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Research article

Changes on the viral capsid surface during the evolution of porcine circovirus type 2 (PCV2) from 2009 till 2018 may lead to a better receptor binding

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Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of porcine circovirus-associated diseases (PCVAD). Three major PCV2 genotypes (PCV2a, PCV2b, and PCV2d) have been identified globally. Despite their worldwide distribution, the prevalence and genetic evolution of PCV2 in Belgium has not previously been determined. In this study, 319 samples from animals suffering from diseases likely to be associated with PCV2 were collected from 2009 to 2018 and analysed by virus titration. The overall prevalence of PCV2 in PCVAD-suspected cases was 15.7 per cent (50/319). The phylogenetic analysis demonstrated that at least three genotypes (PCV2a, PCV2b, and PCV2d) circulated in Belgium from 2009 till 2018, and that PCV2 evolved from PCV2a to PCV2b and from PCV2d-1 to PCV2d-2. Sequence comparison among the forty-three PCV2 isolates showed that they had 89.7–100 per cent nucleotide-sequence and 88.5–100 per cent amino-acid-sequence identities. Three amino acid sites were under positive selection. Three-dimensional analysis of genotype-specific amino acids revealed that most of the mutations were on the outside of the cap protein with a few conserved mutations present on the inner side. Mutations toward more basic amino acids were found on the upper and tail parts of two connecting capsid proteins which form one big contact region, most probably involved in receptor binding. The lower part was relatively conserved. This polarity change together with the formation of an extruding part drive the virus to a more efficient GAG receptor binding. Taken together, these results showed a genotype shift from PCV2a to PCV2b and later on from PCV2d-1 to PCV2d-2, and a PCV2 evolution toward a better receptor binding capacity.

Key words: porcine circovirus type 2; phylogenetic analysis; genetic diversity; deduced amino acid sequence.

1. Introduction

Porcine circovirus type 2 (PCV2) is one of the most widespread viruses that cause economically relevant infections in swine. After its first identification in the late 1990s, PCV2 is linked with a variety of disease manifestations, such as postweaning multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), and reproductive failure (Allan 1998; Rosell et al. 2000; Kim, Chung, and Chae 2003; Madson and Opriessnig 2011). All these diseases are collectively termed porcine circovirus-associated diseases (PCVAD) (Opriessnig, Meng, and Halbur 2007). Retrospective serological studies performed on

archived samples confirmed PCV2 infections from 1962 in Germany, 1969 in Belgium, 1970 in the UK, 1973 in Ireland, and 1985 in Spain and Canada (Magar, Müller, and Larochelle 2000; Walker et al. 2000; Sanchez, Nauwynck, and Pensaert 2001a; Rodriguez-Arrioja et al. 2003; Grierson et al. 2004; Jacobsen et al. 2009). This demonstrated that PCV2 circulated years prior to its identification.

PCV2 belongs to the *Circovirus* genus of the family *Circoviridae*. It is an icosahedral and non-enveloped DNA virus, with a diameter of only 17 nm. The single-stranded circular PCV2 genome is comprised of 1,766–8 nucleotides (nt) (Guo et al. 2010). It contains two major open reading frames (ORFs): ORF1 encodes two replication-associated proteins (rep and rep′), which is essential for the multiplication of the PCV2 genome but absent in the assembled virion; ORF2 encodes a viral capsid protein (cap), which is the only structural protein. The capsid protein determines the antigenicity and virulence of PCV2 (Hamel, Lin, and Nayar 1998; Nawagitgul et al. 2000; Lefebvre et al. 2008; Saha et al. 2012b). Mutation of one or two amino acids in the cap may affect the virulence and pathogenicity of PCV2 (Huang et al. 2011; Saha et al. 2012a). In addition, ORF2 alone can serve as a phylogenetic and epidemiologic marker for comparative analysis of PCV2 (Olvera, Cortey, and Segalés 2007).

