the molecular detection

of PCV2 DNA in cattle samples affected with respiratory disease and in aborted bovine fetuses has been reported (Nayar et al., 1999). The complete genome of PCV2 from lymph node of calf in group 2 was carried out to analyse possible adaptive mutations of PCV2 in calves. The re-isolated genome sequence revealed an identity of 99.9% compared to the inoculum. Two mutations (C1708T and G365C) did occur during infection. Interestingly, all isolated PCV2 genomes from cattle showed a mutation at amino acid 105 (Met to Ile) (Nayar et al., 1999; Kappe et al., 2010; Halami et al., 2014), suggesting that this location could be an adaptive mutation of PCV2 to replicate efficiently in cattle.

In contrast to the PCR results in the present study, we did not detect PCV2 in the PCV2-infected calves using ISH. It is possible that the detected amounts of PCV2 DNA in different tissues of calves in this experiment were not high enough to give a clear signal in the ISH. This would be consistent with the rather low detection limit of ISH of 7 $\log_{10}$ PCV2 genomic copies/ml (Brunborg et al., 2004; Segalés et al., 2005). The highest mean virus load in blood (day 11 p.i.) was 5.9 $\log_{10}$ genomic copy number/ml in calves of group 2.

5.   Conclusions

In conclusion, moderate clinical signs, viraemia and seroconversion were observed in experimentally PCV2-infected calves demonstrating that host susceptibility of PCV2 is not solely restricted to pigs.

6.   Acknowledgments

7.   References

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Table 1 Experimental design.

Group	Age (day)	Inoculum	Dose of PCV2	Route
			Treatment	
G1 (*n*=2)	32	Bone marrow of BNP calves	5.86 $\log_{10}$ PCV2 genomic copies/ml[1]	iv
G2 (*n*=2)	53	PCV2[2]	10.07 $\log_{10}$ PCV2 genomic copies/ml	iv
G3 (*n*=2)	32	PCV2 vaccine[3]	1 ml[4]	im
G4 (*n*=2)	32	Bone marrow of healthy calves[5]	-	iv

[1]PCV2 determined using real-time qPCR.

[2]PCV2b strain (AFSSA P3 CS, Pfizer Olot, SLU, Spain).

[3]Commercial PCV2a based sub-unit vaccine containing the Cap protein expressed in a baculovirus system (Boehringer Ingelheim GmbH, Germany).

[4]According to the manufacturer's instructions.

[5]PCV2 negative using real-time qPCR.

Table 2

Viral load and mean group $\log_{10}$ PCV2 genomic copies/g of porcine circovirus-2 (PCV2) in tissue from the bone marrow, tonsils, spleen, thymus, lymph nodes, liver, lung, kidney and intestine of calves inoculated with PCV2 (G2) at necropsy (days post inoculation 46).

Organ/tissue	Virus load calf 1*	Virus load calf 2*	Mean ±SD
	PCV2-infected calves (G2)		
Bone marrow	6.16	5.98	(6.07 ± 0.18)
Tonsils	6.95	5.16	(6.06 ± 1.79)
Spleen	7.99	7.77	(7.88 ± 0.22)
Thymus	7.48	7.55	(7.52 ± 0.07)
Lymph nodes	7.12	6.67	(6.90 ± 0.45)
Liver	7.78	6.96	(7.37 ± 0.82)
Lung	8.49	7.17	(7.83 ± 1.32)
Kidney	6.08	5.19	(5.64 ± 0.89)
Ileum	6.63	6.26	(6.45 ± 0.37)

