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Co-infection status of novel parvovirus's (PPV2 to 4) with porcine circovirus 2 in porcine respiratory disease complex and porcine circovirus-associated disease from 1997 to 2012

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Abstract

As global pig health diseases, porcine respiratory disease complex (PRDC) and porcine circovirus-associated disease (PCVAD) generate substantial economic losses despite pigs been vaccinated against the primary causative virus, highlighting the importance of understanding virome interactions and specifically co-factor infections. Established primary endemic pathogens for PRDC include porcine circovirus 2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSv) and swine influenza virus (SIV), and PCV2 aetiology in interaction with other co-infecting viruses can result in PCVAD. Porcine parvovirus (PPV) 1 is a well-characterized virus with an available vaccine preventing reproductive failure in sows. However, whilst novel PPV 2 to 7 viruses have been identified since 2001, their viral pathogenic potential in clinical and subclinical disease remains to be determined. Therefore, this study has sought to develop a better understanding of their potential role as associated co-infections in PRDC and PCVAD by examining archival samples for the presence of PCV2 and the novel parvoviruses PPV2-4 from clinically diseased pigs across production age stages. Epidemiologically, the novel PPV2 was found to be the most prevalent within the fattener age group with PPV2-4 statistically associated with pig respiratory disease and enteric ulcers. Additionally, statistical modelling by latent class analysis (LCA) on veterinary pathology scored pigs found a clustering co-factor association between PPV2 and PCV2, suggesting the novel PPV may be involved in PRDC and PCVAD. Phylogenetic analysis of novel PPVs revealed the PPV2 capsid evolution to be diverged from the original strains with a low nucleotide homology of 88%–96% between two distinct clades. These findings determine that novel PPV 2–4 viruses are statistically associated as co-infectors in a diseased pig population, and significantly detected PPV2 clustering co-infection frequency with PCV2 in PRDC and PCVAD diseased pigs through LCA analysis.

KEYWORDS

LCA, PCV2, PCVAD, phylogenetics, pigs, PPV2, PPV3, PPV4, PRDC, prevalence

1 | INTRODUCTION

The novel porcine parvoviruses (PPV2-PPV4) are emerging viral agents and globally endemic in domestic pig and wild boar populations (Afolabi et al., 2018; Cadar, Csagola, et al., 2013; Cságola et al., 2012; Huang et al., 2010; Zhang et al., 2014). Evidence of emerging PPV association with clinical disease has been broadly reported (Xiao, Gerber, et al., 2013; Opriessnig et al., 2014; Saekhow & Ikeda, 2014). However, as these single-stranded (ss) DNA novel viruses are not cell culture adapted, experimental infection has been hindered, resulting in limited knowledge of viral pathogenesis or the relevance of these viruses both clinically and economically. Porcine circovirus type 2 (PCV2), a ssDNA virus, is the known causative agent of the multi-factorial disease, post-weaning multisystemic wasting syndrome (PMWS) in pigs (Allan et al., 1999; Segales, 2012). PCV2 disease is facilitated by a co-factor infection suppressing the pig immune system allowing PCV2 to replicate to a higher rate. Since the initial characterization of PMWS, further disease associations have been documented and referred to as PCV2-associated disease (PCVAD) to encompass a wide variety of clinical symptoms and pathologies including enteric, respiratory, porcine dermatitis nephropathy syndrome (PDNS) and reproductive and congenital disorders (Opriessnig et al., 2007). Additionally, a multi-factorial disease was recognized as porcine respiratory disease complex (PRDC) and is observed in pig herds where respiratory symptoms with coughing, fever, anorexia and gastric ulcers occur in recently weaned piglets and in older pigs. PRDC can be attributed to a wide range of viruses such as PCV2 and potentially PCV type 3 (Kedkovid et al., 2018), PRRSv and swine influenza virus (SIV) which is known to cause significant damage to the epithelial respiratory airways where secondary bacterial pathogens can then cause infection (Opriessnig et al., 2011). A next-generation sequencing (NGS) study on PRDC porcine lung samples has associated the presence of PPV2, PPV3, PPV6 and torque teno virus to PRDC diseased pigs (Qin et al., 2018).

The novel porcine parvoviruses PPV2-4 may have emerged approximately seventy years ago and possess a high nucleotide substitution rate similar to RNA viruses (Cadar, Lorincz, et al., 2013). Through frequent recombination, the positive selection of porcine parvoviruses has the capacity to further adapt and circulate amongst pig herds, which merits continuous phylogenetic mapping and the monitoring of disease association. PPV1 is considered an endemic virus in pig herds causing enteritis and dermatitis with gilts routinely vaccinated to prevent the vertically transmitted disorder, SMEDI (stillbirth, mummification, embryonic death and infertility) (Cartwright et al., 1969; Dea et al., 1985; Duhamel et al., 1991; Kresse et al., 1985; Mengeling et al., 2000).

PPV2, the earliest novel PPV, was discovered in 2001 during a hepatitis E virus porcine sera study (Hijikata et al., 2001). Subsequently in 2006, a South Eastern China study revealed a more divergent PPV2 strain (CN Strain) originating from archival samples from pigs with clinical symptoms of 'high fever disease' (Wang et al., 2010). Phylogenetically, PPV3 is more closely related to PPV2 (Xiao, Gerber, et al., 2013) and was initially reported in 2007 from pig samples collected in Hong Kong (Lau et al., 2008). PPV4 which has three ORFs, similar to the genus bocaparvovirus, was initially identified in 2010 (Cheung et al., 2010) in lung lavage samples (collected in 2005) of wasting pigs during a PCVAD outbreak in North Carolina (Cheung et al., 2007). PPV5 was discovered in the USA in 2013 and PPV6 in China in 2014 (Ni et al., 2014), and is both identified as closely related to PPV4 although the genomes lack the additional ORF3 found in PPV4 (Xiao, Gimenez-Lirola, et al., 2013). More recently, PPV7 was discovered in 2016 by next-generation sequencing (NGS), a divergent *Parvoviridae* linage, most closely related to turkey parvovirus and Eidolon helvum parvovirus 2 was classified in the proposed genus *Chapparvovirus* (Palinski et al., 2016).

The current study sought to investigate the potential statistical association of the novel PPVs 2–4 with PCV2, the causative agent of PMWS. This was performed through a retrospective prevalence study of the pathological, molecular and phylogenetic analyses of PPV2-4 across different pig production age groups. Clinically, diseased pigs' samples over the period from 1997 to 2012 (either side of the introduction of the PCV2 vaccine in ~2006) with scored veterinary pathology and tested for the presence of PPV2-4 and PCV2 were analysed by LCA statistical modelling. This analysis revealed a clustering statistical association of PPV2 with PCV2 in diseased cohort of pigs with PCVAD and PRDC.

2 | METHOD

2.1 | Samples

A biobank of archival pig samples (n = 695), originating from Northern Ireland, Republic of Ireland, Great Britain and other neighbouring European countries, sourced from 9 different studies and post-mortem submissions (Table S1), dating from 1997 to 2012, was collated. This cohort represented a single sample per animal, comprising of various sample types including lymph tissue, sera, abortion fluid, abortion tissue, organ tissue, nasal swab and faeces (Table S1). Novel PPVs 2–4 are known to be endemic in 'healthy pigs', and as it is difficult to assess subclinical disease in pig herds, ultimately this study focused on clinically diseased pigs to demonstrate the prevalence and statistical association within this grouping (Streck et al., 2013). The PCV2 vaccine introduced in ~2006 reduces PCV2 viraemia and viral shedding, and 65% of the samples from this study were categorized in the pre-vaccine category. These samples were sourced from veterinary diagnosed clinically diseased pigs representing production groups originating from France (n = 337, 48.5%), Northern Ireland (n = 182, 26.19%), Great Britain (n = 106, 15.25%), Republic of Ireland (n = 47, 6.7%) and Belgium (n = 23, 3.3%). All recorded sample data were reviewed, and where possible, included the history of animal, age, post-mortem veterinary gross pathology, diagnostic tests including PRRS and PCV2 indirect fluorescence assay, and veterinary histopathology. The collated sample data were categorized by production age groups as foetus (0 weeks),

pre-weaners (<4 weeks), weaners (>4 weeks to < 10 weeks) and fatteners (>10 weeks).

Samples were processed before nucleic acid extraction. Sera were removed from whole clotted blood and spun at 200 ×*g* for 10 mins to pellet remaining blood cells. 10% w/v faecal samples were prepared in transport media with three freeze-thawed cycles and spun at 200 ×*g* for 30 mins 4°C to clarify the supernatant. 0.2 g of tissue samples was trimmed of fatty tissue and homogenized before clarifying at 200 ×*g* for 30 mins 4°C (TissueLyser II, Qiagen Ltd, Crawley, UK). A 200 µl volume of processed sample was extracted by a Total Nucleic Acid kit on a MagNa Pure LC automated liquid handling platform (Roche, Burgess Hill, UK) alongside positive and negative controls.