Based on phylogenetic analysis of PCV2 genomic or ORF2 sequences, PCV2 can be divided into six subtypes (PCV2a–f). PCV2a and PCV2b have been documented worldwide (Segales et al. 2008). PCV2a was the most prevalent genotype until approximately 2003 while PCV2b became the predominant one after 2004 (Segales, Kekarainen, and Cortey 2013). This indicates an obvious genotype shift from PCV2a to PCV2b, which was described in several countries (Carman et al. 2006; Cheung et al. 2007; Dupont et al. 2008; Timmusk et al. 2008; Wiederkehr et al. 2009; Cortey et al. 2011; Segales, Kekarainen, and Cortey 2013). This shift on genotype prevalence coincided with the advent of the most severe outbreaks of PCVAD. However, under experimental conditions, the virulence of PCV2a and PCV2b does not differ significantly (Opriessnig et al. 2008; Saha et al. 2010). PCV2c was initially detected in archived swine serum samples in Denmark, and has recently been found in a feral pig in Brazil (Dupont et al. 2008; Franzo et al. 2015). However, this genotype is likely of minor importance. The origin of PCV2d strains can be traced back to 1998 in Switzerland and is now widespread in many countries, suggesting a genotype shift from PCV2b to PCV2d (Wei et al. 2013; Xiao, Halbur, and Opriessnig 2015; Kwon et al. 2017; Ramos et al. 2017; Thangthamniyom et al. 2017). PCV2d is divided into two subclades, PCV2d-1 and PCV2d-2 (Xiao, Halbur, and Opriessnig 2015). The majority of PCV2d-1 strains were circulating during 1999–2011 while PCV2d-2 was first identified in 2006, indicating that PCV2d-1 is a possible ancestor of PCV2d-2 (Xiao, Halbur, and Opriessnig 2015). The fifth genotype, PCV2e, was first discovered in Mexican pigs and then in the USA in a retrospective study, with the earliest sequence dating back to 2006 (Harmon et al. 2015; Davies et al. 2016). The last genotype, PCV2f, was first identified in China and is also present in Croatia, India, and Indonesia (Bao et al. 2018).

In Belgium, the presence of PCV2 was demonstrated in 1969 based on a retrospective serological analysis of PCV2 antibodies in domestic and feral pig populations (Sanchez, Nauwynck, and Pensaert 2001a). Large PMWS outbreaks have never been described in Belgium (Labarque et al. 2000; Meerts et al. 2004). It is thought that the use of Piétrain boars for the production of hybrid fattening pigs forms the basis of this low susceptibility. Recent work has demonstrated a more efficient uptake and disintegration of PCV2 by monocytes from purebred Piétrain than those from purebred Landrace and Large White, which may in part explain this better resistance (Wei et al. 2018). Until now, only four Belgian strains were isolated from PMWS-affected piglets: strains 1206 and VC2002-K39 were identified as PCV2b, strain VC2002-K2 as PCV2d-1 (originally identified as PCV2b based on the old classification system), and strain 4D4 as a recombinant strain of VC2002-K2 and VC2002-K39 (Meerts et al. 2004, 2005; Lefebvre et al. 2008, 2009; Saha et al. 2014; Xiao, Halbur, and Opriessnig 2015). Despite continuous reports of newly emerging strains and global genotype shifts, there was little information available regarding the molecular epidemiology and evolution of PCV2 in Belgium. The objectives of this study were to examine the prevalence and genetic evolution of PCV2 in Belgium from 2009 till 2018, and to find the driving force behind PCV2 evolution by analyzing the amino acid differences.

2. Materials and methods

2.1 Sample collection

A total of 319 samples (lungs, kidneys, spleen, lymph nodes, serum, or a mix of organs) were collected from unthrifty pigs with clinical signs of PCVAD in different regions of Belgium from 2009 to 2018. These samples were submitted for diagnostic analysis to the Laboratory of Virology, Faculty of Veterinary Medicine, Ghent University. A piece of the organ samples was homogenized for virus titration and DNA extraction, and the rest of the samples were stored at −70 °C.

