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Detection and genetic typing of type 2 porcine circoviruses in archived pig tissues from the UK*

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Summary. *Porcine circovirus 2* (PCV-2) is implicated as the causative agent of post-weaning multisystemic wasting syndrome (PMWS) and is also associated with porcine dermatitis and nephropathy syndrome (PDNS). The recent emergence of epidemic PMWS in the United Kingdom was predated by sporadic cases of PDNS dating back to the early 1980's. The aim of this study was to investigate whether PCV-2 DNA was present in archival tissues, and if so, to investigate the relatedness of these viruses with contemporary strains of PCV-2. DNA extracted from paraffin wax-embedded tissue blocks ($n = 68$), was subjected to a TaqMan® polymerase chain reaction (PCR) targeting a fragment of ORF1 of PCV-2. Positive results were obtained from 41% (9/22), 31% (4/13) and 32% (8/25) of submissions from the 1990's, 1980's and 1970's respectively. The presence of PCV-2 antigen in some of these tissues was confirmed by immunohistochemistry (IHC). A PCR targeting ORF2 was used to obtain sequence data for phylogenetic analysis. Sequences from 5 archival tissues were unique but showed high genetic identity to PCV-2 sequence obtained from a 2000 PDNS case. These data demonstrate that similar isolates of PCV-2 have been present in the UK pig population for more than 30 years.

Introduction

Porcine circoviruses type 1 and type 2 (PCV-1 and PCV-2) are small, non-enveloped viruses with circular, single stranded DNA genomes of approximately

*Nucleotide sequence data reported in this paper are available in the GenBank database under the following accession numbers; AY325515 (UK 2408/86), AY325516 (UK 3986/91), AY325517 (UK Wa 54/95), AY325518 (UK 03/0118/74), AY325519 (UK 01/0050/76).

1700 nucleotides in length, encoding 2 major ambisense open reading frames [2]. Together with a number of avian viruses with similar molecular characteristics, the porcine circoviruses are classified in the genus *Circovirus* within the family *Circoviridae*. PCV-1 was originally isolated as a contaminant of cultured cells [26]. Although PCV-1 persists in the pig population, the presence of PCV-1 has not been associated with any recognised clinical signs or pathology. In contrast, PCV-2 has been implicated as the causative agent of postweaning multisystemic wasting syndrome (PMWS).

PMWS was first observed in Canada in 1991 [8, 15] and is now a major animal health problem and a source of significant financial loss in affected herds. In the UK, PMWS was first recognised in Northern Ireland in 1998 [17] followed by a series of outbreaks in eastern and southern England in 1999 [14]. Large quantities of PCV-2 can be demonstrated in lesions of pigs exhibiting clinical signs of PMWS [21], suggesting a role for this virus in the aetiology of this syndrome. Furthermore, in order to reproduce disease, all experimental models of PMWS have required the presence of PCV-2 [1, 3, 18]. In parallel to the emergence of PMWS, there have also been dramatic increases in cases of porcine dermatitis and nephropathy syndrome (PDNS) in the UK. Although sporadic cases of this syndrome have been recognised since 1987 [30], it was not until 1999 that the epidemic form of PDNS was reported in the UK [14]. It is speculated that PCV-2 may also be associated with the aetiology of PDNS [24], though PCV-2 is not consistently found in PDNS cases [11]. In addition to PMWS and PDNS, PCV-2 has also been associated with peri-natal myocarditis and reproductive failure [29], proliferative and necrotising pneumonia (PNP) [16], congenital tremor (CT-AII) [25] and exudative epidermitis [28].

Though the clinical signs and pathology of these syndromes are well documented [2, 10, 11, 23], the aetiopathogenesis of PMWS and PDNS is still unresolved. In particular, the factors responsible for the recent emergence of the epizootic forms of PMWS and PDNS are unclear. PCV-2 specific antibodies have been detected in archived porcine sera from Northern Ireland dating back to 1973 [27] indicating that PCV-2 is not a new virus.

The aim of this study was to investigate whether PCV-2 nucleic acid was present in porcine tissues archived prior to the UK onset of epizootic PMWS and PDNS in 1999. Subsequent generation of sequence data for these archival PCV-2 strains allowed a comparative analysis with contemporary strains to be performed.

