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

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Abstract

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

Keywords

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

1. Introduction

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

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

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

2.2. *In situ* hybridisation

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

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

2.3. Polymerase chain reaction and sequencing

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

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

3.3. Histology

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

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

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

3.4. Polymerase chain reaction and sequencing

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

3.5 Additional results

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

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

4. Discussion

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

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

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

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

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

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

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

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

It is well established that various risk factors contribute to exacerbating PCV2 infection and the development of associated lesions. These include infections with PPV, PRRSV, swine influenza virus, and *Mycoplasma* (*M.*) *hyopneumoniae* as well as vaccination against PRRS and mycoplasma (Elbers et al., 2006; Ellis et al., 2004). In 1984 there was an outbreak of CSF predominantly in the northern part of Germany leading to a large-scale vaccination to control the epidemic (Pittler et al., 1986). Whether the epidemic infection as well as the vaccination 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 pathogenecity 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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434

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)	GCACCCTGTAACGTTTGTCA	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 [a]GenBank accession number: AF 027217

437 [b]GenBank 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; lnn., 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 µm); 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 µm).
471

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)	GCACCCTGTAACGTTTGTCA	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]

[a]GenBank accession number: AF 027217

[b]GenBank accession number: NC 001792

for = forward

rev = reverse

Figure 1

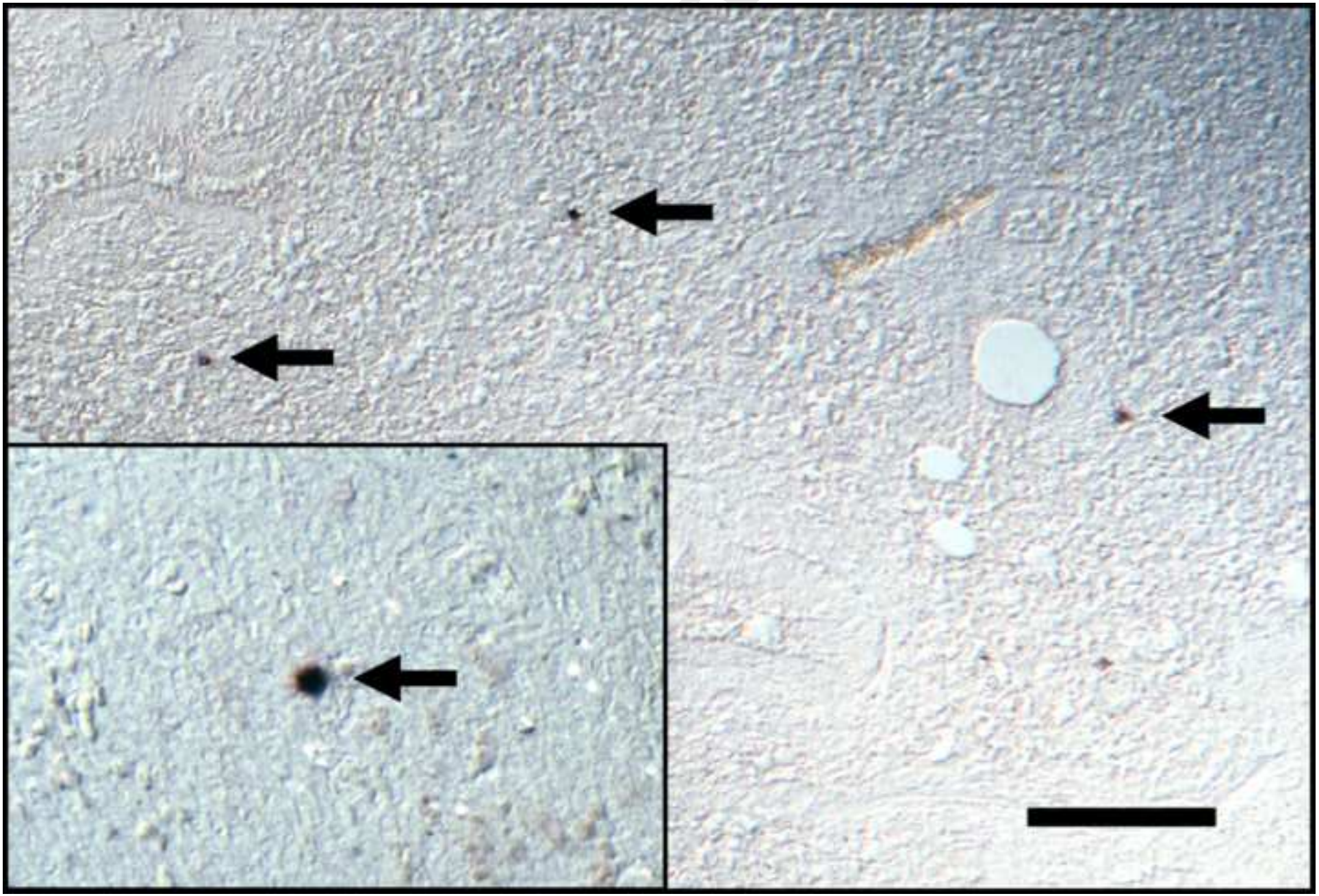


Figure 2

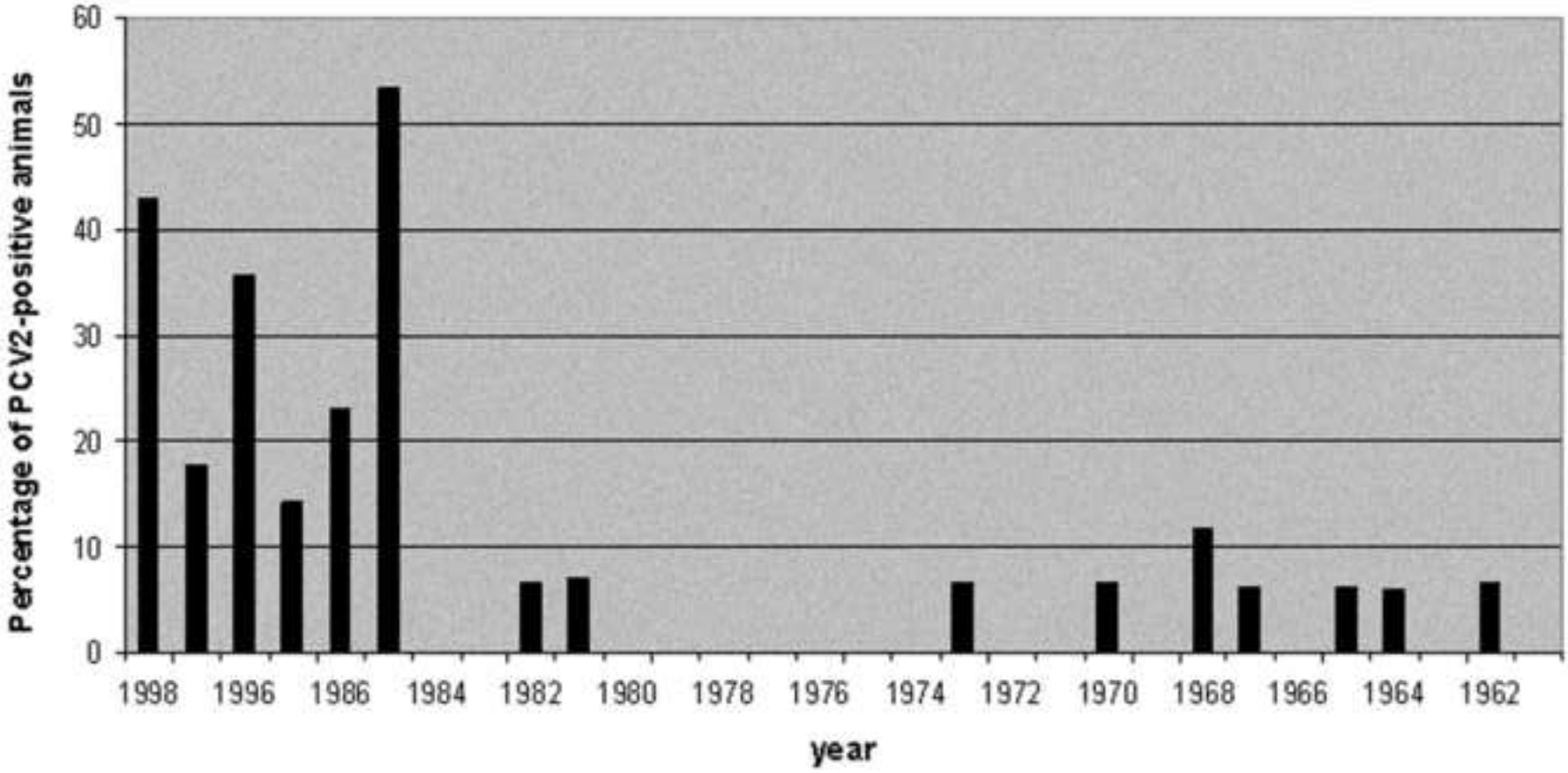

Percentage of PCV2-positive animals
60
50
40
30
20
10
0
1998
1996
1986
1984
1982
1980
1978
1976
1974
1972
1970
1968
1966
1964
1962
year

