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1 Retrospective study on the occurrence of porcine circovirus 2 infection and associated entities
2 in Northern Germany

3

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27 **Abstract**

28 Porcine circovirus type 2 (PCV 2) represents a widespread, globally occurring pathogen
29 with an increasing number of associated entities. To further elucidate the origin, spread and
30 pathogenesis of PCV2 and associated changes archived material of pigs originating from
31 Northern Germany and submitted for necropsy between 1961 to 1998 were investigated by
32 using *in situ* hybridisation and polymerase chain reaction. PCV2 was first detected in a pig
33 from 1962. However, incidence of detectable viral DNA and occurrence of PCV2-associated
34 lesions varied substantially in the following years. The overall incidence of PCV2 infection
35 was low between 1961 and 1984 (0 to 11.7 %) and increased between 1985 and 1998 (14.3 to
36 53.3 %). PCV2-associated pathological changes including postweaning multisystemic wasting
37 syndrome (PMWS) and **most likely** porcine dermatitis and nephropathy syndrome (PDNS)
38 were first observed in 1985. Selected sequence analyses of PCV2 DNA segments revealed
39 high homology with current virus strains. **In summary**, findings showed that PCV2 has been
40 present in the pig population in Northern Germany since 1962. This represents worldwide the
41 earliest report about the detection of the PCV2 genome in pigs. Associated lesions such as
42 PMWS and PDNS were not observed before 1985, indicating that virus infection alone does
43 not seem to be sufficient enough to trigger the development of associated entities. Limited
44 sequence analysis revealed no changes in the viral genome thus suggesting that other factors
45 including environmental changes or co-infections with other agents might play a contributing
46 role in the altered virulence of this pathogen and the occurrence of PCV2-associated lesions.

47

48 **Keywords**

49 Porcine circovirus type 2; *in situ* hybridisation; retrospective study; postweaning
50 multisystemic wasting syndrome; porcine dermatitis and nephropathy syndrome

51

52 1. Introduction

53 Porcine circoviruses (PCV), members of the family *Circoviridae*, are composed of non-
54 enveloped viruses with a single-stranded circular DNA (**ICTVdB Management, 2006**). Two
55 genetically and antigenically different viruses, PCV1 and PCV2, can be distinguished. PCV1
56 was first described 1974 as a contaminant of a porcine kidney cell line (PK 15) and is
57 regarded as being nonpathogenic (Tischer et al. 1986). In 1991 a “postweaning multisystemic
58 wasting syndrome” (PMWS) was reported in Canadian pigs (Harding and Clark, 1998). PCV2
59 was isolated in association with PMWS and the Koch’s postulates were fulfilled (Ellis et al.,
60 1998; Ellis et al., 1999). In the late 1990s, reports of similar disease outbreaks in several
61 countries including France and Spain followed and both diseases as well as PCV2 infection
62 are now found worldwide (Segalés et al., 2005). The first isolation of PCV2 in Germany in
63 association with PMWS was reported in 1998 (Hinrichs et al., 1999). PCV2 is nowadays
64 highly prevalent in swine herds worldwide and infection is often subclinical (Harding et al.,
65 2004). Recent studies indicate the presence of at least two PCV2 genotypes associated with
66 different pathogenic potential (Grau-Roma et al., 2008). However, PCV2 has also been
67 associated with various clinical disease syndromes in swine including PMWS, porcine
68 dermatitis and nephropathy syndrome (PDNS) as well as enteritis, proliferating and
69 necrotising pneumonia (PNP), encephalopathy, congenital tremor and reproductive disorders
70 (Ellis et al., 1999; Harding et al., 2004; Segalés et al., 2004). PMWS is characterised
71 clinically by wasting, dyspnoea, and lymphadenopathy and maybe associated with diarrhoea,
72 pallor, and jaundice (Harding et al., 2004; Rosell et al., 1999). The most prominent
73 histological lesions in PMWS occur in lymphoid organs and consist of extensive lymphocytic
74 depletion, infiltration of macrophages, few multinucleated giant cells, and botryoid basophilic
75 cytoplasmic inclusion bodies. PCV2 antigen and DNA has been demonstrated by
76 immunohistochemistry and/or *in situ* hybridisation (ISH) in various organs in the cytoplasm
77 of macrophages, multinucleated giant cells, dendritic cells in lymphoid tissues, alveolar

78 macrophages, Kupffer cells, and, to a lesser extent, epithelial cells in the lungs, liver,
79 pancreas, and kidneys. Endothelial cells in several organs, including lymph nodes and spleen,
80 are rarely positive for PCV2 (Opriessnig et al., 2006, Rosell et al., 1999). Although vascular
81 lesions are not prominent in PMWS, vasculitis is the hallmark of PDNS (Segalés et al., 2004).
82 Lesions in PDNS are characterised primarily by a cutaneous and subcutaneous necrotising
83 vasculitis as well as glomerulonephritis and are likely to be mediated by immune complex
84 depositions (Sierra et al., 1997). The responsible antigen or antigens triggering this immune
85 complex-mediated disorder is currently unknown. A wide spectrum of factors including
86 drugs, chemicals, food allergens, endogenous antigens, and infectious agents must be
87 considered (Drolet et al. 1999). At present, the role of infectious agents has been suspected
88 more than other factors; among them, PCV2 and porcine respiratory and reproductive
89 syndrome virus (PRRSV) are considered the most likely causative agents (Rovira et al., 2002;
90 Segalés et al., 2004; Thibault et al., 1998). However, to date PCV2 antigen has not been
91 detected within PDNS vascular lesions (Segalés et al., 2004).