2.2 | Detection PCR and sequencing

The detection of PCV2 and the PPVs (PPV1, PPV2, PPV3 and PPV4) (Eurofins, MWG Operon, Germany) (Table S4), primers were used at a concentration of 0.5 µM, in separate 25 µl qPCRs of QuantiTect SYBR® Green (Qiagen Ltd, Crawley, UK) with 2 µl sample template of nucleic acid. Samples were run in duplicate with an in-house PCR inter-run and the extraction controls on the LightCycler 480 (Roche, Burgess Hill, UK) with approximately 10% of samples tested for PPV1. PCR amplification of 45 cycles began after a 15-min activation step, followed by 95°C for 30 s, annealing 30 s (temperature was primer dependent see Table S4), extension at 72°C 30 s with a melt curve before cooling to 40°C. The sample PCR melt–curve analysis was completed with crossing threshold (Ct) >40 considered negative and Ct levels classified into low, medium and high levels (Table S1). The semi-quantitative qPCR data from this study were restrictive as viral loads were not standardized by quantifying in copies per gram and PCR assays were of different specificity and sensitivity. PCR Ct values were accordingly scored as positive or negative for statistical purposes.

Sequencing primer sets were applied (Table S4) to selected samples with a low Ct to amplify the capsid gene. Specifically, the partial variable region of PPV2 VP1 capsid gene and the full VP capsid gene for PPV3 and PPV4 was amplified for phylogenetic analysis. Excised positive amplicons were purified through the Wizard® SV gel and PCR clean-up system (Promega, UK) and sequenced at Eurofins (MWG Operon, Germany). PCV2-positive samples open reading frame 2 from pre- and post-PCV2 vaccination samples ($n = 50$) were also sequenced (Collins & Zaykalova, 2020).

2.3 | Phylogenetic analysis

Multiple sequence alignments (MSA) of novel PPVs were generated using sequences available from Nucleotide-NCBI (National Centre for Biotechnology Information) and sequencing data analysed on Geneious 10v (Biomatters Ltd, Auckland, New Zealand). Sequenced amplicons of the novel PPVs whole and partial capsid were initially aligned on Geneious and then transferred and completed on Mega X (Mega software version 10.0.4) to avail of the alignment tool, multiple sequence comparison by log-expectation (MUSCLE) and phylogenetic tree builder through maximum-likelihood (ML) phylogenetic trees obtaining 1,000 bootstrap replicates. Phylogenetic trees were graphically viewed and edited in Interactive Tree of Life (Letunic & Bork, 2016).

2.4 | Statistical analysis

Initial statistical analysis from the whole cohort of samples evaluated possible association between the detection of virus and disease was completed using permutation tests to calculate the significance probability for the classical chi-square test of the independence of rows and columns in a two-dimensional contingency table using GenStat 16.2 (2013) (VSN International Ltd) (Roff & Bentzen, 1989). All collated results from clinical observations such as wasting, gross pathology, histopathology, diagnostic immunofluorescent assay (IFA) and PCR findings were presented in a scored matrix with positives "1", negatives "0" and no information as a "blank". Chi-squared uni-variate analysis was set with the probability value "*P*" at the statistical significance level of .05.

2.5 | Latent class analysis

LCA was completed using the poLCA package in R (Linzer & Lewis, 2011), which allows for both traditional LCA and latent class regression, where the probability of being in one or another class is influenced by other covariates besides those used to define the classes (Dayton & Macready, 1988). Only UK and Ireland samples for which symptoms were recorded ($n = 272$) were included in these analyses (Supplementary Data S2). Models were run from 200 or 400 random starting points to ensure, as far as possible, that the final fit is a global maximum. A key question for LCA is how many latent classes are present. This was decided by a combination of advice from veterinary experts, who reviewed the sets of symptoms included in analysis, and for the resulting classes, the Bayesian information criterion (BIC) (Dziak et al., 2012) (Supplementary Data S2). As with any other analysis, there is a risk of false-negative and false-positive classifications with LCA, but this is mitigated to the extent possible, by the principled examination of a range of models, and use of expert domain knowledge to guide model construction. All of the models considered are presented in Supplementary Data S2.

3 | RESULTS

3.1 | Viral prevalence by age group and year

PCV2 was determined to be the most frequently detected virus 48% (331/695) across all age groups examined in this study, followed by PPV2 17.3% (120/695) (Figure 1 and Table S3). In foetuses, PCV2 prevalence was 36% (51/143), PPV2 2.8% (4/143), PPV4 0.7%

(1/143) whilst PPV3 was not detected. A similar pattern was also observed in both the pre-weaner and weaner age groups, indicating an increase in PCV2 prevalence from 22% (40/182) to 61% (77/127), respectively, with no major increase in the prevalence of other novel PPVs. Only within the fattener age group did novel parvovirus's prevalence increase to higher levels with PPV2 at 56% (84/150), PPV3 14% (21/150) and PPV4 at 9% (13/150), although PCV2 remained the most frequently detected virus at 77% (115/150).

The profiling of PCV2 detection from 1997 to 2012 found PCV2 consistently present in all years from 1998; however, a decline in viral presence was recorded when the vaccine introduction (~2006) occurred (Table 1, Figure S5). Interestingly, the PCV2 levels also profiled an increased frequency, per year, post-PCV2 vaccine introduction. The earliest detection of co-infections of the novel PPV's was PPV2 in 1998, whilst PPV3 and PPV4 were first detected in 2000 (Table 1). The novel PPVs were detected in samples prior to their initial discovery dates although a study did also detect PPV2 and PPV3 in the USA in 1998 (Opriessnig et al., 2014). A PPV2 sample, not captured in this data analysis, was sequenced from 1996 and included in the phylogenetic analysis. The PPV4 from this study detected in 2000 is the earliest reported PPV4.

3.2 | Viral tissue tropism

In clinically diseased pigs, PCV2 was the most frequently detected virus 48% (331/695) prevalence), followed by PPV2 at 17% (120/695). PPV3 and PPV4 were only detected at a low prevalence of 6% (40/695) and 3% (24/695), respectively (Figure 2 and Table S3). Foetuses and abortion juice tested positive for novel PPVs at a lower prevalence, with only three samples testing positive for PPV2 and one for PPV4. The known vertically transmitted PCV2 virus was present in 24% (21/88) of abortion juice samples and in 53% (16/30) of foetus samples, and although only one placenta sample was tested, it was positive only for PPV2. No PPV2-PPV4 were present in colon, small intestine, thymus and brain samples although these were representative of low sample numbers.

Lymphatic tissue and lymph nodes (LN) were screened for the novel viruses, reporting PPV2, PPV3 and PPV4 prevalence in spleen at 43% (13/30), 3% (1/30) and 7% (2/30), respectively, tonsils at 28% (5/18), 17% (3/18) and 6% (1/18), mesenteric LN at 30% (33/109), 11% (12/109) and 4%, (4/109) and inguinal LN at 52% (31/60), 7% (4/60) and 5% (3/60). Interestingly, PPV2 was the only novel PPV detected in a small subset of bronchial LNs at 33% (1/3) and mediastinal LNs at 29% (2/7) prevalence. All novel PPVs were present in the lungs, with PPV2 having the highest prevalence of 30% (15/50), comparable to the prevalence of PCV2 at 48% (24/50), with PPV3 and PPV4 been detected less frequently at 6% (3/50) and 8% (4/50) prevalence. Nasal swabs provided for a lower detection rate of PPV2 at 2% (1/52), PPV3 at 6% (3/52), PPV4 at 2% (1/52) and PCV2 at 17% (9/52). Sera and faecal samples were positive for PCV2 and the novel PPVs; however, sera had the highest prevalence of PCV2 at 30% (58/193), PPV2 at 6% (12/193), PPV3 at 6% (11/193) and PPV4 at 3%

TABLE 1 Virus prevalence per year

Year	*n* = total samples	PCV2	PPV2	PPV3	PPV4
1997	1	0	0	0	0
1998	3	3 (100%)	2 (67%)	0	0
2000	11	6 (55%)	0	3 (27%)	4 (36%)
2001	8	7 (88%)	0	0	0
2002	25	22 (88%)	14 (56%)	5 (20%)	2 (8%)
2003	2	2 (100%)	0	0	0
2004	135	128 (95%)	41 (30%)	5 (4%)	5 (4%)
2005	16	15 (94%)	3 (19%)	0	0
2006	3	3 (100%)	0	0	0
2008	20	2 (10%)	0	0	0
2009	208	45 (22%)	8 (4%)	5 (2%)	0
2010	219	77 (35%)	39 (18%)	21 (10%)	8 (4%)
2011	19	4 (21%)	3 (16%)	1 (5%)	2 (11%)
2012	25	17 (68%)	10 (40%)	0	3 (12%)
Total	695	331	120	40	24