Figure 3

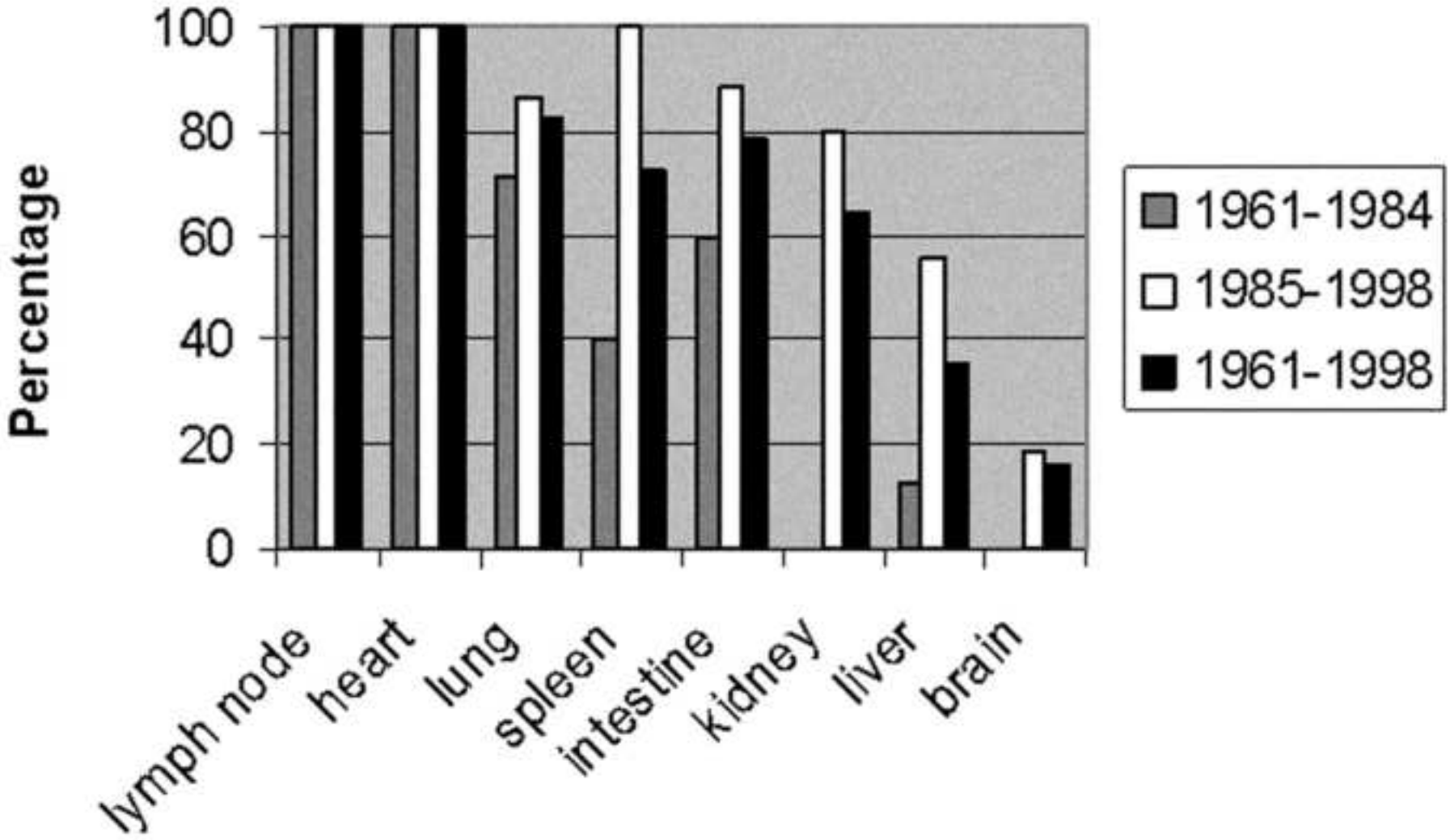