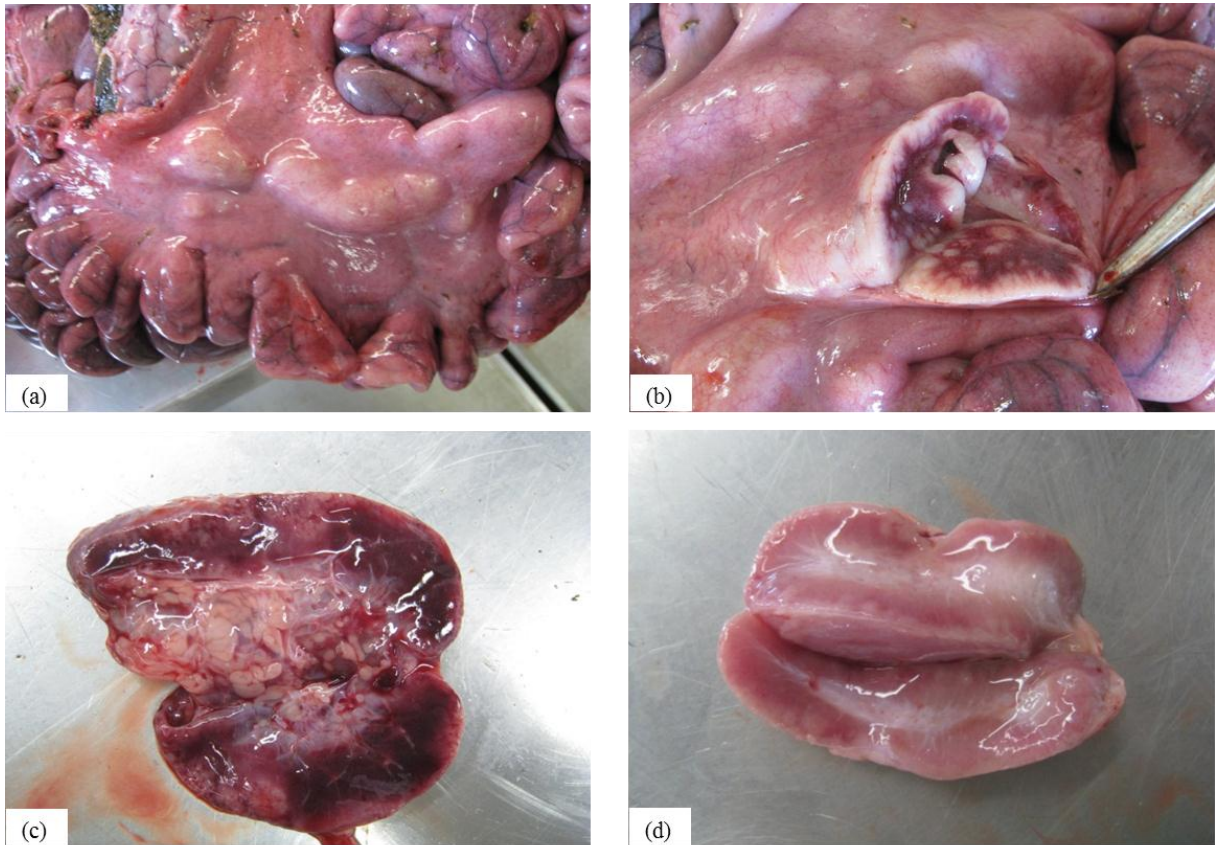* $\log_{10}$ PCV2 genomic copies/g

Fig. 1. Enlargement and haemorrhages of mesenteric lymph node from a PCV2-infected calf (a and b); Haemorrhages in inguinal lymph node from calves infected with bone marrow from BNP-positive calf (c); and superficial inguinal lymph node of control calves (d).
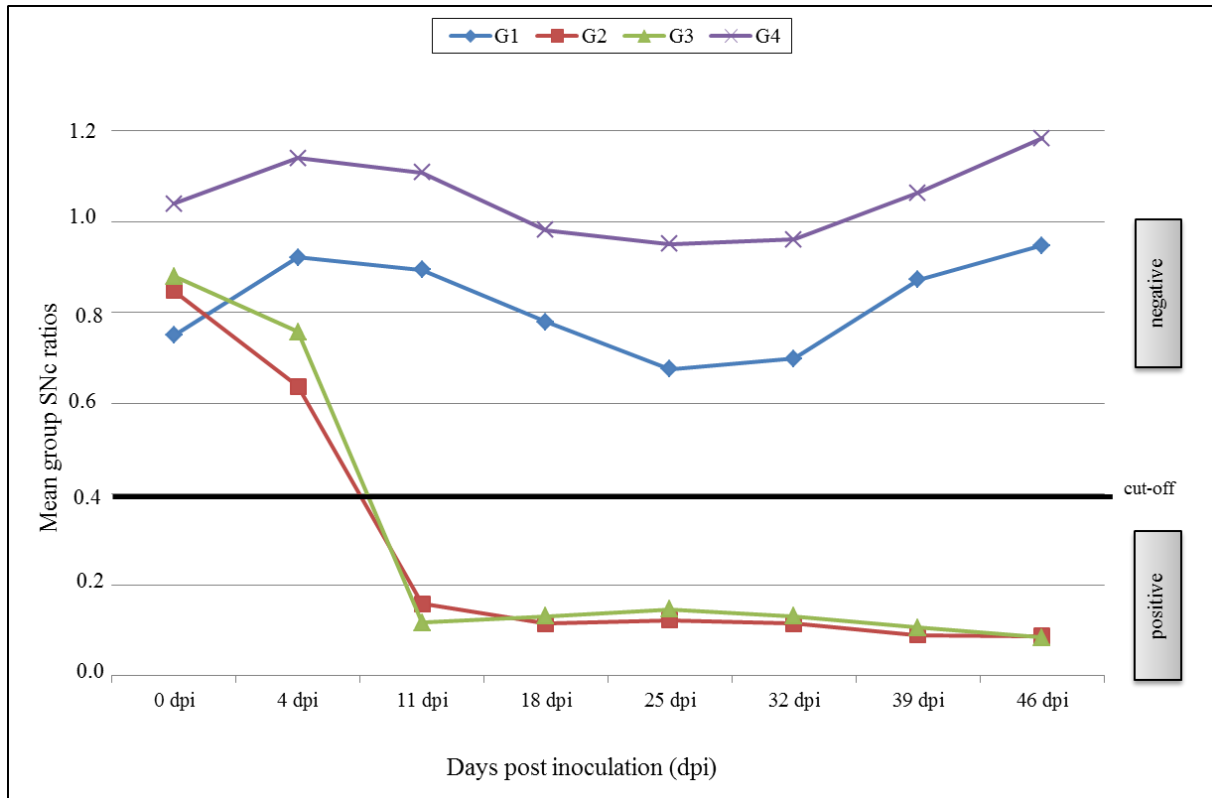
Fig. 2. Antibody production in PCV2 infected calves. Mean group sample to negative corrected (SNc) ratios in serum of calves inoculated with bone marrow of BNP-positive calves (G1), PCV2 (G2), PCV2 vaccine (G3) and non-infected (G4). Samples were considered to be positive for anti-PCV2 antibody if the SNc ratio was equal or less than 0.40 (cut-off). Calves in group 1 and 4 were negative until the end of the experiment (65 days p.i.). Calves in group 2 and 3 were positive until the end of the experiment (65 days p.i.).
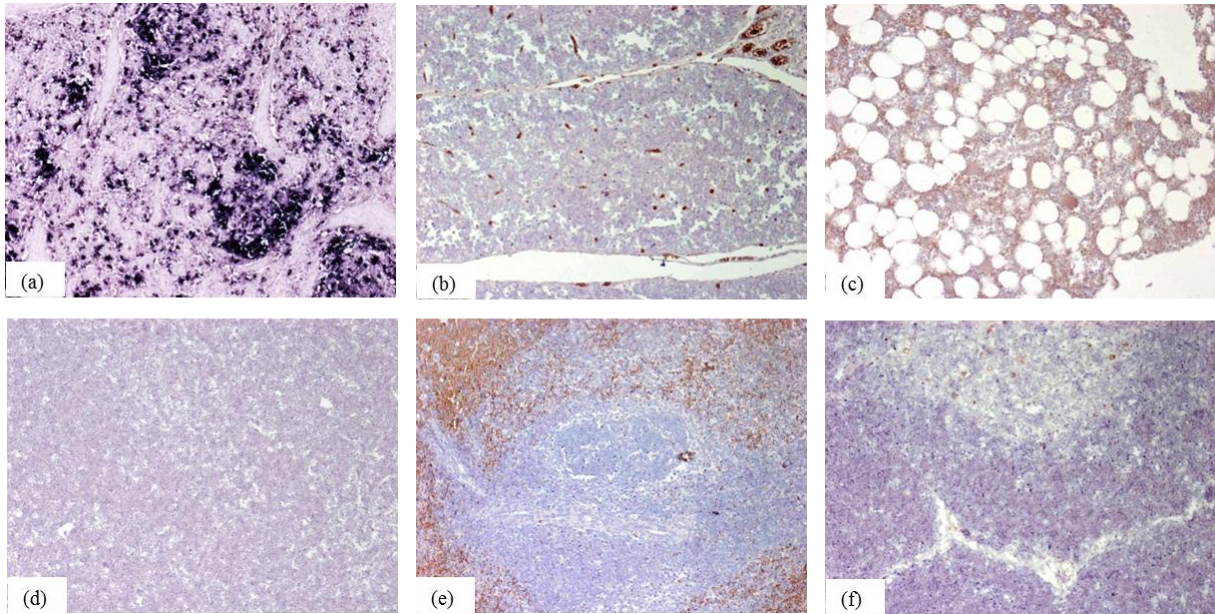
Fig. 3. Demonstration of PCV2 nucleic acid within lymph nodes from a PCV2-positive pig (a). No signal was detected in the ileum (b), bone marrow (c), lymph node (d), spleen (e) and thymus (f) of PCV2-infected calves of group 2 using *in situ* hybridization.

5   GENERAL DISCUSSION

5   GENERAL DISCUSSION

PCV belongs to the genus *Circovirus* within the family *Circoviridae*, and they are the smallest known viruses that replicate autonomously in mammalian cells. Circoviruses are non-enveloped, and they contain characteristic single-stranded, negative-sense, circular DNA (ELLIS et al. 2004). Two distinct PCV genotypes are known; PCV1, a non-pathogenic PK-15 cell line associated, and PCV2, a pathogenic genotype (MEEHAN et al. 1998). The complete genome sequences of a PCV1 strain isolated in 1990 and one isolated in 2011 were obtained and compared to the sequences of other available PCV1 isolates. Phylogenetic analyses revealed very low genetic diversity among these viruses (TOMBACZ et al. 2014). PCV2 has been suggested to play a role in reproductive disorders including PRDC, PDNS and PNP (SEGALÉS et al. 2005; OPRIESSNIG et al. 2007). PCV2 was subdivided into PCV2a, PCV2b and PCV2c (OLVERA et al. 2007; GRAU-ROMA et al 2008; SEGALÉS et al. 2008). PCV2a was the most prevalent subtype in the pig population (ALLAN et al. 2007), while PCV2b was more common in PCVAD outbreaks (GAGNON et al. 2007). PCV2b was the predominant genotype circulating in southern China from 2011 to 2012 (WEI et al. 2013). Current commercial PCV2 vaccines are based on PCV2a and have been shown to be effective in reducing PCV2a and PCV2b viremia and PCV2-associated lesions and disease. Recently, OPRIESSNIG et al. (2014) found that PCV2a based vaccine protected pigs from PCVAD development induced by current PCV2 field viruses. PCV2c was detected in Danish pigs (DUPONT et al. 2008).

Since 2007 a new fatal haemorrhagic diathesis in calves with unknown cause was observed all over Germany, and designated as BNP. The clinicopathological picture resembles clinical signs caused in chicken by infectious anemia virus (haemorrhages due to bone marrow

depletion, severe anemia and atrophy of the thymus), which also is a member of the family *Circoviridae* (KUSCU and GÜREL 2008).