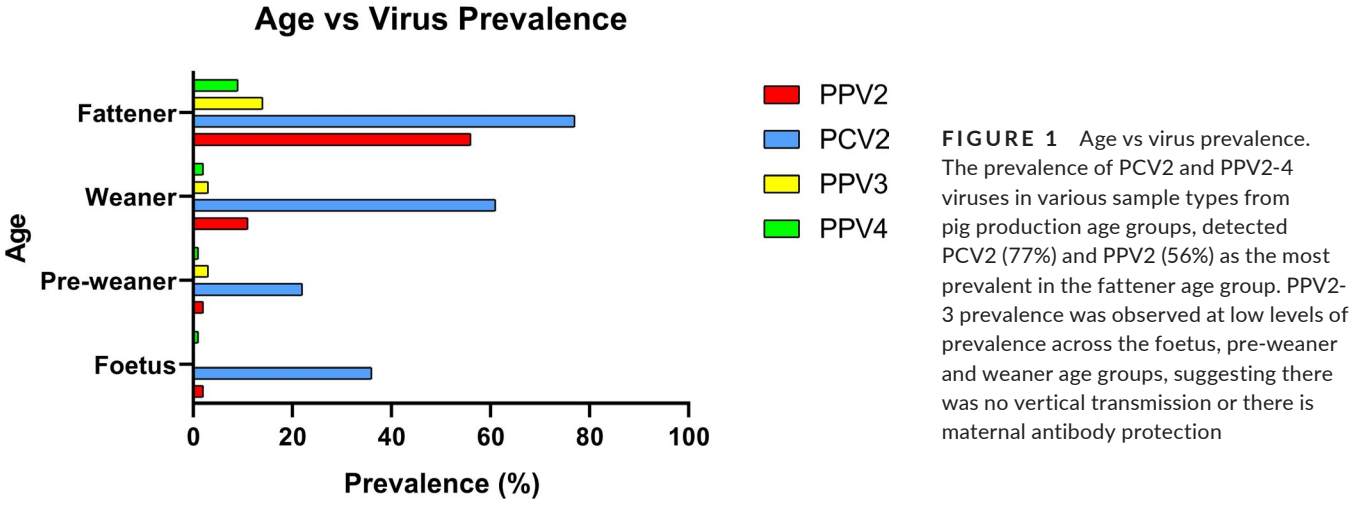

FIGURE 1 Age vs virus prevalence. The prevalence of PCV2 and PPV2-4 viruses in various sample types from pig production age groups, detected PCV2 (77%) and PPV2 (56%) as the most prevalent in the fattener age group. PPV2-3 prevalence was observed at low levels of prevalence across the foetus, pre-weaner and weaner age groups, suggesting there was no vertical transmission or there is maternal antibody protection

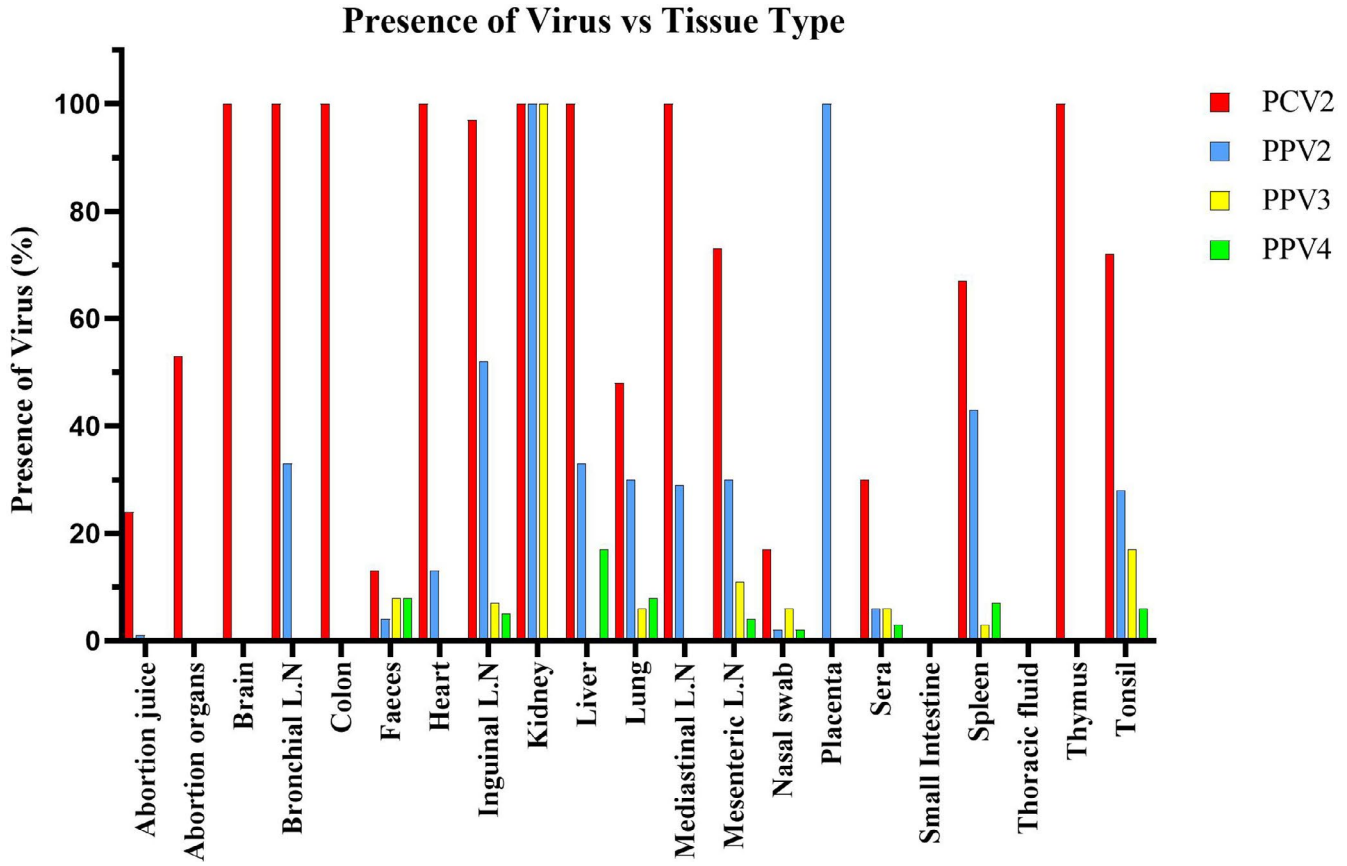

FIGURE 2 Prevalence of virus per tissue. The tissue tropism of PCV2 and PPV2-PPV4 viruses was detected in clinically diseased pigs. PCV2 was systemic and detected in most tissue types (47%), the novel parvoviruses were detected at lower levels of PPV2 (17%), PPV3 (5.8%) and PPV4 (3.5%)

(6/193). Concurrent infections did occur in clinically diseased pigs, with dual infection of PCV2 and novel PPV2 being most frequent at 12.9% (Table 2). PCV2 dual infection with PPV3 only occurred in 3.6% of samples, and PPV2 and PPV3 concurrent infection rates were similar (3%), with all other dual or multiple infections occurring in less than 3% of cases (Table 2 and 3).

TABLE 2 Concurrent infections

Virus	PCV2 *n* (%)	PPV2 *n* (%)	PPV3 *n* (%)	PPV4 *n* (%)
PCV2	331 (47.6%)			
PPV2	90 (12.9%)	120 (17.2%)		
PPV3	25 (3.6%)	23 (3%)	40 (5.8%)	
PPV4	20 (2.88%)	8 (1.2%)	4 (0.6%)	24 (3.5%)

TABLE 3 Multiple concurrent infections

Virus	*n* (%)
PCV2, PPV2, PPV3	17 (2.4%)
PCV2, PPV2, PPV3, PPV4	1 (0.14%)
PCV2, PPV3, PPV4	2 (0.29%)
PPV2, PPV3, PPV4	1 (0.14%)

3.3 | Viral presence and statistical associations with clinical and pathological observations

A total of 25 different parameters including gross post-mortem observations, pathological/histopathological findings and the results of diagnostic assays (such as the IFA for PRRSv) were used as a scoring matrix of clinically diseased pigs (Table 4). The data set ($n = 695$ samples) was initially subjected to chi-squared analysis in order to investigate the presence of virus and statistical association with clinical and pathological presentations. A significant association of virus and lung pathologies was observed for PCV2 ($p = .012$), PPV2 ($p = .001$) and PPV4 ($p = .01$). PCV2 was significantly linked to respiratory pathogenesis with respiratory symptoms ($p = .012$), pneumonia ($p = .017$) and broncho interstitial pneumonia (BIP) ($p = .036$). PPV2 also showed significance to the same respiratory pathogenesis parameters but with an additional pathogenesis of lung oedema and at greater statistical significance ($p = .001$). Notably, PPV3 presented no association with respiratory pathogenesis, and PPV4 had only significant association to pathogenesis and lung oedema. PCV2 was the only virus to demonstrate statistical significance ($p = .001$) with wasting symptoms, enlarged lymph nodes, lymphoid depletion and intracellular inclusions. PPV3 and PPV2 provided none of these

TABLE 4 Count of findings, signs and symptoms used in the latent class analysis

Symptom	Present	Absent	Total	Per cent
Wasting	61	149	210	29
Porcine respiratory and reproductive syndrome virus antibody by IFA (PRRS Ab)	4	206	210	1.90
Lymphoid depletion	68	144	212	32.10
Lymphoedema	22	187	209	10.50
Lymphadenopathy	49	164	213	23
Macrophage syncytia	29	183	212	13.70
intracellular inclusion bodies	26	186	212	12.30
Focal necrosis	17	195	212	8
Kidney pathology	32	173	205	15.60
Respiratory pathology	132	103	235	56.20
Lung consolidation	94	123	217	43.30
Lung oedema	51	163	214	23.80
Broncho interstitial pneumonia (BP-BIP)	49	162	211	23.20
Enteric pathology	155	83	238	65.10
Peritonitis	24	185	209	11.50
Stomach ulcer (gut ulcer)	18	195	213	8.50
Abdominal fluid	19	193	212	9
Porcine dermatitis and nephropathy Syndrome (PDNS)	10	188	198	5.10
Rash	10	202	212	4.70
Organomegaly	10	202	212	4.70
Brain oedema	15	197	212	7.10
Meningitis	16	196	212	7.50
Pericardium pathology	37	175	212	17.50
Eye pathology	5	207	212	2.40
Aborted	4	208	212	1.90

associations but PPV2 was statistically associated with lymphoedema ($p = .002$) with PPV4 also significantly associated with lymphoedema and intracellular inclusions ($p = .001$). In addition, PPV2 and PPV3 were also significantly associated with peritonitis and all novel PPVs were statistically associated with ulcers detected in the stomach, colon and caecum ($p = .001$ to $p = .004$). Kidney disease had the most significant association with PPV4 ($p = .001$), and PPV2 was less ($p = .038$), whereas PCV2 and PPV3 had no association. Porcine dermatitis and nephropathy syndrome (PDNS) were only associated with PPV2 ($p = .001$) and PPV3 ($p = .017$) and interestingly none with PCV2. The novel systemic PPVs and specifically PPV2, statistical analysis reveals, significant association to a range of clinical disease and pathologies; however, these associations are unable to infer the PPV's causal role in the associated pathogenesis.