92 The increasing reports of new PCV2 associated lesions and syndromes including
93 enteritis, PNP, encephalopathy, cardiovascular lesions, reproductive disorders, and congenital
94 tremor raised the question, whether these findings represent truly new emerging entities
95 directly or indirectly associated with PCV2 infection (Opriessnig et al., 2006; Seeliger et al.,
96 2007; Segalés et al., 2004).

97 **The aim of this study was** to determine the presence of PCV2 and associated lesions in
98 archived paraffin-embedded material prior to the first report from 1998 in Northern Germany.

99

100 **2. Material and methods**

101 **2.1. Case selection**

102 Out of 18,855 necropsied pigs originating from Northern Germany between 1998, the
103 year of the first description of PMWS in Germany, and 1961 (excluding the years 1987-1990

104 and 1992-1995), 445 animals, at least 10 pigs per year, were randomly selected for the present
105 study. Material earlier than 1961 was unfortunately not available in the department archive.
106 The criteria for selection included age (between 4 and 16 weeks), weight (under 50 kg),
107 clinical signs, as well as gross and histological findings **such as** lymph node enlargement,
108 lymphocytic depletion in lymphoid tissues, interstitial pneumonia, and interstitial nephritis. In
109 addition, the availability of formalin-fixed and paraffin-embedded tissues of the lymph nodes,
110 spleen, ileum, lung or tonsil was a further requirement. **However, not all tissues were**
111 **available in each case.**

112 Results of previous virological investigations including classical swine fever virus
113 (CSFV) and porcine herpesvirus 1 (PHV-1, Aujeszky's disease) were included. Haematoxylin
114 and eosin (HE) stained sections from all available organs were investigated for
115 histopathological changes.

117 **2.2. *In situ* hybridisation**

118 DNA *in situ* hybridisation (ISH) to detect PCV2 and PCV1 DNA was carried out as
119 described (Rosell et al., 1999; Seeliger et al., 2007). Digoxigenin-labeled, oligonucleotide
120 DNA-probes of 41 basepairs (bp) was designed based on the sequence of the PCV2 and
121 PCV1 open reading frame 1 (ORF1; GenBank accession number AF027217 and NC001792,
122 respectively; Tab. 1). Briefly, tissue sections were dewaxed in xylene, hydrated in graded
123 ethanol and washed in ultrapure, pyrogen-free, DEPC-treated water. After proteolytic
124 digestion, postfixation, acetylation, and prehybridisation, hybridisation was performed
125 overnight in a moist chamber at 56°C with a probe concentration of 100 ng/ml. The detection
126 system consisted of an anti-DIG antibody conjugated with alkaline phosphatase and the
127 substrates nitroblue tetrazoliumchloride (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate
128 (BCIP, X-phosphate), which yielded a bluish precipitate. Formalin-fixed and paraffin-
129 embedded pellets from PK15 cells either infected or non-infected with PCV2 or PCV1 as well

130 as a PCV2 positive and non-infected juvenile pig served as positive and negative controls
131 (Seeliger et al., 2007).

132

133 **2.3. Polymerase chain reaction and sequencing**

134 For detecting the PCV2 genome, DNA was isolated from formalin-fixed and paraffin-
135 embedded tissue using E.Z.N.A® Tissue DNA Mini Kit (PeqLab Biotechnology, Germany)
136 according to the manufacturer's instructions. Additionally, polymerase chain reaction (PCR)
137 using three different primer pairs was performed as described (Ellis et al., 1999; Kim and
138 Chae, 2001). The first two primer pairs targeted amplicons of 481 bp and 225 bp length of the
139 ORF2 and the third pair generated an amplicon of an expected length of 66 bp localised
140 within the ORF1 (Tab. 1). For the latter an optimised protocol with more stringent conditions
141 was applied to exclude PCV1 detection. Hereby, an initial denaturation step for 4 minutes at
142 94°C, followed by 39 cycles denaturation at 94°C for 1 minute, annealing at 56°C for 50
143 seconds, and elongation at 72°C for 30 seconds was used. The reaction ended with a final
144 extension step at 72°C for 10 minutes. Amplicons were visualised by standard gel
145 electrophoresis on a 2% agarose gel. Positive DNA fragments of specific size were cut out
146 from the gel, purified by using a commercial kit (Nucleobond, Macherey & Nagel, Germany)
147 and submitted for sequencing. The latter was performed according to the method of Sanger
148 with fluorescent-labelled BigDye Terminator sequencing kit using automatic sequencers
149 (Applied Biosystems, USA) by an external laboratory (SEQLAB, Germany). Sequences were
150 analysed with LAGLIN online version (<http://www.ch.embnet.org>) to confirm the identity of
151 the obtained sequences. The CLUSTAL W online version
152 (<http://www.ebi.ac.uk/clustalw/index.html>) for comparative alignments was used and data
153 were submitted to DDBJ/EMBL/GenBank database (GenBank accession numbers EU158773,
154 EU158774, EU158775). Formalin-fixed and paraffin-embedded pellets from PK15 cells

155 either infected or non-infected with PCV2 or PCV1 as well as a PCV2 positive and non-
156 infected juvenile pig served as positive and negative controls (Seeliger et al., 2007).