Material and methods

Tissue samples

Archived paraffin wax-embedded tissue blocks of bronchial or mesenteric lymph nodes, lung, liver, intestine or spleen obtained from pigs submitted to the Veterinary Laboratories Agency (VLA) were selected for this study. No specific criteria were used for the selection of these samples, other than age (between 5–12 weeks). In total, 68 tissue blocks, from 68 submissions were tested, comprising 14 submitted to the VLA Aberystwyth regional laboratory in Wales between 1995–1997, and 54 submitted to VLA Weybridge in England between 1970–1995. These tissue blocks had been fixed in 10% buffered formalin, dehydrated through graded alcohol, the tissue cleared with chloroform, and then impregnated and embedded with paraffin wax.

DNA extraction

After facing off the tissue blocks, 10 μm sections were cut using a new microtome blade to minimise the risk of DNA crossover contamination. A bovine tissue was processed for every 10 porcine tissues as an irrelevant negative extraction control. Tissue sections were de-waxed twice with 1 ml volumes of xylene at 55 °C for 15 min, centrifuged for 5 min at 9500 × g (Micro Centaur, MSE) and supernatant discarded. After the second xylene extraction step, the pellets were rehydrated in 1 ml absolute ethanol at 55 °C for 15 min, centrifuged for 2 min at 9500 × g and supernatant discarded. This rehydration step was repeated. The pellets were air dried before being resuspended in 0.5 ml of a 100 mM NaCl, 0.5% SDS, 10 mM Tris-HCl/25 mM EDTA pH 8.0 buffer. Proteinase K (0.5 mg/ml, Gibco BRL) was added to the tube, and the samples were digested at 55 °C for 48 h, with a new supplement of Proteinase K after 24 h. Total DNA was recovered by extracting the digested samples twice with equal volumes of phenol/chloroform using 5 min centrifugation steps at 9500 × g. The aqueous phase was transferred to a new 1.5 ml microtube where the DNA was precipitated by overnight incubation at −20 °C with 0.1 volumes of 3 M sodium acetate (pH 5.2), 2 volumes ethanol (95%) and 0.02 mg glycogen. The DNA was pelleted by centrifugation for 30 min at 9500 × g, supernatant discarded and air-dried. The pellets were dissolved in 10 mM TRIS (pH 8.5), and the DNA concentration and purity was estimated as a ratio of UV light absorbance at 260 and 280 nm (Ultraspec 2000, Pharmacia Biotech). For each tissue sample, a H_2O negative control was extracted in parallel.

Porcine control gene PCR

DNA extracted from paraffin wax blocks is often degraded [13] and can also contain substances inhibitory to PCR [5]. Therefore, a control gene PCR was employed, designed to amplify a fragment of porcine genomic DNA. This identified samples that did not contain amplifiable DNA. This control gene PCR was used in a pilot study, to investigate whether the DNA recovered from the porcine tissues block was suitable for amplification, indicating the potential for success in amplifying longer, PCV-2 targets. Two sets of primers, were designed to amplify the pig tumour necrosis factor-alpha gene (TNF-α: GenBank accession number X54001), designated TNF-FOR and TNF-REV; TNF-FOR3 and TNF-REV (Table 1), yielding 458 bp

Table 1. Primers and probes used in the detection and genetic typing of PCV-2 DNA isolated from archived paraffin wax-embedded pig tissues

	Primer	Sequence (5′ to 3′)	Genome positions
Porcine control gene PCR	TNF-FOR	CAC GTG GAA GGC ACT CAA TG	602–621[a]
	TNF-FOR3	CCT GTC TCT CAG TTC TGA GC	842–861[a]
	TNF-REV	TTA CCT ACA ACG TGG GCG AC	1060–1041[a]
TaqMan® PCR for detection of PCV-2	F867	GCT CT(CT) TAT CGG AGG ATT AC	867–886[b]
	R1004	ATA AAA ACC ATT ACG A(AT)G TGA TA	1004–1026[b]
	TaqMan® probe	CCA TGC CCT GAA TTT CCA TAT GAA AT	960–985[b]
ORF2 PCR for sequencing and genetic typing	PCV-2-2B	TAC ATG GTT ACA CGG ATA TTG TA	1082–1104[b]
	PCV-2-2A	CAC CTT CGG ATA TAC TGT CAA	1582–1562[b]
	PCV-2-2BI2	GTA TGG CGG GAG GAG TAG TTT AC	1289–1311[b]
	PCV-2-OINTR	STG CTG TTA TTC TAG ATG ATA AC	1373–1351[b]