Although PCV caused disease mainly in pigs, there are inconsistent results regarding circovirus infection in cattle. Antibodies against PCV in sera of cattle were reported (TISCHER et al. 1995). Also circovirus DNA was detected in lung tissue samples of bovine respiratory disease and aborted fetuses (NAYAR et al. 1999). Accordingly, the first part of our study was directed to investigate the possible involvement of circoviruses in the etiology of BNP.

5.1 BNP syndrome and detection of circoviruses

BNP was detected in calves in Germany and many countries in Europe (FRIEDRICH et al. 2009; PARDON et al. 2010). Haemorrhages, particularly in skin, subcutis and gastrointestinal tract were the major findings in all BNP calves. Bone marrow of femur and sternal bone, lung, liver, kidney, spleen, and lymph nodes were processed, stained and tested histopathologically (PARDON et al. 2010). In the present study 56 BNP calves were investigated. Samples showed severe hypoplasia or even aplasia of the bone marrow. Lymphocytic depletion (43%) and inflammatory lesions (46%) were less frequently observed. Haematological analysis indicated also an aplastic pancytopenia. To find the cause of BNP, the following causes of haemorrhages in cattle due to thrombocytopenia were described:

1) Hereditary haemorrhagic diathesis, a marked dysfunction of platelets (STEFICEK et al. 1993). However such cause was excluded hence hereditary haemorrhagic diathesis was described only in Simmental cattle while BNP was observed in Simmental and Holstein Friesian calves. Moreover, pedigree analysis indicated no autosomal dominant or recessive hereditary disease.

2) Several toxins and mycotoxins causing bone marrow aplasia and haemorrhages are known also to induce haemorrhages in calves (HARRACH et al. 1983; VALLI 2007). Intoxications with DCVC or furazolidone induce a marked cellularity of bone marrow and extensive haemorrhages (LOCK et al. 1996). Also furazolidone and bracken fern produce fatal haemorrhagic diathesis due to severe bone marrow depletion (HOFFMANN-FEZER et al. 1974; HOFMANN et al. 1974; MAXIE and NEWMAN 2007; VALLI 2007). However, DCVC and its metabolite N-acetyl-DCVC as well as furazolidone yielded negative results and there was no indication for the uptake of bracken fern by calves or cows.

3) Idiopathic thrombocytopenic purpura, an autoimmune disease, may be immune-mediated destruction of platelets (LUNN and BUTLER 1991) or associated with botulism toxoid vaccination or inactivated vaccines against papilloma virus and clostridia (LUNN and BUTLER 1991; YERUHAM et al. 2003). In BNP, affected calves have no history of such vaccinations.

4) Bacterial infections such as *P. multocida* are also known to cause haemorrhagic septicaemia in calves (RIMLER 1978; HORADAGODA et al. 2001). In this study, *P. multocida* was present in only three calves. Microbiological investigation of organ samples revealed a wide spectrum of potentially pathogenic bacteria in diseased calves. However, constant evidence of specific pathogenic bacteria associated with the haemorrhagic disease could not be found. In 29% (n =16) of all cases, no pathogenic bacteria were detected at all. It was assumed that depletion of lymphatic tissue and bone marrow in these calves resulted in severe leukopenia and granulocytopenia associated with immunosuppression and secondary infections.

5) Infection with the non-cytopathic BVDV type 2 may result in severe bleeding tendency due to thrombocytopenia (REBHUN et al. 1989; ELLIS et al. 1998b). However,

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 constant finding in the cases reported in this study. On this account, it seemed reasonable to exclude a BVDV infection. Likewise, infection with BTV was excluded by laboratory examination. Several farmers suspected that vaccination against BTV-8 correlated with BNP. This, however, could not be substantiated

In the tested samples, a circovirus was detected in calves with haemorrhagic disease. No other infectious agent was detected in this animal. The analysis of the whole genome sequence of the circovirus PCV2-Ha08 revealed a close relationship to PCV2b. The hypothesis that PCV2 could infect species other than pigs was postulated. PCV2 sequences had also been detected in beef samples from USA (LI et al. 2011). PCV antibodies have been reported in sera from cattle, humans and mice (TISCHER et al. 1995). In other studies, however, no antibodies to PCV2 were detected in sera from cattle, sheep, horse and humans (ALLAN et al. 2000a; ELLIS et al. 2001), and experimentally PCV2 infected dairy and beef calves did not produce virus-specific antibodies (ELLIS et al. 2001). In this study the detection of PCV2b in BNP calves did not confirm the role of circovirus in BNP, hence the viral genome was even found in one calf of the control group. WILLOUGHBY et al. (2010) found no evidence in either Great Britain or Northern Ireland to suggest a viral cause for BNP or to support a role for PCV2 or other circoviruses in this syndrome.

Recently, the main aetiology of BNP has been well described (PARDON et al. 2011). BNP is induced by colostral alloantibodies binding to peripheral blood-derived leukocytes and platelet antigens of calves (PARDON et al. 2011). Remarkably, calves develop a severe thrombocytopenia and leukocytopenia within few hours after passive transfer of colostral antibodies and die within several days from bleeding disorder and bone marrow depletion (FRIEDRICH et al. 2011; PARDON et al. 2011). Alloantibodies responsible for BNP can

develop in cows previously vaccinated with a leukocytic specific BVDV vaccine (PregSure BVD; Pfizer, Berlin, Germany; vaccine A) (PARDON et al. 2011). Colostra of these cows transfer BNP to healthy calves, indicating a commonly expressed target antigen in responding calves (FRIEDRICH et al. 2011). Alloantibodies are also detectable in blood of respective BNP dams (PARDON et al. 2011). Further immunological characterization of these antibodies revealed that they were of subclass IgG1 (ASSAD et al. 2012).

BNP-inducing colostrum might be associated with alloantibodies directed against MHC class I in response to vaccination with a distinct inactivated viral vaccine. However, the proportion of alloantibody-producing individuals by far exceeds the proportion of clinical BNP cases in the vaccinated population. Therefore, more recently it was suggested that further factors were involved in BNP pathogenesis, e.g., genetic predisposition (JONES et al. 2013; DEMASIUS et al. 2014). BNP has been approved to increase the lethality of common infectious neonatal diseases (HENNIGER et al. 2014).

Although PCV2 was isolated from tested BNP animals, the distinct viral cause of BNP could not be shown. It was important to complete the efforts which implemented in the first part to study the possible cross-species transmission of PCV2 between cattle and pigs. PCV2 was reported to be present in cattle with respiratory disease and from aborted bovine foetuses (NAYAR et al. 1999), but these findings have not been subsequently confirmed (RODRIGUEZ-ARRIOJA et al. 2003). In 2010, PCV2 was detected in muscle tissues of cattle from USA, Pakistan and Nigeria (LI et al. 2010).

Recently, circovirus genomes from tissues of cows were amplified and sequenced and found to be related to PCV2 (LI et al. 2011). The presence of closely related viruses in cattle may reflect recent cross-species transmission.

To confirm the possibility of cross-species transmission of PCV2, the second part of this study was designed to analyse the whole genome sequence of the isolated PCV2 genome from BNP calves.

5.2 Complete genome sequencing of PCV2

Complete genome sequencing was undertaken to verify the presence of PCV2 in cattle. Therefore, a broad-spectrum PCR was performed for amplification of circoviral genomic sequences (HALAMI et al. 2008). A circovirus was detected in five of the BNP-affected calves. Based on the sequence of the PCR products, inverse primers were created which were capable of amplifying the complete circovirus genome from bone marrow, liver, kidney and blood. The strain was designated PCV2-Ha08 and sequenced completely. 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 size and containing the conserved nonamer sequence, is evident in the non-coding region 1.

To confirm our results further, 181 samples from calves affected with BNP in Germany were tested using broad-spectrum PCR (HALAMI et al. 2008). Two out of 181 samples were found positive for PCV2, both from calves with signs typical of BNP. PCV2-Ha09 originated from the blood of a calf in Bavaria, whereas PCV2-Ha10 was detected in lung and brain of a calf in Saxony. The genome of the PCV2-Ha09 is comprised of 1768 nt, however, PCV2-Ha10 contains 1767 nt. Both sequences consist of 3 ORFs (ORF1, ORF2, and ORF3).