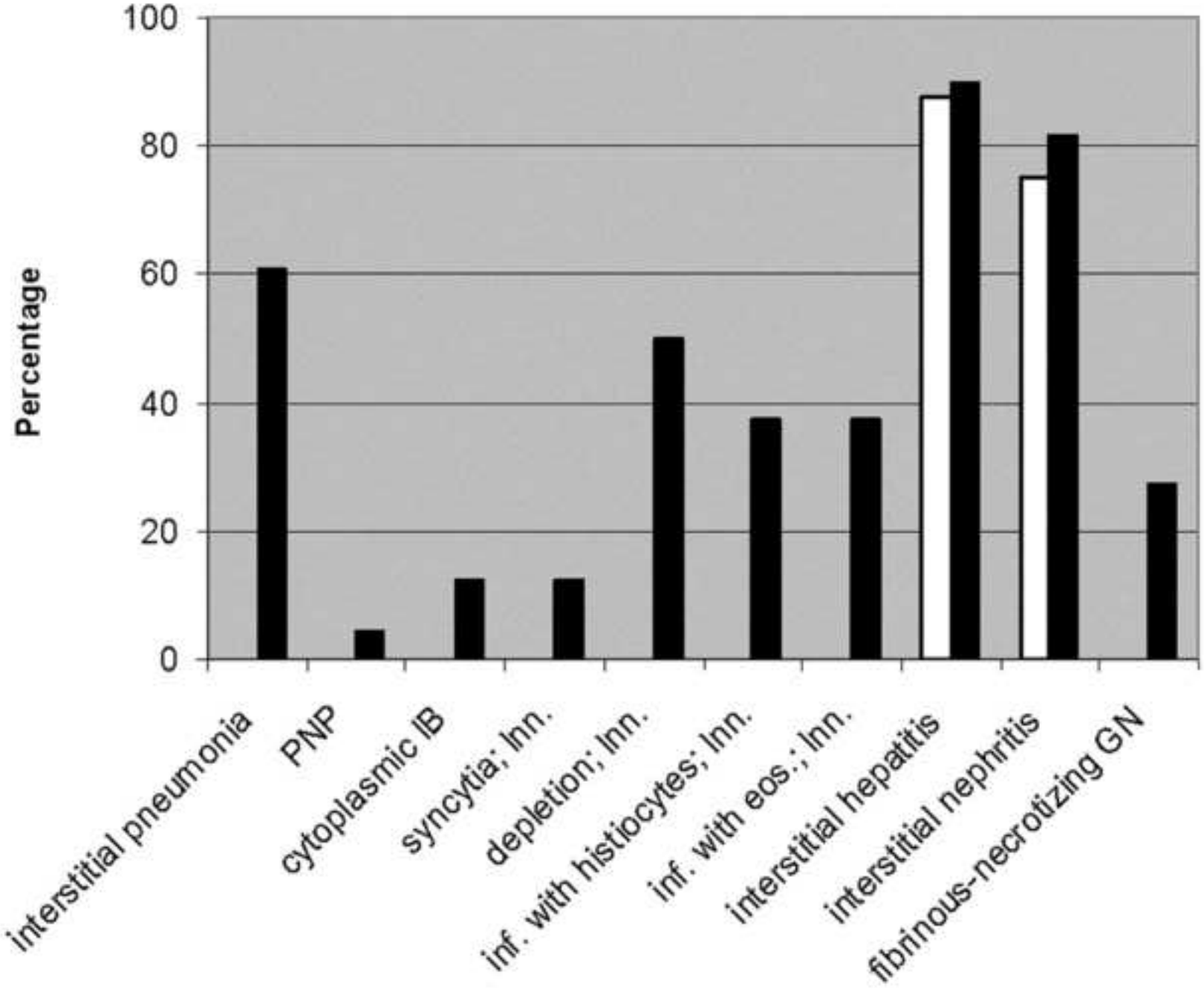