[a]Positions assigned with reference to GenBank accession number X54001
[b]Positions assigned with reference to GenBank accession number AF201897

and 218 bp products respectively. TNF-REV was designed to span a putative intron-exon junction, in order to selectively amplify genomic DNA. PCR reactions were prepared to a final volume of 50 μl, containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 50 pmol of each primer, 0.5 U *Taq* DNA polymerase (Promega), and 500 ng target DNA. Amplification conditions (GeneAmp PCR system 9700, Applied Biosystems) used were 94 °C 2 min, 35 cycles of 94 °C 45 s, 58 °C 40 s, 72 °C 1 min, and a final extension at 72 °C for 10 min. PCR products were analysed by ethidium bromide gel electrophoresis using 2% agarose gels.

TaqMan® PCR for detection of PCV-2

PCR primers targeting a 160 nt fragment, encompassing the 3′ end of ORF1, of both PCV genomes, and a PCV-2 specific TaqMan® probe (Table 1) were designed on the basis of a multiple alignment of four PCV-1 and 34 PCV-2 genomes. Fifty μl PCR reactions were prepared in 96-well optical reaction plates (Applied Biosystems) as described for the control gene PCR, with 4 pmol of the TaqMan probe. Amplification conditions used were 94 °C 2 min, 37 cycles of 94 °C 30 s, 55 °C 1 min, 72 °C 1 min, and a final extension at 72 °C for 7 min. Endonuclease cleavage of the TaqMan® probe associated with successful amplification of the PCV-2 fragment was measured using an ABI PRISM 7200 fluorescence detector (Applied Biosystems).

To determine the analytical sensitivity of the TaqMan® assay, a PCR amplicon was cloned into the pGEM®-T Easy Vector (Promega) and subsequently transformed into DH5α™ competent *E.coli* cells (Max. Efficiency™ Gibco BRL), according to the manufacturer's instructions. Plasmid (pGEM – PCV-2_{160}) DNA was extracted using QiaPrep MiniPrep (Qiagen) and log titrations were assayed.

ORF2 PCR for sequencing and genetic typing

A second PCV-2 specific PCR, targeting a 501 nt fragment of ORF2, was carried out on all DNA samples found to be PCV-2 positive by TaqMan® PCR. PCR reactions with the primers PCV-2-2A and PCV-2-2B (Table 1) were prepared as described for the control gene PCR. Amplification conditions used were 94 °C 2 min, 35 cycles of 94 °C 1 min, 55 °C 1 min, 72 °C 1 min, and a final extension at 72 °C for 7 min. Where it was not possible to amplify the 501 nt fragment of ORF2, 2 sets of primers amplifying 2 overlapping fragments (294 nts: PCV-2-2B and PCV-2-OINTR; 292 nts: PCV-2-2A and PCV-2-2BI2) were used to attempt to obtain this same sequence information (Table 1). The same amplification conditions were used except for PCV-2-2A and PCV-2-2BI2, which required a lower annealing temperature of 50 °C. PCV-2 specific amplicons were recovered from agarose gels, and purified using the Qiaquick PCR purification kit (Qiagen), and then used as templates in direct dye-termination sequence reactions (Big Dye™ Terminator Cycle Sequencing Ready Reaction, Applied Biosystems). 501 nt sequences were truncated to remove ambiguous alignment positions and the resulting 421 nt sequences were aligned using CLUSTALX (Version 1.81). Phylogenetic analysis was performed with PHYLIP (Version 3.5) using DNADIST, and NEIGHBOR, using a PCV-1 as the outgroup sequence (GenBank Accession number Y09921).

Immunohistochemistry

Tissue sections were de-waxed in 2 baths of xylene, and then rehydrated by immersion in 2 baths of absolute alcohol followed by running tap water. For epitope demasking, the rehydrated sections were pre-treated with Protease XXIV (Biogenex), applied in accordance with manufacturers instructions (1 mg/ml, lyophilized protease powder reconstituted in phosphate buffered saline, pH 7.6) for ten min at room temperature. Sections were then washed with

Tris Buffered Saline (0.005M TBS, pH 7.6) prior to the application of a blocking agent (Vector normal goat serum 1/66 diluted in TBS) for thirty min at room temperature. A mouse monoclonal antibody (Mab), 01902 B1 BC, raised against the putative capsid protein of PCV-2, (Synbiotics, Lyon, France) was used to detect PCV-2. The Mab was applied at 1/1000 dilution in TBS for one hour. Parallel sections incubated with only the Mab diluent were processed to serve as negative controls.