The level of nt identity between the complete sequences of PCV2-Ha08, Ha09 and Ha10 comprises 98.7%-99.8%. They are more closely related to the sequence of PCV2b than to the other genotypes, PCV2a and PCV2c (Figures 4 and 5). The identity scores to PCV2b, based

on the complete sequence of the PCV2-Ha08, Ha09 and Ha10, are 98.9%, 98.9%, and 99.6%, respectively.

Compared with published PCV2b sequences, a detailed analysis of the capsid amino acid sequences revealed four identical amino acid changes in PCV2-Ha09 (Bavaria), and 2 others in PCV2-Ha10 (Saxony). These finding were further supported by phylogenetic analysis of the Rep protein sequences. Data obtained will be helpful for the analysis of the evolutionary characteristics and molecular pathogenesis of PCV2 and its prevalence in non-porcine hosts. The detection and sequence analysis of PCV2 revealed that PCV2 in our investigations clearly could infect animal species other than pigs.

PCV2 uses GAGs, heparansulfate and chondroitin sulfate B as receptors for its attachment to host cells (FINSTERBUSCH and MANKERTZ 2009). The GAG receptors are present on a large number of bovine cells (RUIS-SAENZ et al. 2009), suggesting that PCV2 could infect cattle.

Hence the isolation of circoviruses is difficult (CRUZ et al. 2014), our study was only based on the detection of the PCV2 genome and its complete sequence analysis. However, there are inconsistent results of molecular (NAYAR et al. 1999; ELLIS et al. 2001; KAPPE et al. 2010; LI et al. 2011) and serological investigations (TISCHER ET AL. 1995; ALLAN et al. 2000a; ELLIS et al. 2001). Accordingly, it was important to investigate the susceptibility and immune response of calves to experimental PCV2 inoculation.

5.3   Pathogenicity of PCV2 in cattle

Although the PCV2 genome was detected in calves, limited data exist with regard to the pathogenicity of PCV2 in cattle. Therefore, this part of the present study aimed of investigating the susceptibility and immune response of calves to experimental PCV2

inoculation. Animals were either inoculated intravenously (i.v.) with tissue-culture grown PCV2, with bone marrow from either PCV2 positive or negative calves, or immunized intramuscular (i.m.) with a commercial inactivated PCV2 vaccine. Calves showed to be susceptible to PCV2 based on (i) clinical picture, (ii) histopathological lesions, (iii) haematological findings, (iv) seroconversion and (v) virus genome re-isolation as follow:

PCV2-infected calves showed clinical signs including respiratory dysfunction, enlargement of superficial inguinal and intestinal lymph nodes and diarrhea. Similar signs were reported in PCV2 infected pigs (ALLAN and ELLIS 2000; ELLIS et al. 2001). PCV2-infected calves revealed the presence of follicular and pulpy lymphatic hyperplasia with some sings of lymphadenitis in the spleen, and hyperaemia in several lymph nodes. Similar histopathological lesions were described in pigs affected by PCV2 (ELLIS et al. 1999; GALINDO-CARDIEL et al. 2011). PCV2-infected calves showed a decrease in the total leukocyte count at day 11 p.i. This reduction could be explained by the susceptibility of peripheral blood mononuclear cells (PBMC) from cattle and pigs to PCV (ALLAN et al. 1994b).

Seroconversion was observed using competitive ELISA in PCV2-infected (i.v.) and PCV2-vaccinated (i.m.) calves at day 11 and 7 p.i., respectively. These calves displayed high antibody titres throughout the experiment. However, ELLIS et al. (2001) did not find any seroconversion in calves infected at the age of 1 day or 6 months intranasally with 10^5 TCID$_{50}$ PCV2 isolated from pigs. It can be assumed that the i.v. route of infection as used in this study is more suitable to test the pathogenicity of PCV2 in calves. Interestingly, some studies reported the detection of PCV2 DNA in mosquitoes *(Culex)* and house flies *(Muscadomestica)* on commercial pig farms (BLUNT et al. 2011; YANG et al. 2012). This infection route should be considered in the future if the prevalence of PCV2 in cattle would become more frequent.

PCV2 DNA was detected and quantified in blood and tissue samples by real-time quantitative PCR on day 4 p.i. in PCV2-infected calves, and the calves remained positive for PCV2 throughout the study. Prolonged viraemia with detectable viral DNA up to day 125 p.i. was reported from PCV2-infected pigs (SHI et al. 2008; GILLESPIE et al. 2009; PATTERSON et al. 2011). Also high amounts of DNA can be detected in the lymphatic tissues including spleen, thymus and lymph nodes of PCV2 inoculated and diseased (PMWS) pigs (BRUNBORG et al. 2004). The nasal swab samples from PCV2-infected calves were negative indicating that no shedding of the virus via nasal discharge occurred. However, the molecular detection of PCV2 DNA in cattle samples affected with respiratory disease and in aborted bovine fetuses has been reported (NAYAR et al. 1999).