157 Statistical analysis for a correlation between detection of PCV2 DNA and histological
158 lesions was performed using Fisher's exact test with significance at $p \leq 0.05$.

159

160 **3. Results**

161 **3.1. Case selection**

162 The selection criteria, met by 445 randomly selected animals, resulted in the
163 investigation of about 10 to 21 pigs per year (on average 15) with a mean body weight of
164 21.9 kg (ranging from 2.5 to 50 kg) and a mean age of 9.7 weeks (ranging from 4 to
165 16 weeks). However, not all organs were available for each case (lung: $n = 305$; liver: $n =$
166 157 ; lymph node: $n = 138$; spleen: $n = 103$; intestine: $n = 102$; kidney: $n = 89$; brain: $n = 49$;
167 heart: $n = 29$; tonsils: $n = 2$).

168

169 **3.2. *In situ* hybridisation**

170 Among these 445 pigs, 37 revealed positive hybridisation signals for PCV2 in at least
171 one organ. The earliest detection of PCV2 in tissue was in the year 1962 with **a few positive**
172 **cells being detected in the spleen and lung tissue** (Fig. 1) followed by a positive animal
173 from the year 1964. Only 2.77 % (ranging from 0 to 11.7 %) of the animals were positive for
174 PCV2 (10 of 361 pigs) between 1961 and 1984. Surprisingly, in the years 1985 to 1998 the
175 percentage of infected animals increased (27 out of 84 pigs, 32.14 %, ranging from 14.3 % to
176 53.3 %) (Fig. 2). No animal showed positive hybridisation signals with the PCV1-specific
177 probe. PK15 cells infected with PCV1 and PCV2, non-infected cells and various tissues from
178 control pigs showed positive and negative results as expected.

179 Hybridisation signals were most frequently found within the lung (24 out of 305;
180 7.87 %), followed by the intestine (13 out of 102; 12.75 %), lymph node (12 out of 138;

181 8.70 %), spleen (8 out of 103; 7.77 %), kidney (8 out of 89; 8.99 %), liver (6 out of 157;
182 3.82 %), heart (3 out of 29; 10.34 %), brain (3 out of 49; 6.12 %) and tonsil (1 out of 2;
183 50 %). PCV2 DNA was found most frequently in the lymph node (12 out of 12; 100 %), heart
184 (3 out of 3; 100 %), lung (24 out of 29; 82.7 %), intestine (11 out of 14; 78.6 %) and spleen (8
185 out of 11; 72.7 %). Overall, fewer PCV2 positive organs were found between 1961 and 1984
186 compared to 1985 to 1998 (Fig. 3). The signals were found predominantly within
187 macrophages varying from single to numerous positive cells. Other cells with positive
188 hybridisation signals include alveolar macrophages, endothelial cells of blood vessels,
189 fibroblasts and -cytes, Kupffer cells, hepatocytes, and tubular epithelial cells of the kidney.

190

191 3.3. Histology

192 Microscopically, PCV2 positive as well as PCV2 negative animals exhibited a variety of
193 pathological lesions including lymphohistiocytic interstitial pneumonia, suppurative
194 bronchopneumonia, lymphocytic depletion in the spleen and lymph nodes, infiltration of
195 lymph nodes with histiocytes, lympho-histiocytic interstitial hepatitis and nephritis as well as
196 necrotising and fibrinous glomerulonephritis and necrotising dermatitis.

197 Fisher's exact test revealed that interstitial pneumonia (*rightsided-p-*
198 values (p) = 0.0037), hepatitis ($p < 0.0001$) and nephritis ($p < 0.0001$), infiltration of lymph
199 nodes with histiocytes ($p = 0.0128$), and necrotising and fibrinous glomerulonephritis
200 ($p = 0.007$) were found significantly more frequently in PCV2-infected pigs
201 ($p \leq 0.05$ = significant). Only interstitial hepatitis and nephritis were significantly associated
202 with PCV2 infection prior to 1985 (Fig. 4). However, no virus was detectable in the lesions
203 within these kidneys, though some animals exhibited PCV2 DNA intralesionally in the liver.
204 Other PCV2 associated lesions as described for PDNS or PMWS were not found in pigs
205 positive for PCV2 prior to 1985. In contrast, lymphocytic depletion, histiocytic infiltration,
206 cytoplasmic inclusion bodies, multinucleated giant cells and interstitial pneumonia were

207 observed in pigs infected with PCV2 from 1985 onwards. Four of these animals displayed
208 changes characteristic for PMWS with intralesional detection of PCV2. The first animal
209 originated from 1985, one from the year 1996 and two from the year 1998 (Fig. 5).
210 Additionally, three animals exhibited lesions resembling PDNS showing ulcerative dermatitis
211 with vasculitis, and fibrinous and necrotising glomerulonephritis (Fig. 6). Though only few or
212 no cells were positive for PCV2 within the lesions, viral DNA was detected within the lymph
213 nodes, lungs and spleen of these animals. The first animal displaying such alterations also
214 originated from 1985 and the other two from the year 1998.