PCV-2 antigen was visualized by incubation with biotinylated goat anti-mouse IgG (1/200 dilution, with normal goat serum 1/66 dilution in TBS supplemented with 3% normal swine serum, for 30 min at room temperature), an avidin-biotin-peroxidase conjugate (Vector ABC Elite 1/25 dilution in TBS, for 30 min) and citrate-buffered diaminobenzidine for 10 min at room temperature. Each reagent step was preceded by three 5 min TBS washes. The sections were then counterstained using Mayers' haematoxylin, before dehydrating in absolute alcohol, clearing in xylene and coverslipping using DPX mountant. Sections were examined by light microscopy.

Results

Porcine control gene PCR

A preliminary trial was performed to assess whether DNA degradation had occurred and whether this would influence the success of PCRs of differing target sizes. By using the TNF-FOR and TNF-REV primers, only 49% (24/49) of samples gave a detectable 458 bp product. When the primers targeting the smaller 218 bp amplicon were used, the amplification success rate increased to 92% (45/49). A subset ($n = 19$) of these results is depicted in Fig. 1.

Optimisation of TaqMan® PCR for detection of PCV-2

Based on the results from the control gene trial PCR, a 160 nt fragment was selected as a target for the PCV-2 TaqMan® PCR. The TaqMan® assay was optimised for annealing temperature and optimal magnesium ion concentration (data not shown). By using DNA extracted from a PCV-1 contaminated PK-15 cell line as PCR target, the PCV-2 specificity of the TaqMan® PCR was verified by it's inability to detect PCV-1 DNA (data not shown). Using $\log_{10}$ titrations of a

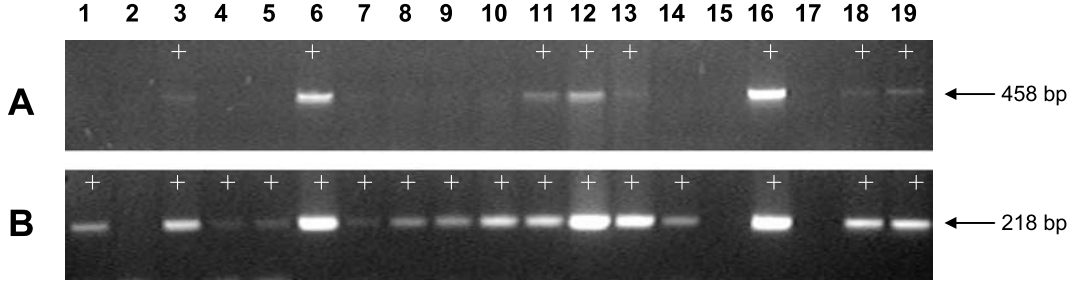


Fig. 1. Amplification of the control porcine genomic DNA fragment by PCR from archival paraffin embedded blocks ($n = 19$). 2% agarose gel shows preferential amplification of (**B**) a 218 bp fragment (16/19 samples positive) compared with (**A**) a larger 458 bp amplicon (8/19 samples positive)