3.4 | Latent class analysis

Viral co-infections were examined using logistic regression analysis and graphical 95% confidence interval level (Table 5 & Supplementary Data S2) and the clinical signs and pathologies plotted (Figure 3 and Supplementary Data S2). The frequency of clinical signs varied greatly, with, for example, enteric pathologies being recorded over 125 times whereas symptoms such as meningitis and rash were rarely seen across four studies (Figure 3). Data were excluded where animals had no clinical or pathological data recorded, whilst animals on which data were recorded were included, even if the data were recorded with the absence of a symptom, sign or finding. An important question addressed as part of the analysis was the choice of symptoms to include and the number of latent classes to be identified. There are 25 different symptoms reported, and the use of low-frequency symptoms is expected to be uninformative. To address these, each LCA model was run on the six symptoms occurring 50 times or more, the 14 symptoms occurring 20 times or more, and the 22 symptoms occurring 10 times or more, as well as all 25 symptoms. Each model was fit to between 1 and 6 latent classes, and these models were then reviewed, both to see whether there were major substantive differences between them, and, by veterinary experts, based on the clustering of clinical pathologies. For most models, the lowest

TABLE 5 Confidence interval

Detection of virus	Co-infection detection	Estimate	Lower confidence interval	Upper confidence interval
PCV2	PPV2	1.88	1.03	3.46
	PPV3	1.89	0.884	4.12
	PPV4	5.65	1.68	24.5
PPV2	PCV2	1.91	1.06	3.48
	PPV3	7.74	3.36	19.1
	PPV4	1.28	0.448	3.55
PPV3	PCV2	1.86	0.854	4.15
	PPV2	8.49	3.6	21.6
	PPV4	2.21	0.564	7.13
PPV4	PCV2	6.24	1.95	25.3
	PPV2	1.34	0.468	3.75
	PPV3	2.42	0.619	7.78

BIC was at 3 latent classes, and for one, the model fit to symptoms occurring 20 times or more, the lowest BIC was for 4 latent classes. A veterinary expert opinion established a substantively meaningful set of symptoms were those occurring ten times or more, for which 3 latent classes were the best fitting model. Our initial model (Figure 4, Supplementary Data S2) identified three groups of animals: those with predominantly respiratory symptoms, but no wasting, and fewer GI symptoms; those with a predominance of GI symptoms, and little respiratory symptoms and wasting; and a third group with prominent wasting, respiratory and gut symptoms. The ordering of these three groups is arbitrary within any given model.

To examine the effect of other covariates, specifically the age of the animal and evidence of viral infection, a series of latent class regression models were fitted with covariates representing the age group of the animal, and evidence of infection with PCV2 and PPV2-PPV4 (Supplementary Data S2). Age and PCV2 did contribute to class membership, and there was little evidence of any contribution of PPV2, PPV3 or PPV4 on their own to class membership. However, there was good evidence for a joint effect of PCV2 and PPV2, and some evidence for an interaction between them (Table 6). Once both viruses were added to the model, there was little remaining evidence for an effect of age (Table 7). The confidence intervals for these effects are wide, reflecting the relatively small number of animals, but the direction and approximate magnitude of the main effects appear to be robust to a range of modelling assumptions.

3.5 | Phylogenetic analysis

The partial PPV2 variable region of the VP1 capsid gene sequence from 33 selected samples with low Ct values was determined and edited to a 330-bp fragment to incorporate the equivalent sequenced regions deposited in GenBank for phylogenetic analysis (Figure 5) (Saekhow & Ikeda, 2014). The ML tree displays two distinct clades inclusive of 81 sequences downloaded from GenBank and PPV2 sequences from this study with an overall nucleotide percentage homology identity between the clades at 88 to 96%. The p-distance within clade 1 was 0 to 0.08, clade 2 at 0 to 0.06 and between clades p-distance of 0.04 to 0.13. The majority of PPV2 sequences in this study and dating from 1996 to 2012 clustered in clade 2 comprising of mainly European samples with a 6% homology difference. Only three PPV2 sequences from this study (MK639161 isolate 5, MK639164 isolate 8, MK639169 isolate 13), with nucleotide homology difference at 7%, and dating from 2004 and 2005, were positioned in the upper clade 1 with an assortment of sequence origins from South Korea (KY018938), USA (JX101461), China (MG345013), Brazil (KF725661) and other European countries. Wild boar sequences were situated in both clades of the PPV2 phylogenetic tree.

The complete viral capsid gene of 9 PPV3 UK samples with low Ct values was sequenced for phylogenetic analysis (Figure 6), situated at 2,180 to 4957bp on reference KY586145 PPV3 sequence and was aligned with an additional sixty full capsid sequences. The range of the nucleotide difference in the PPV3 phylogenetic tree was 5% across samples from both domestic pigs and wild boar originating from China, USA, South America and Europe creating three main clades with a p-distance between the clades of 0.01 to 0.05. The sequences from this study positioned within clade 3 (p-distance of 0 to 0.03) nearest to European nested clades. PPV3 MK649766 isolate 16 from 2010 had the lowest nucleotide homology with the Chinese strain JQ177082 from clade 2 at 95%. The clades 1 and 2 represent a small number of Chinese PPV3 sequences dating from 2005 to 2007 and 2010 to 2011, respectively.

The nearly complete capsid sequences for 3 PPV4 isolates, at the position of 3,430 to 5308bp of reference sequence KC701334, were phylogenetically analysed (Figure 7). The PPV4 ML tree split into two clades with high genetic nucleotide homology of 97% to 100% and nucleotide p-distance at 0.01 to 0.02. The PPV4 isolate 01 (MK609918) positioned in clade 1 with European sequences dating