The complete genome sequence of the re-isolated PCV2 from lymph node of PCV2-infected calves, which had been designated as PCV2-Ha11, showed an identity of 99.9% compared to the inoculum, with presence of possible adaptive mutations of PCV2. In the phylogenetic analysis, the virus was clustered with the PCV2b genomes (Figures 4 and 5). Two mutations (C1708T and G365C) did occur during infection (Appendix I). Interestingly, all isolated PCV2 genomes from cattle showed a mutation at amino acid 105 (Met to Ile) (NAYAR et al. 1999; KAPPE et al. 2010; HALAMI et al. 2014), suggesting that this location could be an adaptive mutation of PCV2 to replicate efficiently in cattle (Appendix II).
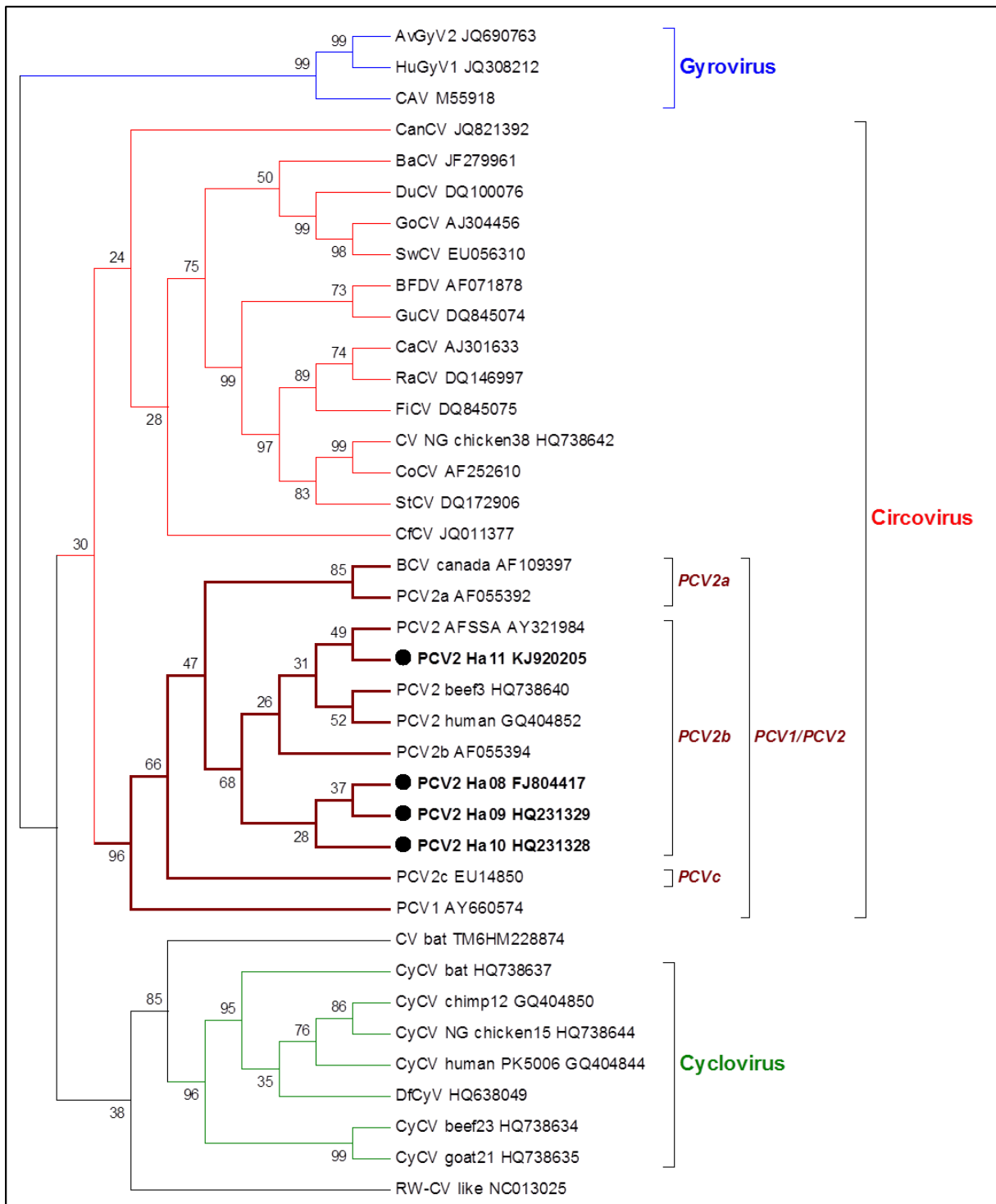
Figure 4: Phylogenetic analysis of the members of the family *Circoviridae* based on the complete nucleotide sequence. The tree was generated by the distanced-based neighbor-joining method using MEGA 5.10. The reliability of the tree was assessed by bootstrap analysis with 1,000 replications.
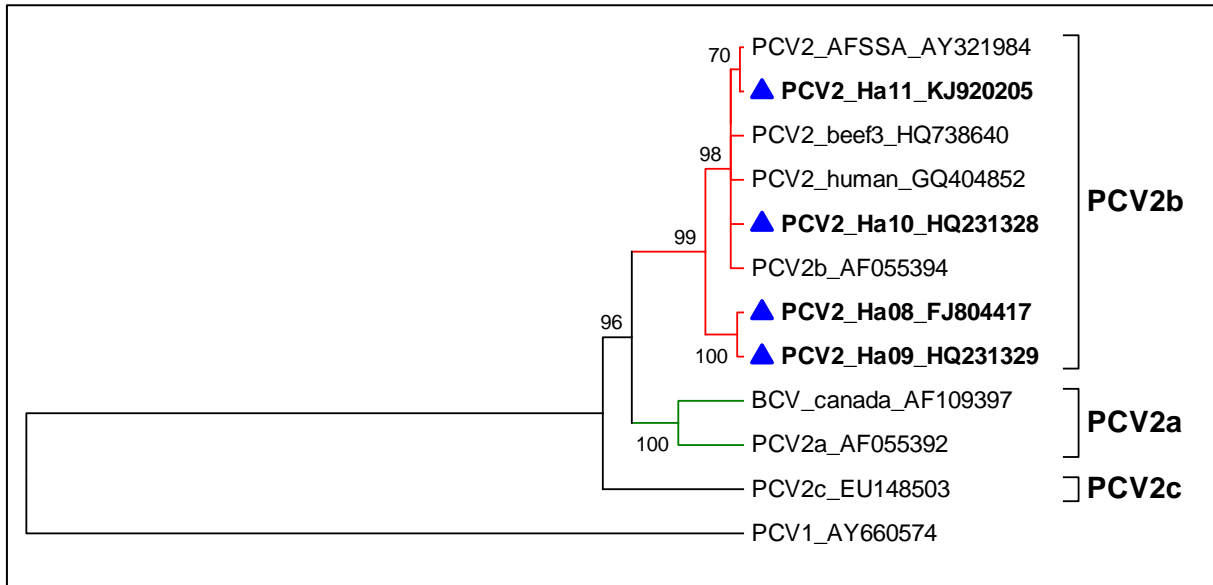
Figure 5: Phylogenetic analysis of PCV2 genomes isolated from pig, cattle, and human. Phylogenetic analysis was based on the nucleotide sequences of the complete genome. The tree was generated by the distanced-based neighbor-joining method using MEGA 5.10. The reliability of the tree was assessed by bootstrap analysis with 1,000 replications.

5.4 Potential risk of PCV in humans

Attention for the possible interspecies transmission of PCV to humans should be also considered. Retrospective studies have since shown that PCV2 antigen was identified as early as 1962 in tissues from a pig (JACOBSEN et al. 2009). After 30 years a severe wasting syndrome in pigs was characterized (DAFT et al. 1996; HARDING and CLARK 1997; LECANN et al. 1997). Obviously, the virus needs almost 30 years to adapt to pig tissues to cause distinct manifestations. The virus was found to contaminate pork products (LI et al. 2010). It is possible that PCV2 could infect humans through contaminated pork products (Figure 6) hence PCV2 was detected in a colon biopsy from an ulcerative colitis patient and in human stool samples (BERNSTEIN et al. 2003; LI et al. 2010).

The detection of PCV2 in cattle tissues in this study and in beef products (LI et al. 2011) supports our hypothesis that humans could be exposed to PCV2 through ingestion of

contaminated food or contact with infected animals. The contamination of human vaccines (e.g., Rotarix®, GlaxoSmithKline, Belgium and RotaTeq®, Merck and Co., USA) with PCV1 and PCV2 (VICTORIA et al. 2010; McCLENAHAN et al. 2011a; McCLENAHAN et al. 2011b) also provides ample opportunities for circovirus transmission to humans. Regarding to the detection of PCV2 DNA in mosquitoes *(Culex)* and house flies *(Musca domestica)* on commercial pig farms (BLUNT et al. 2011; YANG et al. 2012), insects should be considered as an important vector for transmission of the virus into animals (e.g., pig and cattle) and humans (Figure 6).