215

216 **3.4. Polymerase chain reaction and sequencing**

217 In order to substantiate the ISH findings, PCR for PCV2 and PCV1 with different
218 primer pairs was performed. The PCR specific for PCV2 revealed 481 and 225 bp long
219 amplicons in pigs from the year 1985 onwards. Unfortunately, this PCR design failed to
220 confirm the ISH results prior to the year 1985 probably due to storage-related DNA damage.
221 However, using a third primer pair generating a short 66 bp long amplicon the ISH results
222 could be confirmed including the case from 1962. Sequence analysis of the amplicons of the
223 isolates from 1962 (GenBank accession number EU158774) and 1967 (GenBank accession
224 number EU158775) revealed 98 % and 100 % homology to current PCV2 isolates (GenBank
225 accession number AF027217), respectively. Additionally, sequence analysis of the amplicon
226 of the isolate from 1985 (GenBank accession number EU 158773) revealed 100 % identity to
227 current PCV2 isolates (GenBank accession number AY325515).

228

229 **3.5 Additional results**

230 Two of the 37 PCV2 positive pigs exhibited a concurrent infection with the classical
231 swine fever virus (CSFV). In one animal from the year 1967 the diagnosis was confirmed by
232 immunofluorescence testing and in the other animal from the year 1985 by virus isolation.

233 The animal from the year 1967 exhibited only mild lesions characterised by infiltration of
234 plasma cells and eosinophilic granulocytes in the lamina propria of the intestine, a mild
235 lympho-histiocytic periportal infiltration in the liver and a mild infiltration with neutrophilic
236 granulocytes in the lymph node. In the latter a mild to moderate amount of PCV2 positive
237 macrophages was detected. The animal from the year 1985 showed a mild vasculitis in the
238 leptomeninx and the spleen, severe lymphocytic depletion in the spleen and lymph node, mild
239 interstitial nephritis and severe necrotising lymphadenitis with PCV2 characteristic
240 cytoplasmic basophilic inclusion bodies. PCV2 was detectable in all organs predominantly in
241 macrophages with numerous positive cells in spleen and lymph node (Fig. 5). This animal
242 represented the first pig with an unusual high PCV2 DNA load at that time point. Similar
243 amounts were only found in pigs of the late 1990s. No animal was infected with PHV-1.

244

245 **4. Discussion**

246 This retrospective study on the occurrence of PCV2 and associated lesions revealed
247 three significant findings: (i) PCV2 infection has been present in the swine population in
248 Northern Germany at least since 1962, (ii) single cases of PMWS and PDNS had already been
249 observed in 1985, and (iii) associated lesions were not detectable prior to 1985. Though PCR,
250 using various primer pairs, confirmed PCV2 infection by demonstrating a 481 and a 225 bp
251 long amplicon of the ORF2 in the samples collected between 1985 and 1998, the same
252 protocol failed to confirm the results of the ISH prior to the year 1985. This may be due to a
253 time-dependent increased DNA-degradation in formalin-fixed and paraffin-embedded tissues
254 (Grierson et al., 2004; Lisowski et al., 2001). Though the PCR with the primer pair for the
255 amplification of a 66 bp long amplicon of the ORF1 was successful, the obtained amplicon
256 could have also originated from PCV1. The primers possess 100% and 80% identity to the
257 PCV1 genome, respectively. The specificity of the PCR for PCV2 was ensured by changing
258 the annealing temperature allowing the detection of PCV2 but not of PCV1 in infected cells

259 (Ishii and Fukui, 2001). The obtained sequences displayed a 98-100 % homology to other
260 PCV2 strains. However, obtained sequences were too short for a concluding statement
261 whether differences in tissue distribution and pathological findings are related to different
262 PCV 2 strains obtained in various decades. Recent studies revealed two PCV 2 genotypes
263 based on differences in the ORF2 **with PCV2 genotype 1 possibly being more pathogenic**
264 **than PCV2 genotype 2** (Grau-Roma et al., 2008). In addition, a Danish study showed a
265 temporally genotypic shift of PCV2 within a pig population from a third PCV2 genotype,
266 termed PCV 2 genotype 3, via the known PCV2 genotype 2 towards PCV2 genotype 1
267 (Dupont et al., 2008). Unfortunately, in the present study used primers were not able to detect
268 PCV2 within tissues obtained prior to 1985. Therefore, possible differences with respect to
269 different PCV 2 genotypes could not be investigated. The PCV2 sequence from a pig from
270 1985 displayed a sequence similar to PCV2 genotype 2 (primer pair p285/p286; GenBank
271 accession number EU 158773).