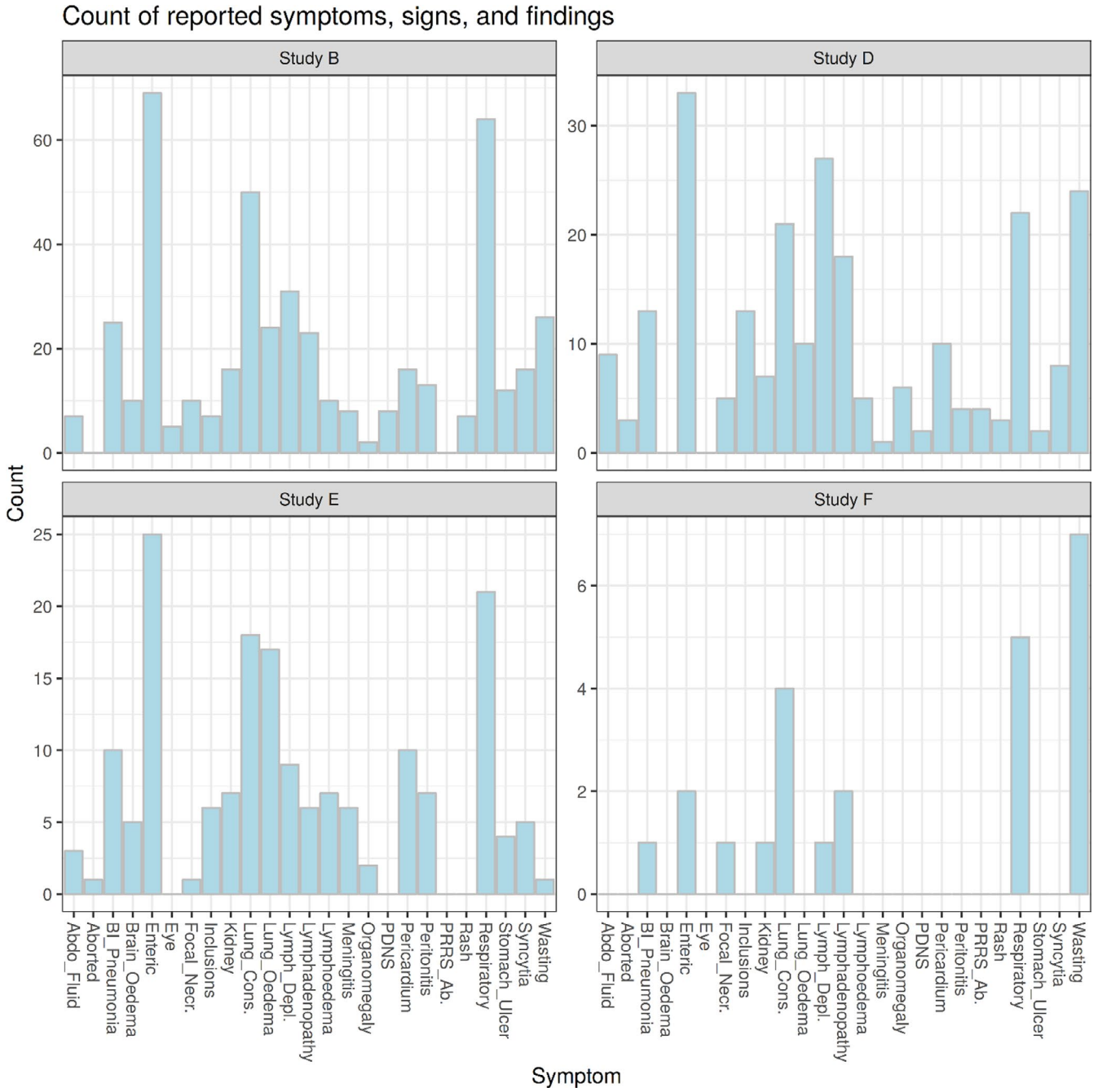

FIGURE 3 Count of reported symptoms, signs and findings per study Count of reported symptoms, signs and findings for each of the four studies that recorded a range of symptoms and physical findings on post-mortem, the number of occasions on which each was recorded

2004 to 2011 whereas clade 2 sequences were of mixed global origins from China and Brazil with isolate 07 (MK613834) and 05 (MK609919) nested within European clades.

Positive PCV2 samples ($n = 50$) were sequenced for PCV2 capsid open reading frame 2 in a parallel study revealing a genotype PCV2a shift to PCV2b occurring prior to the introduction of PCV2 vaccination (Collins & Zaykalova, 2020).

All sequences were deposited in NCBI GenBank with accession numbers; PPV2 MK639157-MK649760, PPV3 MK0649761-MK649769 and PPV4 MK609918, MK609919 and MK613834.

4 | DISCUSSION

There is a scientific premise concerning pig herd health issues attributed to viral aetiology in cases of multi-factorial PCVAD and PRDC which remains unexplained (Opriessnig et al., 2011; Qin et al., 2018). Parvovirus plays an important role as a viral co-factor in PCVAD and PRDC, and with compounding factors of high nucleotide substitution rate, positive selection from vaccination pressure, co-infection and recombination all contribute to the continual adaption and emergence of new parvovirus strains (Parrish & Kawaoka, 2005;

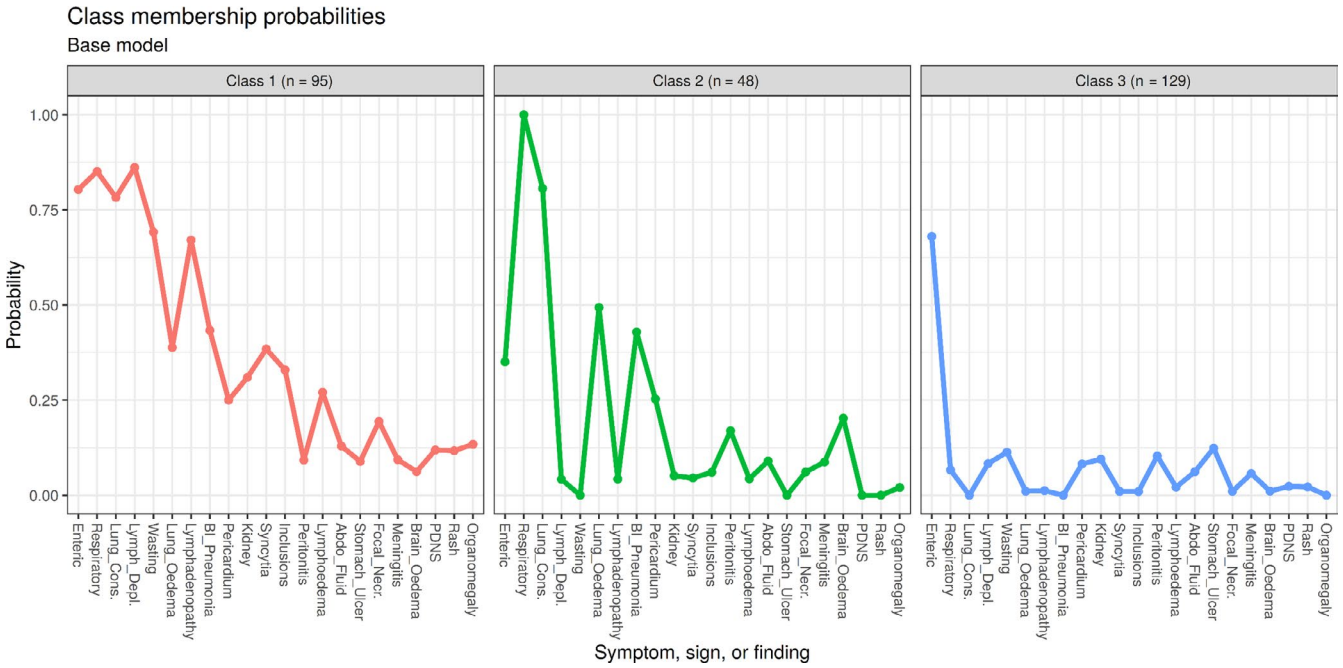

FIGURE 4 Latent class model membership probabilities. The estimated probability from the base latent class model which a particular symptom, sign or finding occurs amongst the members of each of the three latent classes occurs. Symptoms, signs and findings are ordered in terms of their overall frequency across the whole study sample

TABLE 6 Covariates of PPV2 and PCV2

Term	Class	Value coefficient	Value standard error	Odds ratio	Odds ratio lower	Odds ratio upper
Intercept	Class 2:1	0.58	0.41			
PCV2 count	Class 2:1	1.19	0.77	3.28	0.73	14.9
PPV2 count	Class 2:1	−0.29	0.91	0.75	0.13	4.41
PCV2 count.PPV2 count	Class 2:1	−2.06	1.24	0.13	0.01	1.45
Intercept	Class 3:1	−1.88	0.89			
PCV2 count	Class 3:1	3.78	1.09	43.7	5.14	370
PPV2 count	Class 3:1	1.49	1.3	4.45	0.34	57.4
PCV2 count.PPV2 count	Class 3:1	−3.17	1.48	0.04	0	0.77

TABLE 7 Covariates of PCV2 and PPV2 with age

Term	Class	Value coefficient	Value standard error	Odds ratio	Odds ratio lower	Odds ratio upper
Intercept	Class 2:1	−3.89	1.67			
PCV2 count	Class 2:1	2.21	1	9.11	1.28	64.9
PPV2 count	Class 2:1	0.26	0.68	1.3	0.34	4.91
Ages	Class 2:1	0.79	0.5	2.21	0.83	5.88
Intercept	Class 3:1	−2.27	1.29			
PCV2 count	Class 3:1	−0.52	0.61	0.59	0.18	1.96
PPV2 count	Class 3:1	0.83	0.87	2.3	0.42	12.6
Ages	Class 3:1	0.68	0.56	1.98	0.67	5.87

Shackelton & Holmes, 2006). The current study cohort represented clinically diseased animals from a range of production age groups, with samples dating from 1997 to 2012 of which 65% were acquired prior to the introduction of the PCV2 vaccine in ~2006. PPV2-PPV4 were found to exhibit similar infection prevalence profiles in pre-weaner (2%, 3% and 1%) and weaner (11%, 3% and 2%) age groups,