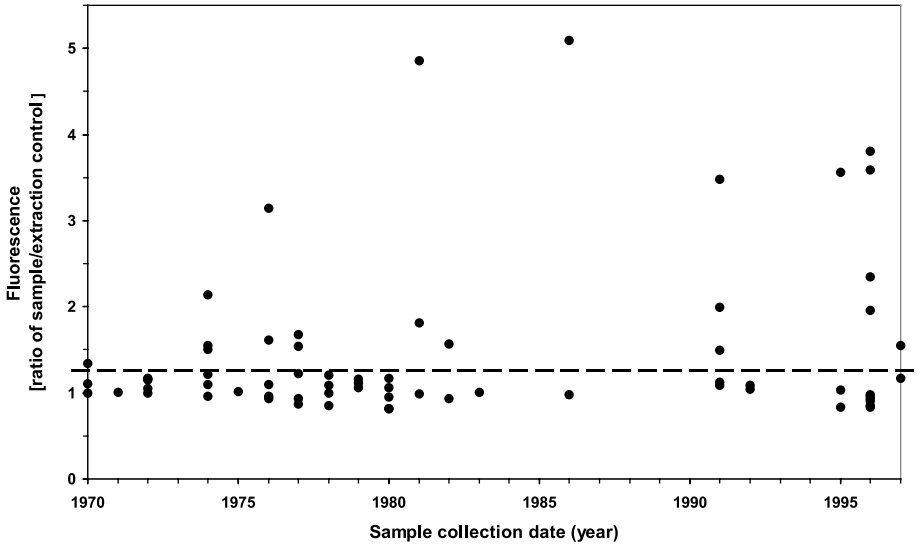


Fig. 2. TaqMan® Fluorescence for the 68 submissions examined. Fluorescence is given as a ratio of the intensities obtained with samples and extraction controls processed in parallel

Table 2. Detection of PCV-2 DNA by TaqMan® PCR in archived paraffin wax-embedded tissue blocks (1970–1997)

Decade	Number of submissions	Control gene PCR % positive	PCV-2 TaqMan % positive[b]
1990's	22	95 (21/22)[a]	41 (9/22)[c]
1980's	13	100 (13/13)	31 (4/13)
1970's	33	67 (22/33)	32 (8/25)[d]
Total	68	82 (56/68)	35 (21/60)