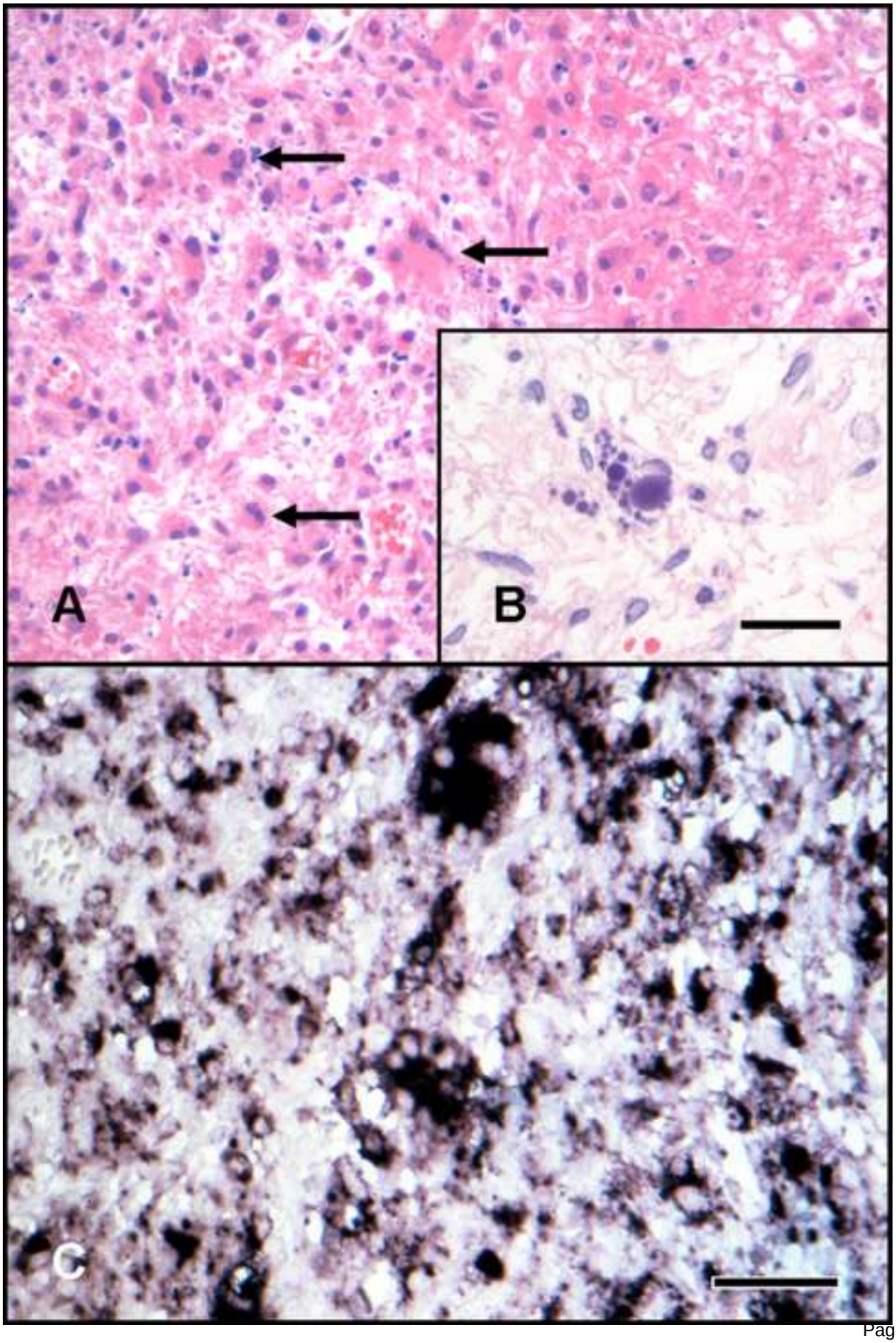