Although there are no indications about the pathogenicity of PCV2 or circovirus like particles in humans at this time, the evolution of circoviruses in humans due to frequent exposure could pose a potential risk for humans on the long term.
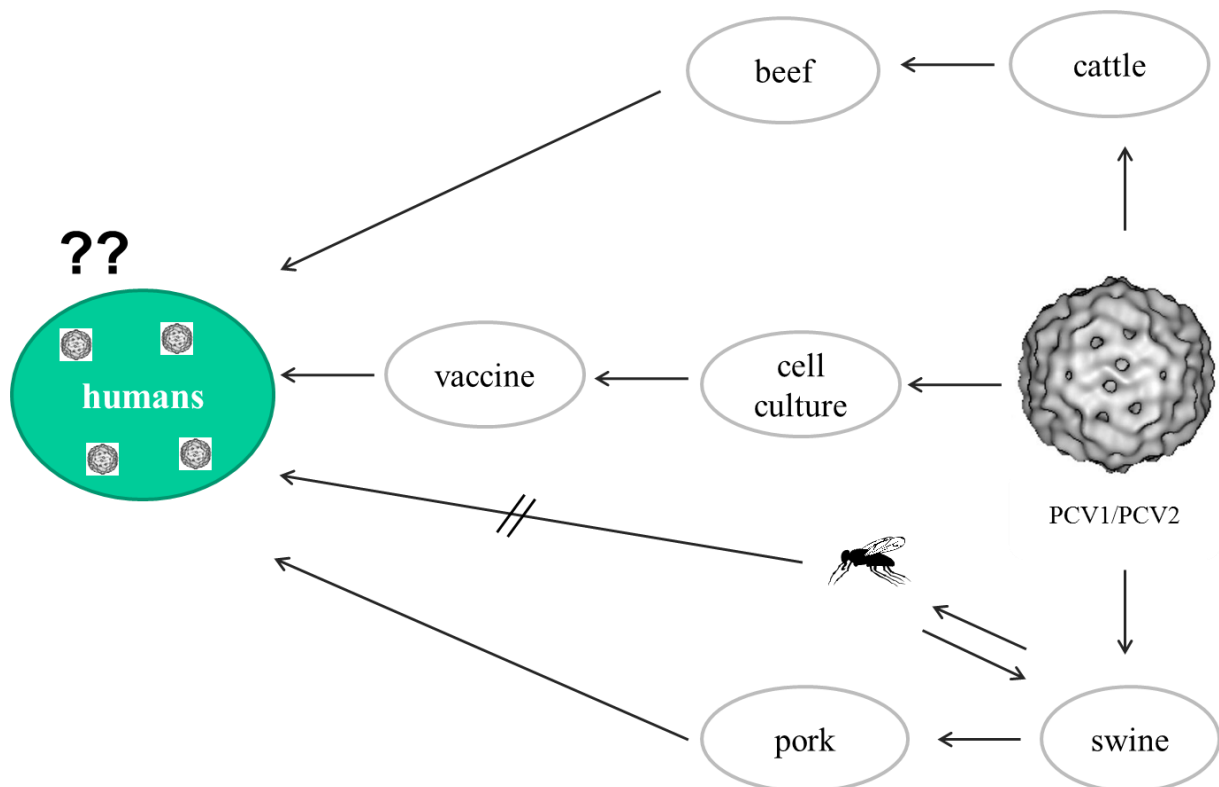
Figure 6: Possible interspecies transmission of PCV.

6 SUMMARY

6   SUMMARY

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Circovirus Infection in Cattle

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Circoviruses are small, non-enveloped, spherical viruses with a circular single-stranded DNA genome of 1.7 to 2.4 kb in size. Porcine circovirus type 2 (PCV2), which belongs to the genus *Circovirus*, has been associated with a number of disease manifestations, today known as porcine circovirus associated disease (PCVAD) in pigs. Circovirus infection in cattle has not been convincingly described so far. Serological investigations on circovirus-specific antibodies led to contradictory results. In 2007, Bovine neonatal pancytopenia (BNP) was reported in Europe with unknown cause. The clinicopathological picture and haemorrhagic condition of BNP strongly resembled chicken infectious anemia, caused by circovirus, in chickens. Therefore, in this study, a broad-spectrum PCR was performed for amplification of circoviral genomic sequences. Circoviral DNA was detected in 5 of 25 BNP-affected calves. The whole viral genome was sequenced. The detected genome has a length of 1768 nucleotides and revealed a high similarity (up to 99%) with PCV2 genotype b (Publication 1). Although the aetiology of BNP was recently described as a result of the transfer of colostral alloantibodies binding to peripheral blood-derived leukocytes and platelet antigens of calves it was important to complete the efforts implemented in the first part regarding the detection of PCV2 genome in cattle to study the susceptibility, and immune response of calves to experimental infection to PCV2.

SUMMARY

For this purpose further 181 samples from calves affected with BNP in Germany were tested using a broad-spectrum PCR. Two out of 181 samples were found positive for PCV2, both from calves with signs typical for BNP. The genomes of the two viruses were comprised of 1768 nt and 1767 nt, respectively (Publication 2).

Furthermore, the susceptibility and immune response of calves to experimental PCV2 inoculation and the possibility to develop a seroconversion after vaccination with a PCV2 commercial vaccine were investigated. PCV2-specific antibodies were detected in the PCV2-immunized animals and in the tissue-culture grown PCV2-infected animals from day 7 and 11 p.i. onwards, respectively. PCV2 was detected by real-time qPCR only in blood samples of the tissue-culture grown PCV2-infected animals and in various tissues (e.g., spleen, lymph nodes, thymus).

The complete genome of the re-isolated PCV2 genome from lymph node of PCV2-infected calves showed an identity of 99.9% compared to the inoculum with presence of possible adaptive mutations of PCV2. Two mutations (C1708T and G365C) did occur during infection. The sequence analysis of the re-isolated PCV2 genome showed a mutation, possibly an adaptive mutation, at amino acid 105 within the *rep* gene (Met to Ile; Publication 3).

In conclusion, the detection of PCV2 genome and experimentally induced seroconversion revealed that host susceptibility of PCV2 is not solely restricted to pigs, and a cross-species transmission of PCV2 between pigs and cattle is possible.

7 ZUSAMMENFASSUNG

7   ZUSAMMENFASSUNG

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Circovirus-Infektion beim Rind

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Schlüsselwörter: Porcine Circovirus Type 2, Bovine Neonatale Panzytopenie, Serokonversion, qPCR

Circoviren sind kleine, unbehüllte Viren mit einem einzelsträngigen zirkulären DNA Genom mit eine Größe von 1,7 bis 2,4 kb. Das Porcine Circovirus Typ 2 (PCV2), welches zum Genus *Circovirus* gehört, ist mit einer Anzahl von Krankheitsmanifestationen verbunden worden, die heute als Porcine Circovirus Assoziierte Krankheiten (PCVAD) zusammengefasst sind. Die PCV2-Infektion bei Rindern ist bis zum jetzigen Zeitpunkt marginal erforscht worden. Serologische Untersuchungen auf Circovirus spezifische Antikörperführten zu widersprüchlichen Ergebnissen. Im Jahr 2007 wurde von der Bovinen Neonatalen Panzytopenie (BNP) in Europa mit unklarer Genese berichtet. Das klinisch - pathologische Bild der Hämorrhagien ähnelte dem Krankheitsbild der Infektiösen Anämie, welche durch ein Circovirus bei Hühnern verursacht wird. Deshalb wurde in dieser Studie eine Breitspektrum PCR zum Nachweis von Cirocvirus-Genomen durchgeführt. In 5 von 25 BNP betroffenen Kälbern konnte circovirale DNA nachgewiesen werden. Das komplette Genom wurde nachfolgend amplifiziert, kloniert und sequenziert. Das nachgewiesene Genom (PCV2-Ha08) hat eine Länge von 1768 Nukleotiden und zeigte eine hohe Homologie (bis zu 99%) mit PCV2-Genotyp b (siehe Publikation 1). Als Ursache der BNP ist vor kurzen die Übertragung von Alloantikörpern über das Kolostrum beschrieben wurden, welche die Zerstörungen von

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Leukozyten und Thrombozyten sowie deren Vorläuferzellen bewirken. Ungeachtet dessen war es wichtig, die Empfänglichkeit und Immunantwort von Kälbern nach experimenteller Infektion mit PCV2 zu studieren. Für diesen Zweck wurden weitere 181 Proben von BNP-Kälbern aus Deutschland mit Hilfe einer Breitspektrum-PCR getestet. In zwei von 181 Proben wurde PCV2 DNA nachgewiesen. Die vollständigen Sequenzen konnten amplifiziert werden. Während das erste Genom aus einer Blutprobe eines Kalbs in Bayern stammte (PCV2-Ha09), stammte das zweite nachgewiesene Genom aus Lunge und Gehirn von einem Kalb in Sachsen (PCV2-Ha10). Das Genom (PCV2-Ha09) besteht aus 1768 nt, währenddessen das Genom (PCV2-Ha10) aus 1767 nt aufgebaut ist (siehe Publikation 2). Weiterhin wurden die PCV2 Empfänglichkeit und die Immunantwort von Kälbern durch experimentellen PCV2 Inokulation sowie die Möglichkeit, eine Serokonversion nach Impfung mit einer kommerziellen PCV2 Vakzin zu entwickeln, untersucht. PCV2-spezifische Antikörper wurden in den PCV2-infizierten Tieren und in den PCV2-immunisierten Tieren im Tag 11 und 7 nach Inokulation (p.i.) nachgewiesen. PCV2-Genome wurden durch quantitative Realtime-PCR zwischen Tag 4 und Tag 46 p.i. nur in den Blutproben sowie in verschiedenen Geweben (z.B. Milz, Lymphknoten, Thymus) der PCV2-infizierten Tiere nachgewiesen. Das Genom, welches von den Lymphknoten der PCV2-infizierten Kälber erneut isoliert wurde, zeigt eine Identität von 99,9% gegenüber dem Inokulum. Dies weist möglicherweise auf adaptierte Mutationen im PCV2 Genom hin. Die Mutationen C1708T und G365C sind während der Infektionen aufgetreten. Die Sequenzanalyse zeigt eine mögliche adaptierte Mutation an der Aminosäure Nr. 105 in Replikationsgen (Met zu Ile) (siehe Publikation 3). Zusammenfassend kann geschlussfolgert werden, dass der Nachweis der PCV2 Genomen und eine experimentell induzierte Serokonversion möglich war. Es konnte gezeigt werden, dass die Empfänglichkeit von PCV2 nicht allein auf Schweine begrenzt ist und eine Übertragung von PCV2 auf Rinder möglich ist.