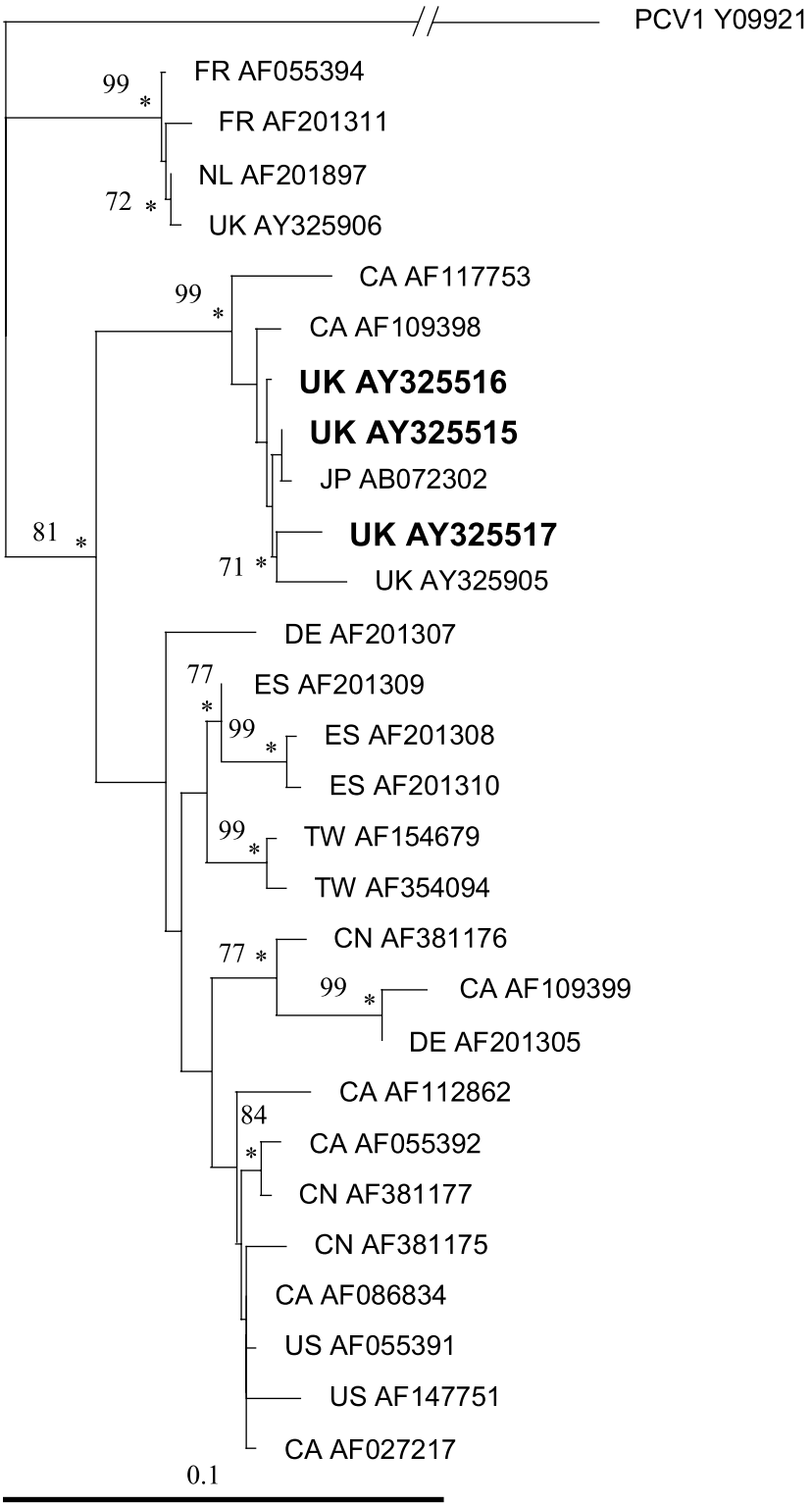
[a]Number positive/number tested

[b]% positive for PCV-2 TaqMan® calculated as: (number positive/number containing amplifiable DNA)*100

[c]1 sample was PCV-2 TaqMan® positive but did not give a detectable band for the control gene PCR

[d]3 samples were PCV-2 TaqMan® positive but did not give a detectable band for the control gene PCR

Fig. 3. Phylogenetic dendrogram depicting genetic distance between 28 PCV-2 isolates and a PCV-1 isolate, based on partial nucleotide sequence of ORF2 region (421 nt). Country of origin and Genbank accession numbers are indicated. Genetic distances were calculated using PHYLIP (Version 3.5) using DNADIST and NEIGHBOR. Bootstrap confidence values were calculated using SEQBOOT and CONSENSE (number of replicates = 2000). Bootstrap values over 70% are considered significant and are indicated by *


PCV1 Y09921
99
*
FR AF055394
FR AF201311
NL AF201897
72
*
UK AY325906
CA AF117753
99
*
CA AF109398
UK AY325516
UK AY325515
JP AB072302
81
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UK AY325517
71
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UK AY325905
DE AF201307
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ES AF201309
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ES AF201308
ES AF201310
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TW AF154679
TW AF354094
CN AF381176
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CA AF109399
DE AF201305
CA AF112862
84
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CA AF055392
CN AF381177
CN AF381175
CA AF086834
US AF055391
US AF147751
CA AF027217
0.1

cloned amplicon (pGEM – PCV-2_{160}), the analytical sensitivity of this assay was estimated at between 70–700 copies (data not shown).

TaqMan® PCR for detection of PCV-2

Of the 68 submissions tested by the TaqMan® assay, a total of 21 were PCV-2 positive (Fig. 2). The PCV-2 detection rates in tissues from each decade are given in Table 2. The earliest PCV-2 TaqMan® positive sample dated back to a tissue block from 1970. All bovine tissue controls and H_2O extraction controls were negative.

ORF2 PCR for sequencing and genetic typing

The 501 nt fragment of ORF2 (corresponding to nt 153–653 of the ORF2 transcript) was amplifiable from three of 21 TaqMan® positive samples (2408/86, 3986/91, Wa 54/95). When truncated to 421 nt for analysis, these sequences showed 98.6–99.8% identity to each other. They also showed high genetic identity with a case of PDNS in England in 2000 (97.1% to 97.9%), relative to genetic identity between year 2000 PDNS case and other PCV2 isolates (88.4% to 97.6%).

Phylogenetic analysis supported the close relationship of these archival PCV-2s to the PCV-2 isolated from a case of PDNS in England in 2000 (GenBank Accession number AY325905) (Fig. 3). From the 18 TaqMan® positive samples which failed to give 501 bp PCV-2 amplicons, two samples from the 1970's (03/0118/74, 01/0050/76) were successfully amplified by the PCV-2-2B/PCV-2-OINTR primer pair. Alignment and subsequent phylogenetic analysis of 246 nt ORF2 sequences of the latter samples with the corresponding regions of PCV-2 isolates used to construct Fig. 3, showed that although together they appeared to form a discrete group, these older samples were also closely related to the other archival UK PCV-2 samples (data not shown).