2.2 Virus isolation and titration

Twenty per cent (w/v) suspensions of each tissue sample or pools were made in cold PBS. Afterward, the PCV2 titers were determined by virus titration on PK-15 cells as described elsewhere (Sanchez et al. 2001b). The virus titers were expressed as $\log_{10}TCID_{50}$/g of tissue ($TCID_{50}$ = tissue culture infectious dose with 50% endpoint). Samples were categorized into three groups with high ($\geq$4.5 $\log_{10}TCID_{50}$/g), medium (3–4.5 $\log_{10}TCID_{50}$/g), and low ($\leq$3 $\log_{10}TCID_{50}$/g) virus titers according to previously defined criteria (Sanchez et al. 2004). The detection limit of this technique was $10^{1.7}$ $TCID_{50}$/g tissue (Saha et al. 2011).

2.3 DNA extraction, PCR, and sequencing

Total DNA was extracted from tissue homogenates or serum using QIAamp Cador Pathogen mini kit (Qiagen) following the manufacturer's instructions. The viral DNA extracted from PCV2 strain 1121 virus stock and double distilled water were used as positive and negative controls during PCR analysis, respectively. To amplify the ORF2 gene, a conventional PCR assay was performed using two primer pairs (ORF2-fw: 5′-gcgca cttcttttcgttttcag-3′, ORF2-rev: 5′-gaatgcggccgcttatcacttcgtaatg gtttttattattca-3′; pgPCV2-fw: 5′-ggctgtggcctttgktac-3′, pgPCV2-rev: 5′-tgtrgaccacgtaggcctcg-3′) (Lefebvre et al. 2008; Wei et al. 2018). The PCR reaction (25 µl) contained 5 µl of 5 × PCR buffer, 0.2 µl of 100 mM dNTPs, 1 µl of 10 µM primers each, 5 µl of DNA template, 0.5 µl of Herculase II fusion DNA polymerase, and 12.3 µl water. The thermal cycling programme for the PCR reaction was 94 °C for 2 min, then 30 cycles of 94 °C for 15 s, 54 °C for 30 s, and 68 °C for 1 min (ORF2-fw/rev) or for 2 min (pgPCV2-fw/rev), followed by 68 °C for 10 min. The amplification products were examined by agarose gel electrophoresis and were visualized by ethidium bromide staining and UV light. The amplicons were sent to GATC (Constance, Germany) for Sanger sequencing. The sequences of the DNA fragments were then assembled

using EditSeq programme of the DNASTAR version 7.0 (DNASTAR Inc., Madison, WI, USA).

2.4 Bioinformatics analysis

Forty-three ORF2 nucleotide sequences obtained in this study and twenty-nine published PCV2 ORF2 sequences available in GenBank (Supplementary Table S1) were included in the phylogenetic analysis. All sequences were aligned using the Clustal W method of the MegAlign programme of the DNASTAR version 7.0. Nucleotide and amino acid sequence similarities were analysed based on the aligned dataset. A phylogenetic tree was constructed using the neighbour-joining method in MEGA 7 software (Kumar, Stecher, and Tamura 2016). Reliability of the neighbour-joining tree was calculated using 1,000 bootstrap replicates. Sequences obtained were deposited in GenBank.

2.5 Selection pressure analysis

An analysis of selection pressure on the cap protein was evaluated using different methods in the Datamonkey (http://www.datamonkey.org), based on the ratios between non-synonymous and synonymous substitution rates (dN-dS) (Pond and Frost 2005). Pervasive and episodic diversifying/purifying selection was estimated using single-likelihood ancestor counting (SLAC), fixed effects likelihood (FEL), mixed effects model of evolution (MEME), and fast unconstrained Bayesian approximation (FUBAR) (Pond and Frost 2005; Murrell et al. 2012, 2013). The positive signals were accepted when the *P*-value was below 0.1 in SLAC, FEL, and MEME and when the posterior probability (Post. Pr.) was above 0.9 in FUBAR. Sites were assumed to be under positive selection pressure when the positive signals were detected by at least two methods.