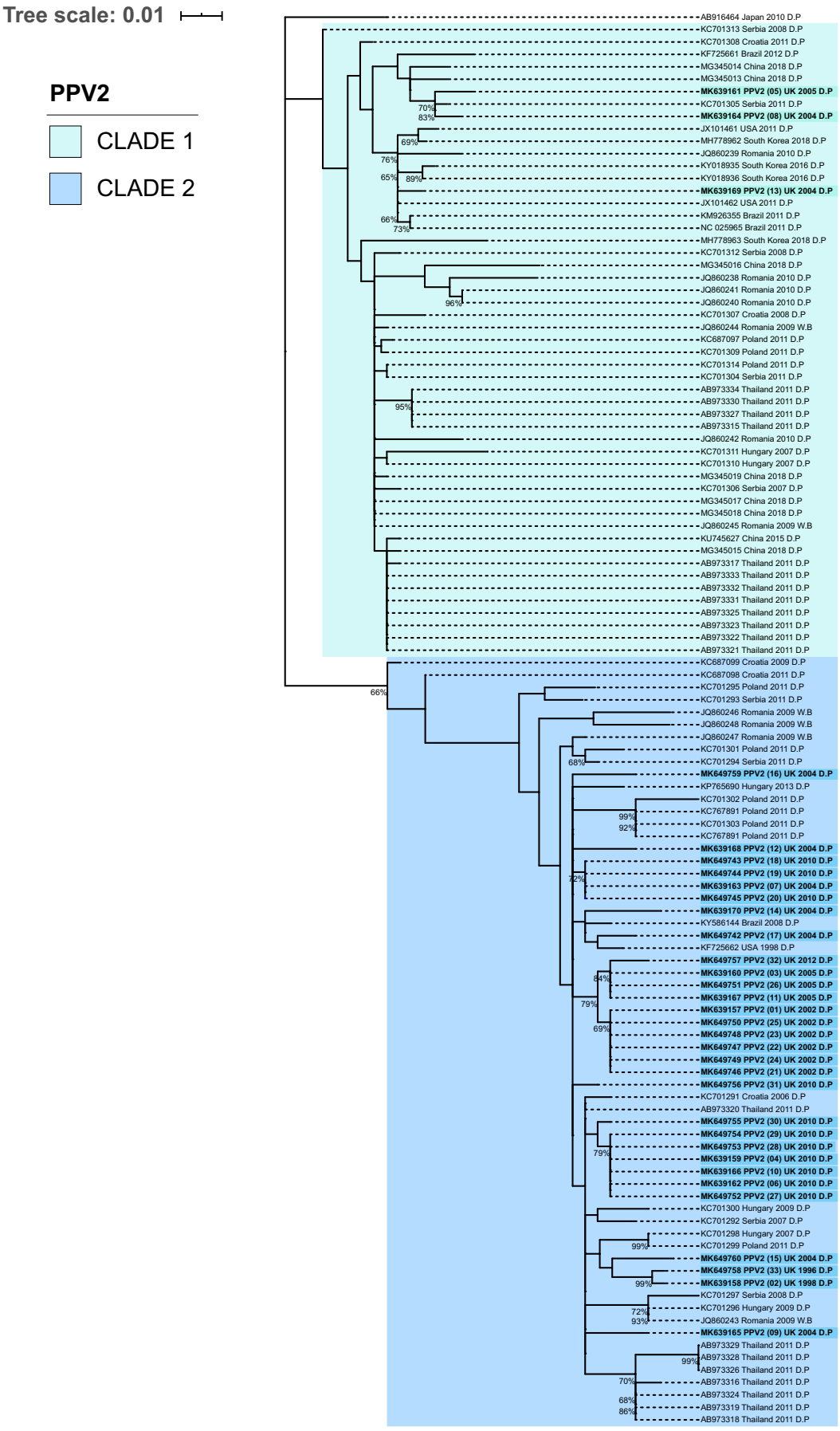
Tree scale: 0.01
PPV2
CLADE 1
CLADE 2
AB916464 Japan 2010 D.P
KC701313 Serbia 2008 D.P
KC701308 Croatia 2011 D.P
KF725661 Brazil 2012 D.P
MG345014 China 2018 D.P
MG345013 China 2018 D.P
MK639161 PPV2 (05) UK 2005 D.P
KC701305 Serbia 2011 D.P
MK639164 PPV2 (08) UK 2004 D.P
JX101461 USA 2011 D.P
MH778962 South Korea 2018 D.P
JQ860239 Romania 2010 D.P
KY018935 South Korea 2016 D.P
KY018936 South Korea 2016 D.P
MK639169 PPV2 (13) UK 2004 D.P
JX101462 USA 2011 D.P
KM926355 Brazil 2011 D.P
NC 025965 Brazil 2011 D.P
MH778963 South Korea 2018 D.P
KC701312 Serbia 2008 D.P
MG345016 China 2018 D.P
JQ860238 Romania 2010 D.P
JQ860241 Romania 2010 D.P
JQ860240 Romania 2010 D.P
KC701307 Croatia 2008 D.P
JQ860244 Romania 2009 W.B
KC687097 Poland 2011 D.P
KC701309 Poland 2011 D.P
KC701314 Poland 2011 D.P
KC701304 Serbia 2011 D.P
AB973334 Thailand 2011 D.P
AB973330 Thailand 2011 D.P
AB973327 Thailand 2011 D.P
AB973315 Thailand 2011 D.P
JQ860242 Romania 2010 D.P
KC701311 Hungary 2007 D.P
KC701310 Hungary 2007 D.P
MG345019 China 2018 D.P
KC701306 Serbia 2007 D.P
MG345017 China 2018 D.P
MG345018 China 2018 D.P
JQ860245 Romania 2009 W.B
KU745627 China 2015 D.P
MG345015 China 2018 D.P
AB973317 Thailand 2011 D.P
AB973333 Thailand 2011 D.P
AB973332 Thailand 2011 D.P
AB973331 Thailand 2011 D.P
AB973325 Thailand 2011 D.P
AB973323 Thailand 2011 D.P
AB973322 Thailand 2011 D.P
AB973321 Thailand 2011 D.P
KC687099 Croatia 2009 D.P
KC687098 Croatia 2011 D.P
KC701295 Poland 2011 D.P
KC701293 Serbia 2011 D.P
JQ860246 Romania 2009 W.B
JQ860248 Romania 2009 W.B
JQ860247 Romania 2009 W.B
KC701301 Poland 2011 D.P
KC701294 Serbia 2011 D.P
MK649759 PPV2 (16) UK 2004 D.P
KP765690 Hungary 2013 D.P
KC701302 Poland 2011 D.P
KC767891 Poland 2011 D.P
KC701303 Poland 2011 D.P
KC767891 Poland 2011 D.P
MK639168 PPV2 (12) UK 2004 D.P
MK649743 PPV2 (18) UK 2010 D.P
MK649744 PPV2 (19) UK 2010 D.P
MK639163 PPV2 (07) UK 2004 D.P
MK649745 PPV2 (20) UK 2010 D.P
MK639170 PPV2 (14) UK 2004 D.P
KY586144 Brazil 2008 D.P
MK649742 PPV2 (17) UK 2004 D.P
KF725662 USA 1998 D.P
MK649757 PPV2 (32) UK 2012 D.P
MK639160 PPV2 (03) UK 2005 D.P
MK649751 PPV2 (26) UK 2005 D.P
MK639167 PPV2 (11) UK 2005 D.P
MK639157 PPV2 (01) UK 2002 D.P
MK649750 PPV2 (25) UK 2002 D.P
MK649748 PPV2 (23) UK 2002 D.P
MK649747 PPV2 (22) UK 2002 D.P
MK649749 PPV2 (24) UK 2002 D.P
MK649746 PPV2 (21) UK 2002 D.P
MK649756 PPV2 (31) UK 2010 D.P
KC701291 Croatia 2006 D.P
AB973320 Thailand 2011 D.P
MK649755 PPV2 (30) UK 2010 D.P
MK649754 PPV2 (29) UK 2010 D.P
MK649753 PPV2 (28) UK 2010 D.P
MK639159 PPV2 (04) UK 2010 D.P
MK639166 PPV2 (10) UK 2010 D.P
MK639162 PPV2 (06) UK 2010 D.P
MK649752 PPV2 (27) UK 2010 D.P
KC701300 Hungary 2009 D.P
KC701292 Serbia 2007 D.P
KC701298 Hungary 2007 D.P
KC701299 Poland 2011 D.P
MK649760 PPV2 (15) UK 2004 D.P
MK649758 PPV2 (33) UK 1996 D.P
MK639158 PPV2 (02) UK 1998 D.P
KC701297 Serbia 2008 D.P
KC701296 Hungary 2009 D.P
JQ860243 Romania 2009 W.B
MK639165 PPV2 (09) UK 2004 D.P
AB973329 Thailand 2011 D.P
AB973328 Thailand 2011 D.P
AB973326 Thailand 2011 D.P
AB973316 Thailand 2011 D.P
AB973324 Thailand 2011 D.P
AB973319 Thailand 2011 D.P
AB973318 Thailand 2011 D.P

FIGURE 5 PPV2 phylogenetic analysis. The tree with the highest log-likelihood (−2324.94) is shown and analysis involved 114 nucleotide sequences. There were a total of 330 positions in the final data set and formed two main clades. Figures 5 to 7 phylogenetic analysis. The molecular phylogenetic analysis by maximum-likelihood method for PPV2-PPV4 virus's evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, obtaining 1,000 bootstrap replicates and then selecting the topology with superior log-likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

whilst fattener (>10 weeks) group profiles demonstrated higher levels of PPV2 (56%, 14% and 9%), signifying PPV2 to be the most prevalent novel PPV in this study. In contrast, PCV2 had a high level of prevalence at foetus level (36%) through vertical transmission which dipped at the pre-weaner age due to maternal antibody and/or vaccine effects, and which then increased to 61% and 77% in weaner and fattener groups, respectively. Statistical analysis in diseased pigs demonstrated the presence of PPV2 and PPV4 in pigs with lung disease and lymph node oedema pathologies. PPV2 detection significantly identified in pigs with pneumonia and BIP, whilst PPV4 was more specifically associated with lung oedema. However, although no statistical association of PPV2-4 and PCVAD wasting symptoms was identified, all had significant association with enteric ulcers with PPV2 and PPV3 significantly associated with peritonitis. Veterinary clinical scored data composed of 25 parameters from the diseased pig cohort were also applied to the detection of novel parvoviruses (PPV2-PPV4) utilizing LCA, indicating that PCV2 and PPV2 clustering, as a concurrent infection, was observed as significantly associated. Moreover, this analysis also found that no statistical significant association with PCVAD could be drawn from single viral infections or other combinations apart from PPV2 and PCV2. This observed PCV2 and PPV2 co-infection confirms findings from other studies using different statistical approaches (Opriessnig et al., 2013, 2014; Saekhow & Ikeda, 2014).