Immunohistochemistry

The 15 TaqMan® positives from the 54 submissions to VLA Weybridge between 1970 and 1995, were selected for staining by immunohistochemistry (IHC). Four of these were also found to be PCV-2 positive by IHC. Intense staining for PCV-2 antigen was detected in two cases from 1986 and 1991. From the 1986 case, sections of a mesenteric lymph node and jejunum showed PCV-2 specific staining (Fig. 4). In the latter section, histiocytes in primarily submucosal lymphoid follicles were intensely stained for PCV-2 antigen, whilst in the lymph node there was patchy labelling of histiocytes within depleted follicles and sinusoids, but less intensely than seen in cells within the jejunal submucosa. In the submission from 1991, intense PCV-2 specific staining was seen in individual cells in alveolar septa. In two further submissions, dating from 1977 and 1991, sparse PCV-2 specific staining of occasional cells was also evident in lung and spleen sections, respectively.

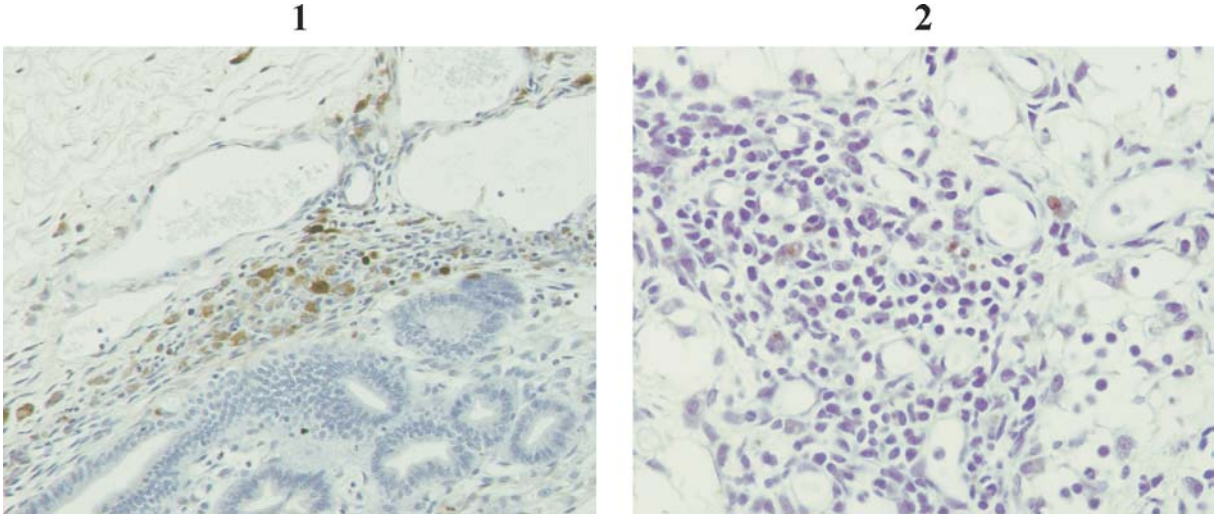

Fig. 4. Detection of PCV-2 by IHC in paraffin wax-embedded tissue sections of: *1* intestinal submucosal tissue (magnification ×200), and *2* a mesenteric lymph node (magnification ×400), both from submission UK 2408/86

Discussion

This paper describes the detection and genetic typing of PCV-2 DNA in archived paraffin wax-embedded tissues from UK pigs. These data confirm that PCV-2 has been present in the UK pig population since at least 1970, findings consistent with results from archival serological studies in Northern Ireland [27] and Spain [22]. Of the tissue blocks shown to contain amplifiable DNA, 41% of those assayed from the 1990's, 31% from the 1980's and 32% from the 1970's were PCV-2 positive, and indicating that PCV-2 was not uncommon in the UK pig population over this time period. No definitive conclusions regarding prevalence can be drawn however, since this data set is a skewed representation of the UK pig population.