272 The year 1962 represented the earliest confirmed detection of PCV 2 worldwide.
273 Although tissue samples collected before 1961 were not available, it seems reasonable to
274 assume that PCV2 was already present in the pig population prior to this time point.

275 In previous investigations the earliest detection of PCV2 in archived formalin-fixed
276 material originated from the 1970s in the UK using TaqMan®-PCR and
277 immunohistochemistry; however, no information was provided as to whether tissues
278 displayed histological lesions (Grierson et al., 2004). In Swiss archived material the earliest
279 PCV2 infection was found in 1986 by using immunohistochemistry with the earliest
280 histopathological lesions typical for PMWS in 1986 (Staebler et al., 2005). Similarly, a study
281 from Spain revealed the presence of PCV2 in archived material from 1985 onwards, and the
282 occurrence of typical PMWS lesions as early as 1986 (Rodriguez-Arriola et al., 2003).

283 The first animal showing light microscopic characteristics of PMWS originated from
284 1985 in the present study; at least 10 years prior the first reported description of this entity in

285 Germany (Hinrichs et al., 1999). In Canada the disease has been known since the early 1990s,
286 but the association to PCV2 was first reported in 1998 (Ellis et al., 1998).

287 Two pigs infected with PCV2 exhibited a co-infection with CSFV. One animal from
288 1985 revealed a high virus load of PCV2 in various organs. Lesions consisting of vasculitis,
289 lymphadenitis with botryoid cytoplasmic inclusion bodies, and interstitial nephritis were
290 present. These pathologic changes differed substantially from other PCV2-infected pigs
291 during this time period. The altered type of lesions indicates that coinfection with CSFV
292 seemed to play a substantial role in the development of increased PCV2-associated changes.
293 Though pathological alterations in CSF include vascular changes with lymphocytic
294 perivascular cuffing (Jones et al., 1997), it remains unclear whether observed lesions are due
295 to the infection with CSFV or PCV2 or a combination of both. However, the high virus load
296 of PCV2 indicates an exacerbation of the infection in combination with CSF.

297 The presented data show a sudden increase in the incidence of PCV2 infection from
298 2.77% to 32.14% between 1962-1984 and 1985-1998, respectively. It remains unclear which
299 factors contributed to this increased appearance of PCV2 infection and associated lesions. The
300 first reports of PCV2 and associated lesions occurred almost simultaneously on both sides of
301 the Atlantic (Ellis et al., 1998; Hinrichs et al., 1999). This suggests that PCV2 was already
302 within the pig population for a long time, and possible similar or different changes may have
303 contributed to the increased virulence of PCV2 on both continents.

304 It is well established that various risk factors contribute to exacerbating PCV2 infection
305 and the development of associated lesions. These include infections with PPV, PRRSV, swine
306 influenza virus, and *Mycoplasma (M.) hyopneumoniae* as well as vaccination against PRRS
307 and mycoplasma (Elbers et al., 2006; Ellis et al., 2004). In 1984 there was an outbreak of CSF
308 predominantly in the northern part of Germany leading to a large-scale vaccination to control
309 the epidemic (Pittler et al., 1986). Whether the epidemic infection as well as the vaccination
310 had an impact on PCV2 incidence and pathogenesis remains speculative but must be

311 considered. Whether vaccination against *M. hyopneumoniae*, commercially available since
312 1992, also played a role in the increased PCV2 prevalence and lesion development remains
313 speculative. In the same time period other nowadays important swine diseases were noticed
314 for the first time including PRRS (Dea et al., 1992; Wensvoort et al., 1991) or increased in
315 prevalence such as proliferative enteropathy due to *Lawsonia intracellularis* (Kroll et al.,
316 2005).

317 In summary, the presented data revealed that PCV 2 infection could be detected as
318 early as 1962 in pigs and associated lesions such as PDNS and PMWS were already observed
319 in 1985. Whether increased virus spread and pathogenicity are due to environmental factors,
320 changes in the genetic make-up of the virus or host or due to immunomodulatory effects
321 needs to be investigated in further studies.