The observed concurrent infections of novel PPV and PCV2 resulted in PCV2 and PPV2 being the most predominant (12.9%), whilst other co-infections occurred at a much lower rate (<3.6%) similar to findings from a previous reported USA study (Opriessnig et al., 2014), with the viral distribution across age groups also similar to previous reports (Cságola et al., 2012; Xiao, Gerber, et al., 2013; Xiao et al., 2012). The low-level prevalence of novel PPVs in the pre-weaner and weaner age groups may indicate that the viruses were not vertically transmitted or that piglets acquired protection from maternally derived antibody and is comparable to the epidemiological serological profile study of PPV2 infection with severe respiratory disease, where onset occurred from 28 days post-decline of PPV2 protective maternal antibodies (Cságola et al., 2016). A subset of samples (10%) from this cohort were initially screened for PPV1 resulting in negative or very weakly positive results, with remaining samples not tested considering that the widely administered PPV1 vaccine would result in low prevalence. The most recent European PPV prevalence studies undertaken in Poland (2015–2017) on 19 pig farms detecting PPV1-6 genotypes reported a high prevalence of PPV2 (69%), PPV5 (28%) and PPV6 (32%) in sera with lower levels of PPV3 and PPV4, and due to vaccination a very low level of PPV1 notably, however, oral fluid levels detected higher levels of virus (Miłek et al., 2019). A study on 8 farms with non-acute PCVAD pig serum samples (2014–2017) reported a significant higher level of PCV2 virus detected when co-infected with PPV7 (Miłek et al., 2020). Assessments of the prevalence of novel PPVs and correlation to PCV2 genotypes showed a high prevalence of PPV2 occurring where the vaccine evading PCV2d genotype was detected (Opriessnig et al., 2014). Parallel analysis of this sample cohort detected only PCV2a and PCV2b genotypes, with PCV2b being the dominant strain from 2003 onwards whilst other PCV2 genotypes were not detected (Collins & Zaykalova, 2020). The global prevalence rates of novel PPVs are considerably variable, specifically the prevalence of PPV3 and PPV4 virus, and this variation is compounded by factors not fully understood and may affect the viral epidemiological dynamics, for example the country of origin, sample type, study design, environmental factors and year of sampling. A recent retrospective study (Sun et al., 2015) on PCVAD suspected pigs (mixed samples) in China revealed a similarly higher occurrence of PPV3 (45.11%) and PPV4 (21.56%), with a South African prevalence study of healthy and sick pigs (2015–2016) reporting a higher rate of PPV4 prevalence (43.6%) (Afolabi et al., 2018). Whilst these recent reports are indicative that prevalence rates for PPV3 and PPV4 may have increased, validated standardized assays, phylogenetic analysis, sample age and tissue type would be beneficial in enabling data comparisons to be made from any future studies.

The retrospective yearly virus prevalence, first detected PCV2 in 1998, and whilst profiling a high prevalence, a decline in frequency occurred with the introduction of the PCV2 vaccine (~2006) (Table 1, Figure S5). The impact of vaccine reduced the PCV2 frequency, which continued over several years, followed by a yearly incline of prevalence from 2009 onwards to 2012. The most consistently present novel PPV, PPV2, detection occurs years from 1998 to 2012; however, all novel PPV2-4 were undetectable from 2006 to 2008. The correlation of novel PPV absence is possibly linked with the PCV2 vaccine introduction and impact of reduction of PCV2 frequency (Table 1, Figure S5). It is well established that the suppression of the immune system by PPV1 co-infection allows the increases PCV2 viral replication and hence greater viral load, conversely, the presence of virus per year has highlighted, that PCV2 may also be essential for the interdependent existence of the novel PPV's warranting further investigations. The PCV2 and PPV2

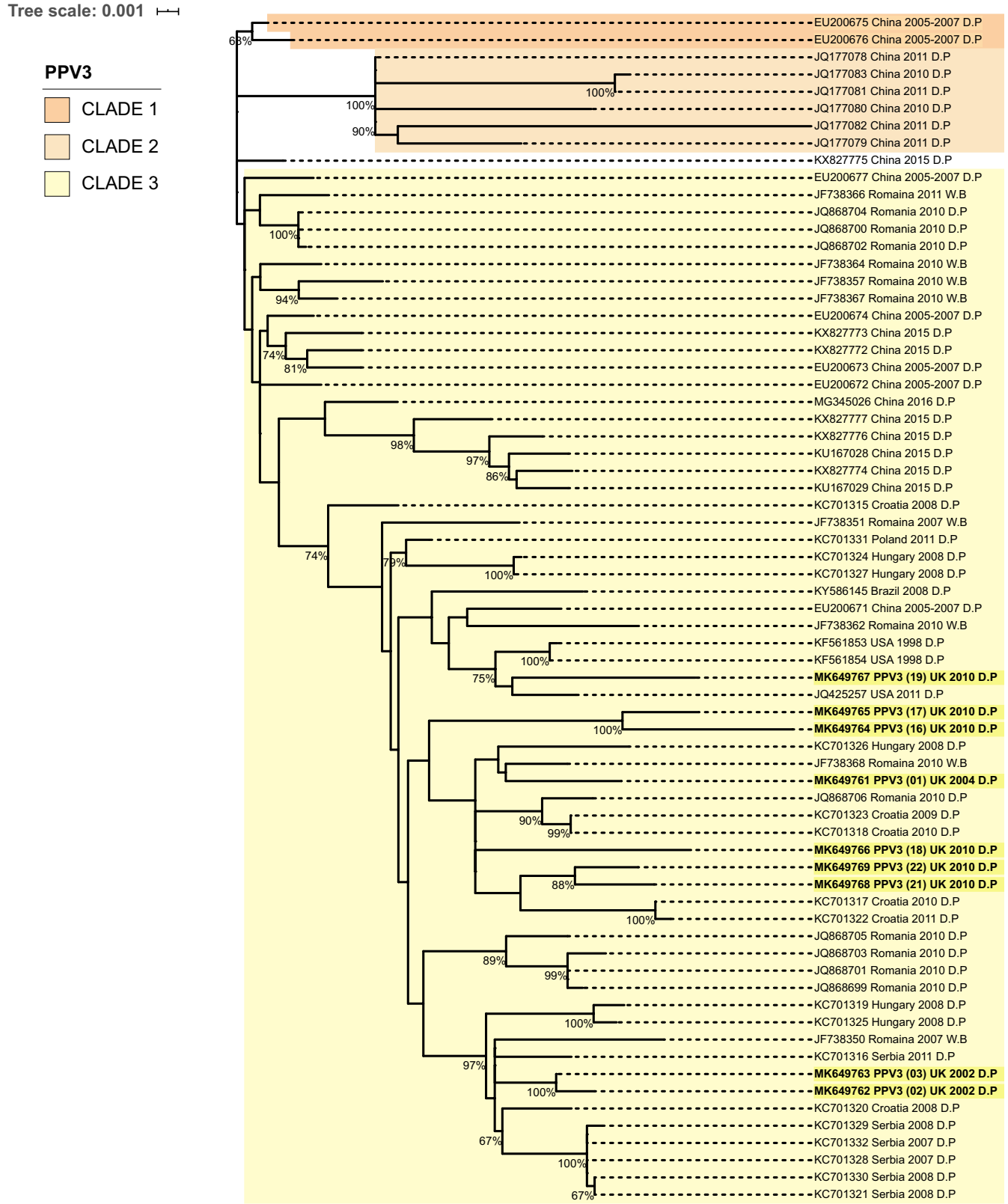

FIGURE 6 PPV3 phylogenetic analysis. The tree with the highest log-likelihood (−10311.93) is shown and analysis involved 69 nucleotide sequences. There were a total of 2,787 positions in the final data set and formed three clades

in this study detected virus from 1998 although an additional PPV2 sample was sequenced from 1996 and included in the phylogenetic sequencing analysis which may be the earliest reported detection. A previous retrospective study established PCV2 circulated in pig herds since 1962 and before the observation of clinical PMWS cases, and in the USA a study detected the PCV2 in 1998 (Jacobsen