We were able to detect a larger fragment of PCV-2 (for genetic typing of the TaqMan® positives) in only 3/21 of the PCV-2 TaqMan® positive tissue blocks. This is likely to be due to degradation of the DNA in older paraffin wax-embedded blocks, as suggested by results from the control gene PCR (*TNF-α*); we were able to amplify a 218 bp fragment of *TNF-α* from 100% and 95% of DNA extracted from tissue blocks from the 1980's and 1990's respectively, but from only 67% from the 1970's. This apparent 'age effect' on the quality of the extracted DNA may be influenced by several factors described in previous studies, including 1) sample quality prior to fixation and embedding [12], 2) formalin fixative method [6, 7] and/or 3) length of time stored as paraffin wax-embedded tissue [9, 12]. Instances where DNA extracted from tissue blocks did not contain amplifiable TNF-α DNA but did contain amplifiable PCV-2 DNA may be due to relatively high PCV-2 copy numbers per cell and/or the protection provided to the viral genome by the viral capsid postmortally and during the fixation and embedding process.

Despite evidence provided here, and elsewhere, that PCV-2 has been present in the pig population for some time, PMWS and PDNS have only recently emerged as significant disease syndromes. Early speculation proposed changes to the PCV-2 genome to account for an apparent increase in virulence. A 421 nt fragment of ORF2 was used for phylogenetic analysis in this study, ORF2 being the most variable region of the PCV-2 genome. Since only a quarter of the PCV-2 genome was characterised, changes in other areas of the genome could have occurred. Complete genome sequencing and analysis would be required to determine whether virulence modifying changes in other areas of the genome have occurred. However, the recent reproduction of PMWS using a Swedish PCV-2 isolate (Sweden is free of PMWS) provides strong evidence against the emergence of mutated PCV-2s being responsible for PMWS [4]. Current scientific evidence suggests the involvement of extraneous agents/factors in combination with PCV-2 in pathogenesis, including co-infection with other viral agents (PPV – [1, 4, 18]; PRRS – [3]), non-viral agents/factors including various microbial agents, and components of modern intensive farming practices.

The detection of PCV-2 nucleic acid by PCR was supported with the detection of PCV-2 antigen in four samples, and included the detection of PCV-2 antigen in a submission dating back to 1977. The IHC protocol was not optimised for the purposes of this study, and therefore no comparison can be made with the TaqMan® assay with regard to the relative sensitivity of the two assays.

Intense positive staining for PCV-2 antigen may be suggestive of PMWS, as opposed to a subclinical infection with PCV-2, which is more likely to be detected by PCR. To address this question, we reviewed the submission histories of the samples that were strongly positive for PCV-2 by IHC. The submission from 1986 involved one of seven, 6-week old suckling piglets that had died after a period of scouring. At autopsy the piglet presented in fair condition, with severe oedema of the mesenteries and some jaundice. The intestinal walls appeared thickened, and the mesenteric lymph nodes were enlarged. Histopathological findings included lymphocytic depletion and histiocytic infiltration of the lymph node, with variable staining of inclusion bodies in macrophages. The intestinal sections showed congestion, villous atrophy with hyperplastic crypts, and ulceration of the mucosa over Peyer's patches. In the submucosa histiocytic infiltration was noted together with depletion of gut-associated lymphoid tissue follicles. No other pathogens beyond *E.coli* were isolated, and a diagnosis of chronic enteropathy was made. The submission from 1991 was one of sudden death in an 11-week old piglet, which on autopsy was diagnosed with acute bronchointerstitial pneumonia and splenic granuloma. Whilst the described pathological changes are compatible with those seen in submissions of PMWS, the clinical presentation of these two submissions would today not have qualified as PMWS. However, modern husbandry practises such as early weaning have been suggested as contributory factors to the incidence of PMWS today, and it cannot be excluded that immunological or other stress might have triggered a full PMWS-like clinical picture in these pigs infected with PCV-2 twenty to thirty years ago.

We have also reported the development of a TaqMan® PCR to detect PCV-2 DNA. This TaqMan® PCR assay is a valuable addition to existing PCR detection methods from paraffin wax-embedded blocks [19, 20], reducing the risk of sample contamination with the elimination of post-PCR tube manipulation, and increasing the speed of analysis with the replacement of the time consuming step of gel electrophoresis.

In summary, PCV-2 has been present in the UK pig population dating back to at least 1970. In addition it would appear that PCV-2 was not uncommon in the UK population over this period. The high genetic similarity observed among five samples characterised in this archival study and a contemporary UK PCV-2 isolate provide evidence against the involvement of a 'new' or modified virus in the aetiology of PMWS/PDNS.

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