Percentage
100
80
60
40
20
0
lymph node
heart
lung
spleen
intestine
kidney
liver
brain
1961-1984
1985-1998
1961-1998

Figure 4


100
80
60
40
20
0
Percentage
interstitial pneumonia
PNP
cytoplasmic IB
syncytia; Inn.
depletion; Inn.
inf. with histiocytes; Inn.
inf. with eos.; Inn.
interstitial hepatitis
interstitial nephritis
fibrinous-necrotizing GN



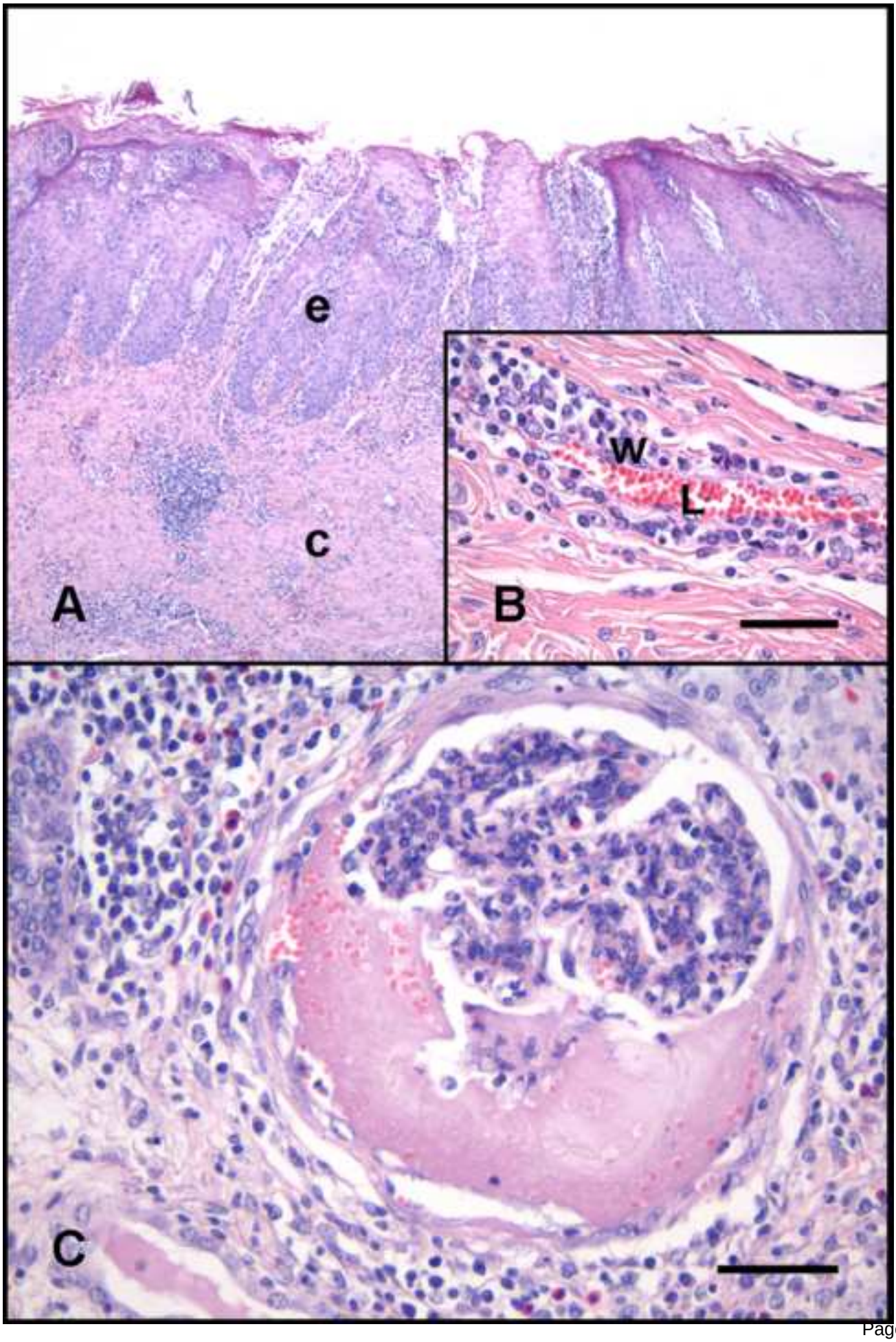


Figure 1 black-white