322

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327

328

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435 **Table 1: Primers and probes used for detecting PCV2 and PCV1 DNA from archived material**

Designation	Reference	Sequence (5' to 3')	length	Genome position
p196 – PCV2 for	Ellis et al. (1999)	CGGATATTGTAGTCCTGGTCG	481 bp	1094-1114 ^a
p197 – PCV2 rev		ACTGTCAAGGCTACCACAGTCA		1570-1549 ^a
p285 – PCV2 for	Kim and Chae (2001)	GATTGTATGGCGGGAGGAGT	225 bp	1286-1305 ^a
p286 – PCV2 rev		ATTGACGACTTTGTTCCCCC		1510-1491 ^a
p325 – PCV2 for	(this work)	GCACCCTGTAACGTTTGTC A	66 bp	464-483 ^a
p327 – PCV2 rev		ATTTTCCCGCTCACTTTCAA		529-510 ^a
PCV2 probe	Seeliger et al. (2007)	CCTTCCTCATTACCCTCCTCGCCAACAATAAAATAATCAAA	41 bp	208-168 ^a
PCV1 probe	Seeliger et al. (2007)	CCCTCTTCCAAACCTTCCTCTCCGCAAACAAAATAATCAAA	41 bp	195-155 ^b

436 ^aGenBank accession number: AF 027217437 ^bGenBank accession number: NC 001792

438 for = forward

439 rev = reverse

440

441 **Figure Caption**

442 **Fig. 1:** Positive PCV2 *in situ* hybridisation (ISH) signals in the lung of a pig from 1962
443 (DNA ISH, bar = 200 µm). Insert: Positive PCV2-ISH signal in the spleen of the
444 same animal (DNA ISH, bar = 50 µm).

445
446 **Fig. 2:** Percentage of PCV2 positive animals between 1961 and 1998 (excluding the years
447 1987-1990 and 1992-1995).

448
449 **Fig. 3:** Percentage of PCV2 *in situ* hybridisation positive organs of PCV2-infected
450 animals divided into early investigated period (1961-1984), late investigated
451 period (1985-1998) and total period (1961-1998).

452
453 **Fig. 4:** Percentage of virus associated lesions in PCV2 positive pigs: White columns, time
454 period 1961-1984; black columns, time period 1985-1998; PNP = proliferative-
455 necrotising pneumonia; IB, inclusion bodies; Inn., lymph nodes; inf., infiltration;
456 eos., eosinophilic granulocytes; GN, glomerulonephritis.

457
458 **Fig. 5:** **Lymph node** from a pig from 1985 (co-infected with classical swine fever virus)
459 displaying lesion characteristic for postweaning multisystemic wasting syndrome
460 (PMWS): A. severe lymphocytic depletion and multinucleated giant cells (arrows,
461 HE, bar = 40 µm); B. botryoid cytoplasmic inclusions (HE, bar = 20 µm); C.
462 lymphocytic depletion and high PCV2 load (DNA *in situ* hybridisation,
463 bar = 50 µm).

464
465 **Fig. 6:** **Skin (A, B) and kidney (C)** from a pig from 1985 with lesions characteristic for
466 porcine dermatitis and nephropathy syndrome (PDNS): A. Skin with ulcerative

467 dermatitis and vasculitis; e = epidermis; c = corium (HE, bar = 200 μ m); B. detail
468 of A. with lympho-histiocytic vasculitis; L = vessel lumen; w = vessel wall (HE,
469 bar = 45m); C. Severe fibrinous and necrotising glomerulonephritis and moderate
470 lymph-histiocytic infiltrates in the adjacent stroma (HE, bar = 50 μ m).
471

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Table 1: Primers and probes used for detecting PCV2 and PCV1 DNA from archived material

Designation	Reference	Sequence (5' to 3')	length	Genome position
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PCV1 probe	Seeliger et al. (2007)	CCCTCTTCCAAACCTTCCTCTCCGCAAACAAAATAATCAAA	41 bp	195-155 ^b