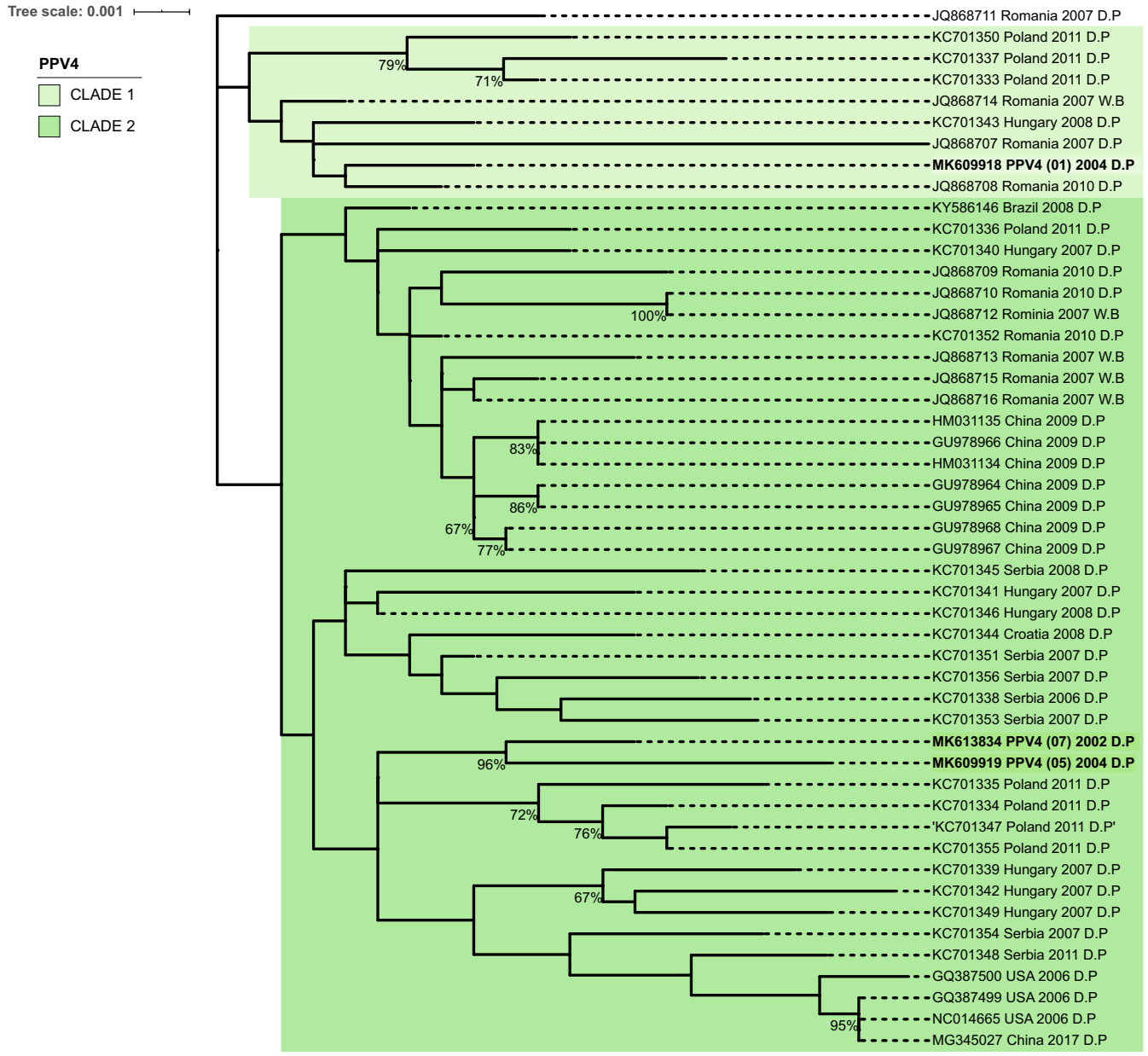

FIGURE 7 PPV4 phylogenetic analysis. The tree with the highest log-likelihood (−4422.01) is shown. The analysis involved 49 nucleotide sequences, and there were a total of 1731 positions in the final data set formed two clades

et al., 2009; Opriessnig et al., 2014). Both PPV3 and PPV4 were only detected from 2000 and are earlier than they were originally discovered although a previous study identified PPV3 in USA in 1998 whereas this may be the earliest reporting of PPV4.

Empirically, tissue sampled from a variety of sample types allowed for an insight into the tropism of these viruses with an overall prevalence of PCV2 (47%), PPV2 (17.3%), PPV3 (5.8%) and PPV4 (3.5%). PPV2 to 4 caused systematic infection in pigs with virus detected mainly in the lymphatic tissue, lungs, nasal tract, faeces and sera. Subsequent analysis showed that the lung tissue prevalence of PPV2 was high (28%) although PCV2 had the highest prevalence (45%). In the current study, bronchial and mediastinal lymph nodes situated near the respiratory system were only positive for PPV2 which supports evidence that PPV2 is involved in respiratory infection. PPV2 to 4 were found in a kidney sample tested meriting further investigation to better understand the role of PPV2-4 in kidney pathology and disease. It was also observed that PPV2 was detected at a much higher rate in lymphatic tissue such as tonsil (28%), spleen (43%), mesenteric LN (30%), mediastinal LN (29%), inguinal LN (52%) and bronchial LN (33%) than PPV3 and PPV4, indicating that in this cohort PPV2 is more virulent and better adapted to cause systemic infection. A USA study reported lung tissue prevalence of PPV2 (42.7%), PPV3 (9.1%) and PPV4 (4.3%) (Opriessnig et al., 2014) whereas a Japanese study in both clinically diseased and healthy slaughter age pigs' tonsils found elevated PPV2 prevalence of 100% and 58%, respectively

(Saekhow & Ikeda, 2014). A 2010 German study of hearts and tonsils of slaughter age pigs detected PPV2 in 55% of tonsils and 78% of hearts, with PPV3 and PPV4 detected at lower rates in tonsils only (Streck et al., 2013). This prevalence level demonstrated that the PPV2 virus could exist at subclinical levels in relatively healthy pigs, with lymphatic and heart tissue targeted by PPV2 particularly as parvovirus is known to favour mitotically active cells that enter the S-phase for their replication. Recent fundamental pathogenesis studies on PPV2 have provided further crucial information on viral tropism and infection dynamics. The pathology of PCV2 and PPV2 infection indicated that PPV2 may contribute to the pathologies of pneumonia observing significant association with lung lesions (Novosel et al., 2018). Opriessnig (2016) performed an experimental infection of PCV2 and PPV2 (via a tissue homogenate) finding infected pigs developed microscopic lesions with severe lymphoid depletion, consistent with PCVAD, demonstrating that PPV2 can act as the trigger for PCVAD in the presence of PCV2 infection.

Phylogenetic analysis of novel PPVs in this study showed the greatest genetic diversity occurred within PPV2 sequences where 114 PPV2 sequences of global origin from both domestic and wild boar formed two distinct clades as reported previously (Figure 5) (Cságola et al., 2012; Saekhow & Ikeda, 2014). A high nucleotide divergence of 88 to 96% with a p-distance of 0.04 to 0.13 between each clade in this study was determined. The VP1 PPV2 gene is a potential genotype subtyping region with a cut-off p-distance value of 0.04. Clade 1 sequences were comprised of chronologically more recent (up to 2018) PPV2 isolates, although it also included three early sequences isolated in this study (from 2004 and 2005 samples) demonstrating continual evolution and adaption of the PPV2 capsid. The evolutionary diversity of PPV2 capsid region mutations resulting from antigenic escape, host adaption or the role of viral pathogenicity remains to be established and highlights the need to continue to phylogenetically map current circulating strains assessing the divergence of the PPV2 clade 1. The PPV3 phylogeny maps three main clades with the majority of strains positioned within clade 3 originating from global strains although predominantly strains isolated from Europe. The clades 1 and 2 comprise of PPV3 strains from China isolated in 2007 to 2011 showing further divergence of PPV3 with an overall nucleotide homology difference of 5% across all clades. The PPV3 strains from this study are nested clades within clade 3 with the most similar strains to the European strains, including wild boar although one sequence mapped more closely to a USA strain from 1998 (Figure 6). The PPV4 capsid remains highly conserved in comparison with PPV2 (Cadar, Csagola, et al., 2013; Cadar, Lorincz, et al., 2013) with a high 97% nucleotide homology similarity. It would be advantageous to phylogenetically map more recently isolated PPV2, PPV3 and PPV4 viruses to ascertain whether further genetic drifting or recombination has occurred.

The findings of this study on clinically diseased pigs have demonstrated that PPV2, a genetically diverse virus, is a co-factor to PCV2 infection in PCVAD and PRDC. In the absence of isolated and cultured virus, statistical modelling by LCA analysis has indicated that cluster co-infection of PPV2 and PCV2 occurs in a diseased pig population, whereas PPV3 and PPV4 showed no cluster association with PCV2. Further investigations establishing viruses attributed to PRDC disease utilizing quantifiable PCR assays for viral load and including PPV5 to PPV7, PCV3 plus porcine bocaparvoviruses would be beneficial. Additionally, inclusion of swine influenza, an important primary pathogen of PRDC and a causative agent of lung lesions, should be considered as a critical parameter in statistical modelling analysis. Future work by viral metagenomics in longitudinal studies is an advantageous tool in understanding disease associations, co-infection dynamics of parvoviruses and PRDC. Successful viral isolation/propagation and the development of serological neutralization assays for PPV2-7 will be necessary in order to determine Koch's postulates, and characterize viral serotypes and cross-protection with the ultimate aim of developing a suitable vaccine candidate with good antigenic variability to protect pig herds. This study confirms the novel PPV 2–4 virus's co-infection occurs with PCV2 and can be statistically associated with respiratory disease and specifically, PPV2 co-infects with PCV2 in PRDC and PCVAD.

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CONFLICT OF INTEREST

There is no conflict of interest.

ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal guidelines, have been adhered to. The study included sampling of animals for diagnostic purposes and therefore did not require approval from the ethic committee.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the supplementary material of this article, and the sequenced data are deposited in NCBI GenBank.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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