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9 APPENDIX

9   APPENDIX

Alignment of the re-isolated virus (PCV2_Ha11_KJ920205) from lymph node of PCV2-infected calves and the inoculum virus (PCV2_AFSSA_AY321984).

Appendix I:

Alignment report based on the whole nucleotide sequence (1767 nt) of both viruses.

```
               10        20        30        40        50        60        70
1    ACCAGCGCACTTCGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGG  PCV2_AFSSA_AY321984
1    ACCAGCGCACTTCGGCAGCGGCAGCACCTCGGCAGCACCTCAGCAGCAACATGCCCAGCAAGAAGAATGG  PCV2_Ha11_KJ920205

               80        90       100       110       120       130       140
71   AAGAAGCGGACCCCAACCCCATAAAAGGTGGGTGTTCACTCTGAATAATCCTTCCGAAGACGAGCGCAAG  PCV2_AFSSA_AY321984
71   AAGAAGCGGACCCCAACCCCATAAAAGGTGGGTGTTCACTCTGAATAATCCTTCCGAAGACGAGCGCAAG  PCV2_Ha11_KJ920205

              150       160       170       180       190       200       210
141  AAAATACGGGATCTTCCAATATCCCTATTTGATTATTTTATTGTTGGCGAGGAGGGTAATGAGGAAGGAC  PCV2_AFSSA_AY321984
141  AAAATACGGGATCTTCCAATATCCCTATTTGATTATTTTATTGTTGGCGAGGAGGGTAATGAGGAAGGAC  PCV2_Ha11_KJ920205

              220       230       240       250       260       270       280
211  GAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCAGACTTTTAATAAAGTGAAGTGGTATTT  PCV2_AFSSA_AY321984
211  GAACACCTCACCTCCAGGGGTTCGCTAATTTTGTGAAGAAGCAGACTTTTAATAAAGTGAAGTGGTATTT  PCV2_Ha11_KJ920205

              290       300       310       320       330       340       350
281  GGGTGCCCGCTGCCACATCGAGAAAGCGAAAGGAACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAA  PCV2_AFSSA_AY321984
281  GGGTGCCCGCTGCCACATCGAGAAAGCGAAAGGAACAGATCAGCAGAATAAAGAATACTGCAGTAAAGAA  PCV2_Ha11_KJ920205

              360       370       380       390       400       410       420
351  GGCAACTTACTGATGGAGTGTGGAGCTCCTAGATCTCAGGGACAACGGAGTGACCTGTCTACTGCTGTGA  PCV2_AFSSA_AY321984
351  GGCAACTTACTGAT␊GAGTGTGGAGCTCCTAGATCTCAGGGACAACGGAGTGACCTGTCTACTGCTGTGA  PCV2_Ha11_KJ920205

              430       440       450       460       470       480       490
421  GTACCTTGTTGGAGAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTT  PCV2_AFSSA_AY321984
421  GTACCTTGTTGGAGAGCGGGAGTCTGGTGACCGTTGCAGAGCAGCACCCTGTAACGTTTGTCAGAAATTT  PCV2_Ha11_KJ920205

              500       510       520       530       540       550       560
491  CCGCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACTAATGTACAC  PCV2_AFSSA_AY321984
491  CCGCGGGCTGGCTGAACTTTTGAAAGTGAGCGGGAAAATGCAGAAGCGTGATTGGAAGACTAATGTACAC  PCV2_Ha11_KJ920205

              570       580       590       600       610       620       630
561  GTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTGCAGACCCGGAAACCACAT  PCV2_AFSSA_AY321984
561  GTCATTGTGGGGCCACCTGGGTGTGGTAAAAGCAAATGGGCTGCTAATTTTGCAGACCCGGAAACCACAT  PCV2_Ha11_KJ920205

              640       650       660       670       680       690       700
631  ACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGGTGAAGAAGTGGTTGTTATTGATGACTT  PCV2_AFSSA_AY321984
631  ACTGGAAACCACCTAGAAACAAGTGGTGGGATGGTTACCATGGTGAAGAAGTGGTTGTTATTGATGACTT  PCV2_Ha11_KJ920205

              710       720       730       740       750       760       770
701  TTATGGCTGGCTGCCCTGGGATGATCTACTGAGACTGTGTGATCGATATCCATTGACTGTAGAGACTAAA  PCV2_AFSSA_AY321984
701  TTATGGCTGGCTGCCCTGGGATGATCTACTGAGACTGTGTGATCGATATCCATTGACTGTAGAGACTAAA  PCV2_Ha11_KJ920205

              780       790       800       810       820       830       840
771  GGTGGAACTGTACCTTTTTTGGCCCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACT  PCV2_AFSSA_AY321984
771  GGTGGAACTGTACCTTTTTTGGCCCGCAGTATTCTGATTACCAGCAATCAGACCCCGTTGGAATGGTACT  PCV2_Ha11_KJ920205

              850       860       870       880       890       900       910
841  CCTCAACTGCTGTCCCAGCTGTAGAAGCTCTTTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGC  PCV2_AFSSA_AY321984
841  CCTCAACTGCTGTCCCAGCTGTAGAAGCTCTTTATCGGAGGATTACTTCCTTGGTATTTTGGAAGAATGC  PCV2_Ha11_KJ920205
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                920       930       940       950       960       970       980
911 TACAGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAATTTCCATAT PCV2_AFSSA_AY321984
911 TACAGAACAATCCACGGAGGAAGGGGGCCAGTTCGTCACCCTTTCCCCCCCATGCCCTGAATTTCCATAT PCV2_Ha11_KJ920205

                990      1000      1010      1020      1030      1040      1050
981 GAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCATTAAGGGTTAAGTGGGGG PCV2_AFSSA_AY321984
981 GAAATAAATTACTGAGTCTTTTTTATCACTTCGTAATGGTTTTTATTATTCATTAAGGGTTAAGTGGGGG PCV2_Ha11_KJ920205

               1060      1070      1080      1090      1100      1110      1120
1051 GTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTACACGGATATTGTATTCCTGGTCGTATATA PCV2_AFSSA_AY321984
1051 GTCTTTAAGATTAAATTCTCTGAATTGTACATACATGGTTACACGGATATTGTATTCCTGGTCGTATATA PCV2_Ha11_KJ920205

               1130      1140      1150      1160      1170      1180      1190
1121 CTGTTTTCGAACGCAGTGCCGAGGCCTACGTGGTCTACATTTCCAGCAGTTTGTAGTCTCAGCCACAGCT PCV2_AFSSA_AY321984
1121 CTGTTTTCGAACGCAGTGCCGAGGCCTACGTGGTCTACATTTCCAGCAGTTTGTAGTCTCAGCCACAGCT PCV2_Ha11_KJ920205

               1200      1210      1220      1230      1240      1250      1260
1191 GGTTTCTTTTGTTGTTTGGTTGGAAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAGTAGCG PCV2_AFSSA_AY321984
1191 GGTTTCTTTTGTTGTTTGGTTGGAAGTAATCAATAGTGGAATCTAGGACAGGTTTGGGGGTAAAGTAGCG PCV2_Ha11_KJ920205

               1270      1280      1290      1300      1310      1320      1330
1261 GGAGTGGTAGGAGAAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTGAGG PCV2_AFSSA_AY321984
1261 GGAGTGGTAGGAGAAGGGCTGGGTTATGGTATGGCGGGAGGAGTAGTTTACATAGGGGTCATAGGTGAGG PCV2_Ha11_KJ920205

               1340      1350      1360      1370      1380      1390      1400
1331 GCTGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCACTGGAGCCCACTCCCCTGTCACCCTGGG PCV2_AFSSA_AY321984
1331 GCTGTGGCCTTTGTTACAAAGTTATCATCTAGAATAACAGCACTGGAGCCCACTCCCCTGTCACCCTGGG PCV2_Ha11_KJ920205

               1410      1420      1430      1440      1450      1460      1470
1401 TGATCGGGGAGCAGGGCCAGAATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGCACAGAGCG PCV2_AFSSA_AY321984
1401 TGATCGGGGAGCAGGGCCAGAATTCAACCTTAACCTTTCTTATTCTGTAGTATTCAAAGGGCACAGAGCG PCV2_Ha11_KJ920205

               1480      1490      1500      1510      1520      1530      1540
1471 GGGGTTTGAGCCCCCTCCTGGGGGAAGAAAGTCATTAATATTGAATCTCATCATGTCCACCGCCCAGGAG PCV2_AFSSA_AY321984
1471 GGGGTTTGAGCCCCCTCCTGGGGGAAGAAAGTCATTAATATTGAATCTCATCATGTCCACCGCCCAGGAG PCV2_Ha11_KJ920205

               1550      1560      1570      1580      1590      1600      1610
1541 GGCGTTCTGACTGTGGTTCGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTGAAGATGCCAT PCV2_AFSSA_AY321984