^aGenBank accession number: AF 027217

^bGenBank accession number: NC 001792

for = forward

rev = reverse

Figure 1

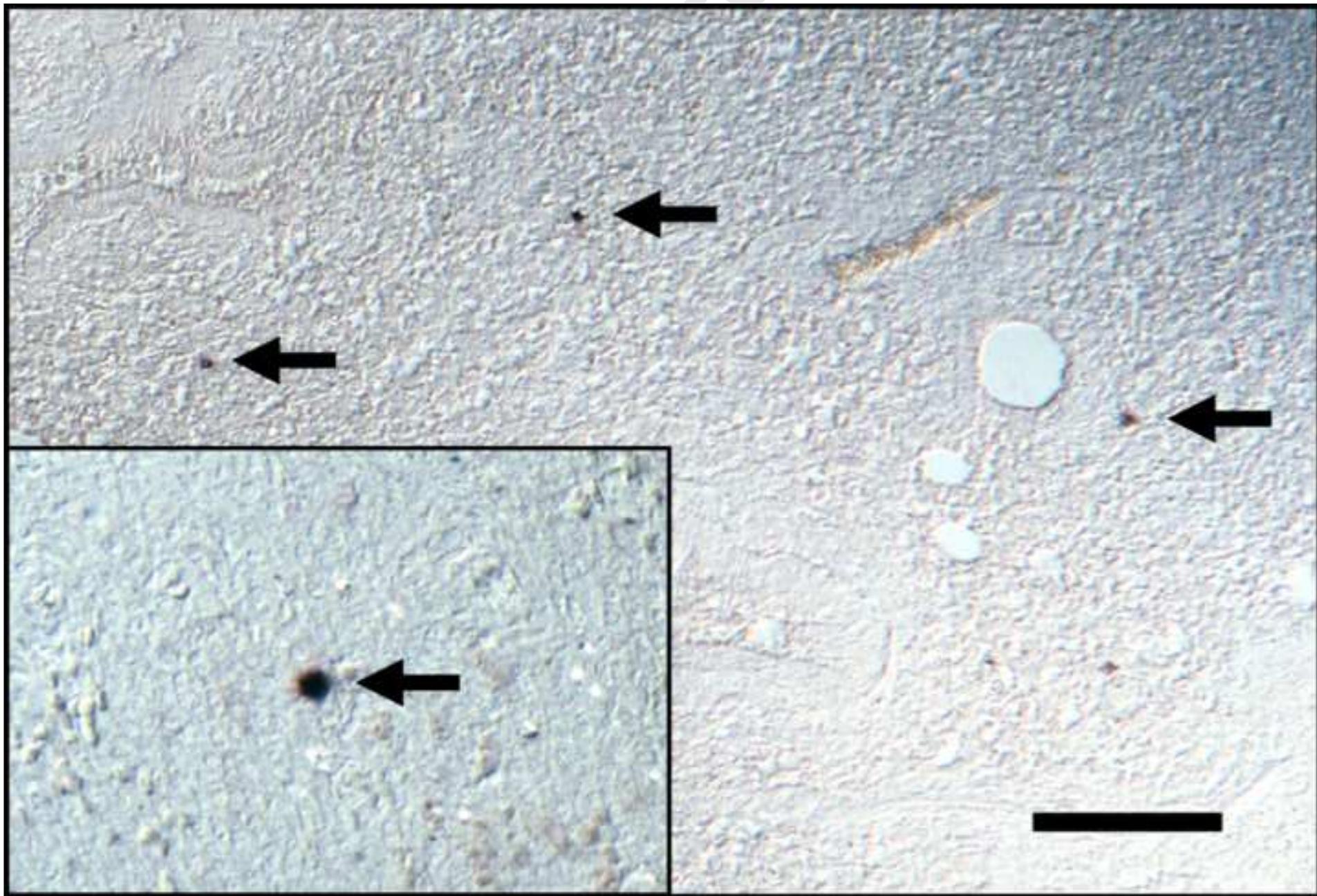


Figure 2

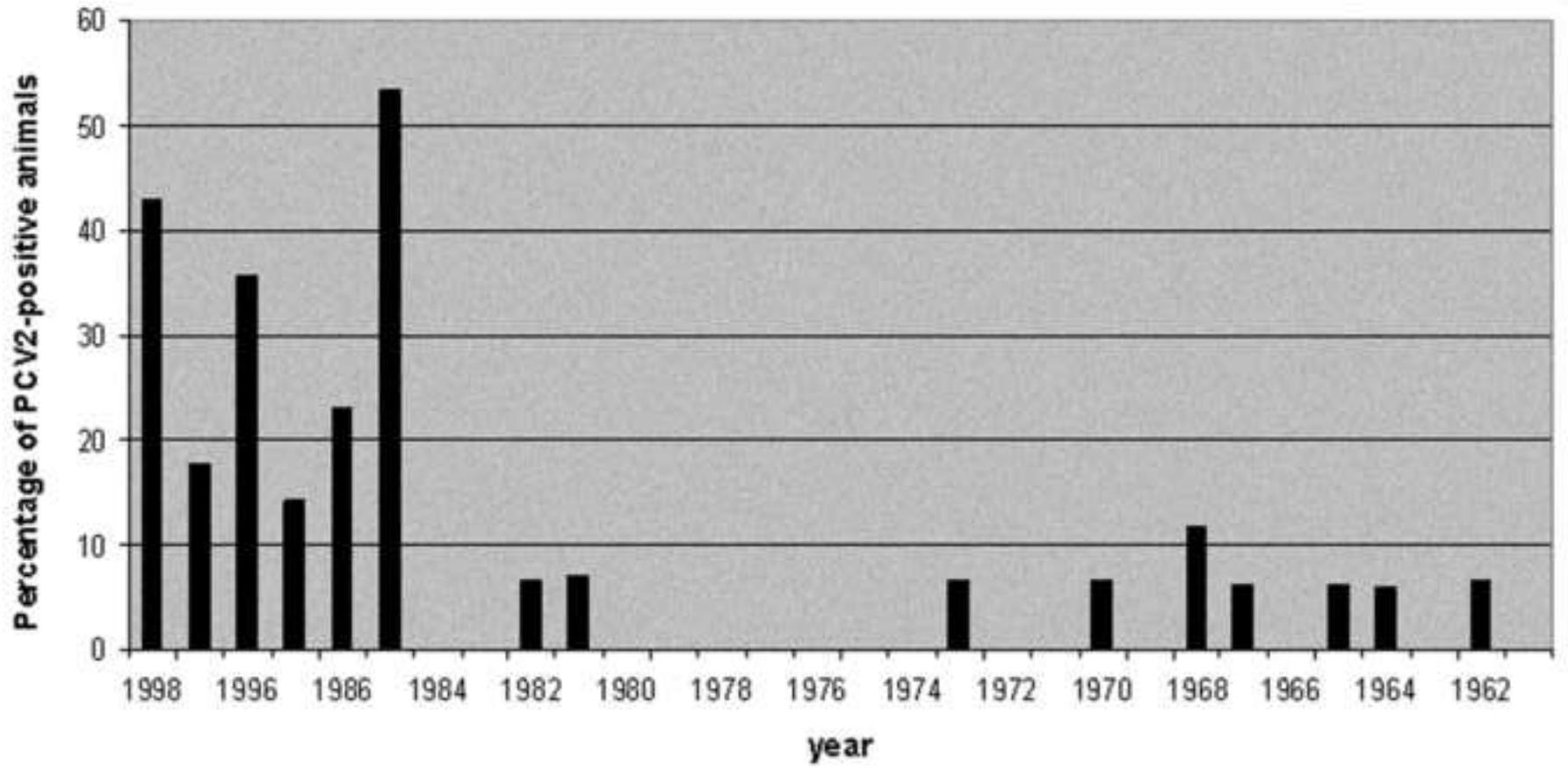


Figure 3

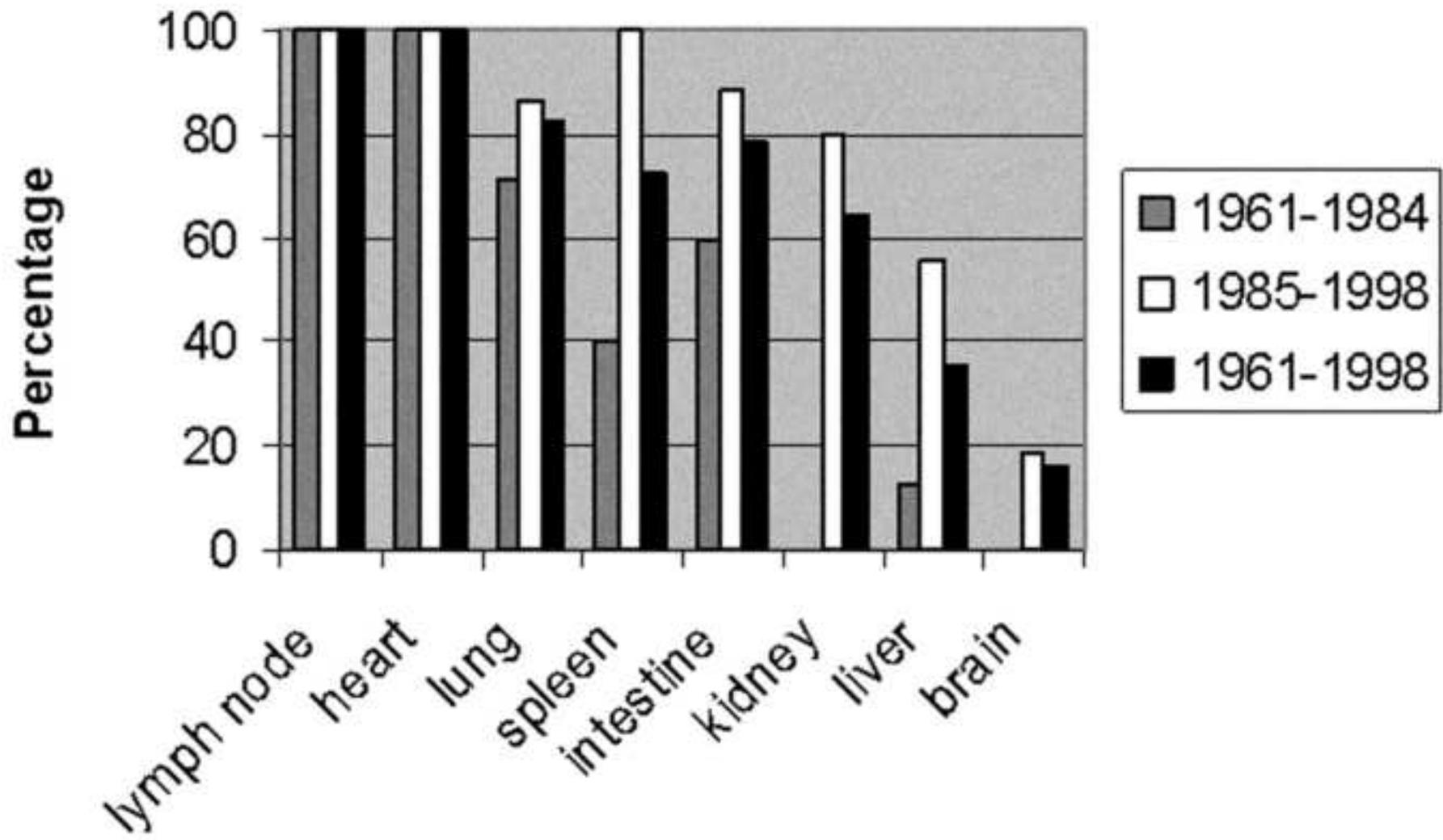


Figure 4