1541 GGCGTTCTGACTGTGGTTCGCTTGACAGTATATCCGAAGGTGCGGGAGAGGCGGGTGTTGAAGATGCCAT PCV2_Ha11_KJ920205

               1620      1630      1640      1650      1660      1670      1680
1611 TTTTCCTTCTCCAGCGGTAACGGTGGCGGGGGTGGACGAGCCAGGGGCGGCGGCGGAGGATCTGGCCAAG PCV2_AFSSA_AY321984
1611 TTTTCCTTCTCCAGCGGTAACGGTGGCGGGGGTGGACGAGCCAGGGGCGGCGGCGGAGGATCTGGCCAAG PCV2_Ha11_KJ920205

               1690      1700      1710      1720      1730      1740      1750
1681 ATGGCTGCGGGGGCGGTGTCTTCTTCTCCGGTAACGCCTCCTTGGATACGTCATATCTGAAAACGAAAGA PCV2_AFSSA_AY321984
1681 ATGGCTGCGGGGGCGGTGTCTTCTTCTTCGGTAACGCCTCCTTGGATACGTCATATCTGAAAACGAAAGA PCV2_Ha11_KJ920205

               1760
1751 AGTGCGCTGTAAGTATT                                                       PCV2_AFSSA_AY321984
1751 AGTGCGCTGTAAGTATT                                                       PCV2_Ha11_KJ920205
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Appendix II:

Alignment report of the *rep* gene based on the amino acid sequence (314 amino acid) of PCV2 detected in cattle, beef and human including the isolates from this study (Ha08, Ha09, Ha10 and Ha11) compared to the reference isolates (PCV2a, PCV2b and PCV2c).

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                  10        20        30        40        50        60
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG  PCV2a_AF055392
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2b_AF055394
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG  BCV_canada_AF109397
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_AFSSA_AY321984
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_beef3_HQ738640
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_Ha08_FJ804417
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_Ha09_HQ231329
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_Ha10_HQ231328
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_Ha11_KJ920205
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRDLPISLFDYFIVGEEGNEEGRTPHLQG  PCV2_human_GQ404852
1    MPSKKNGRSGPQPHKRWVFTLNNPSEDERKKIRELPISLFDYFIVGEEGNEEGRTPHLQG  PCV2c_EU148503


                  70        80        90        100       110       120
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  PCV2a_AF055392
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLMECGAPRSQGQRSDLS  PCV2b_AF055394
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  BCV_canada_AF109397
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLMECGAPRSQGQRSDLS  PCV2_AFSSA_AY321984
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  PCV2_beef3_HQ738640
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  PCV2_Ha08_FJ804417
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  PCV2_Ha09_HQ231329
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  PCV2_Ha10_HQ231328
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLIECGAPRSQGQRSDLS  PCV2_Ha11_KJ920205
61   FANFVKKQTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNILIECGAPRSQGQRSDLS  PCV2_human_GQ404852
61   FANFVKKKTFNKVKWYLGARCHIEKAKGTDQQNKEYCSKEGNLLMECGAPRSQGQRSDLS  PCV2c_EU148503


                  130       140       150       160       170       180
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2a_AF055392
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2b_AF055394
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  BCV_canada_AF109397
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_AFSSA_AY321984
121  TAVSTLLESGSLATVAEQYPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_beef3_HQ738640
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_Ha08_FJ804417
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_Ha09_HQ231329
121  TAVSTLLASGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_Ha10_HQ231328
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_Ha11_KJ920205
121  TAVSTLLETGSLVTVAEQEPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2_human_GQ404852
121  TAVSTLLESGSLVTVAEQHPVTFVRNFRGLAELLKVSGKMQKRDWKTNVHVIVGPPGCGK  PCV2c_EU148503
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                190       200       210       220       230       240
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2a_AF055392
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2b_AF055394
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK BCV_canada_AF109397
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2_AFSSA_AY321984
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2_beef3_HQ738640
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLGLCDRYPLTVETK PCV2_Ha08_FJ804417
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2_Ha09_HQ231329
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2_Ha10_HQ231328
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2_Ha11_KJ920205
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2_human_GQ404852
181 SKWAANFADPETTYWKPPRNKWWDGYHGEEVVIDDFYGWLPWDDLLRLCDRYPLTVETK PCV2c_EU148503

                250       260       270       280       290       300
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2a_AF055392
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2b_AF055394
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT BCV_canada_AF109397
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_AFSSA_AY321984
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_beef3_HQ738640
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_Ha08_FJ804417
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_Ha09_HQ231329
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_Ha10_HQ231328
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_Ha11_KJ920205
241 GGTVPFLDRSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKNATEQSTEEGGQFVT PCV2_human_GQ404852
241 GGTVPFLARSILITSNQTPLEWYSSTAVPAVEALYRRITSLVFWKTATEQSTEEGGQFVT PCV2c_EU148503

                310
301 LSPPCPEFPYEINY                                              PCV2a_AF055392
301 LSPPCPEFPYEINY                                              PCV2b_AF055394
301 LSPPCPEFPYEINY                                              BCV_canada_AF109397
301 LSPPCPEFPYEINY                                              PCV2_AFSSA_AY321984
301 LSPPCPEFPYEINY                                              PCV2_beef3_HQ738640
301 LSPPCPEFPYEINY                                              PCV2_Ha08_FJ804417
301 LSPPCPEFPYEINY                                              PCV2_Ha09_HQ231329
301 LSPPCPEFPYEINY                                              PCV2_Ha10_HQ231328
301 LSPPCPEFPYEINY                                              PCV2_Ha11_KJ920205
301 LSPPCPEFPYEINY                                              PCV2_human_GQ404852
301 LSPPCPEFPYEINY                                              PCV2c_EU148503
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