2.6 Three-dimensional (3D) mapping of amino acids in the PCV2 cap protein

The accurate 3D structures of PCV2 cap protein of different genotypes were predicted using iterative threading assembly refinement (I-TASSER) server (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) based on the sequence of a PCV2a strain (Fh17; GenBank no. AY322004), a PCV2b strain (1206; GenBank no. EF990644), and a PCV2d strain (K2; GenBank no. EF990645) (Zhang 2008; Roy, Kucukural, and Zhang 2010; Yang et al. 2015). The structures of cap protein were displayed with PyMOL software (South San Francisco, CA, USA).

3. Results

3.1 Percentage of infectious PCV2-positive cases in Belgium from 2009 till 2018

The total number of collected cases and virus isolation positive cases of each year are shown in Table 1. Of the 319 clinical samples, 50 samples (15.7%) were positive for PCV2 by virus titration. The total samples were then categorized by virus titer ($log_{10}TCID_{50}/g$) (Sanchez et al. 2004). Eight per cent of the samples (25/319) contained large amounts of infectious PCV2 ($\geq$4.5 $log_{10}TCID_{50}/g$), 1 per cent of the samples (3/319) contained moderate amounts of infectious PCV2 (3–4.5 $log_{10}TCID_{50}/g$), 7 per cent of the samples (22/319) contained low amounts of infectious PCV2 ($\leq$3 $log_{10}TCID_{50}/g$), and 84 per cent of the samples (269/319) were negative for PCV2 isolation.

3.2 Sequencing of PCV2 ORF2

A total of forty-three samples were subjected to DNA extraction and PCR amplification of the ORF2 gene. The distribution of these PCR-examined samples in each year is shown in Table 1. Sixteen PCV2 isolates from 2009 were randomly selected for further PCR analysis from the twenty-six PCV2-titration positive cases due to a relatively large sample size; all PCV2 isolates from 2010 till 2017 (twelve isolates in 2010, four isolates in 2009, four isolates in 2010, four isolates in 2013 and zero isolate in 2014–7) were analyzed by PCR; three out of six serum samples collected in 2018 that were PCR positive were further sequenced. The detailed information of these PCR-examined samples is shown in Table 2, including the code, year of isolation, tissue origin, PCV2 titer, and GenBank accession number. The complete ORF2 sequences were 702 or 705 base pairs (bp) in length.

3.3 Phylogenetic and homology analyses of PCV2 ORF2 sequences

Genetic and phylogenetic analyses were performed using ORF2 sequences of forty-three Belgian PCV2 isolates and twenty-nine reference sequences deposited in GenBank by neighbour-joining method. The phylogenetic tree showed that the forty-three PCV2 sequences obtained could be classified into three distinct genotypes (Fig. 1). Three of the forty-three (7%) PCV2 isolates belonged to genotype 2a, thirty-one of the forty-three (72%) isolates to genotype 2b, and nine of the forty-three (21%)

Table 1. Number of total collected samples, PCV2 isolation-positive cases, PCR-examined cases, and PCV2 genotypes identified each year in 319 samples collected from 2009 to 2018 in Belgium.

Year	Total collected	Virus isolation positive (%)	PCR examined	PCV2a	PCV2b	PCV2d	
						PCV2d-1	PCV2d-2
2009	141	26 (18.4%)	16/26	3	12	1	0
2010	104	12 (11.5%)	12/12	0	7	5	0
2011	29	4 (13.8%)	4/4	0	4	0	0
2012	18	4 (22.2%)	4/4	0	4	0	0
2013	7	4 (57.1%)	4/4	0	4	0	0
2014	3	0	0	0	0	0	0
2015	7	0	0	0	0	0	0
2016	1	0	0	0	0	0	0
2017	3	0	0	0	0	0	0
2018	6	0	3	0	0	0	3
Total	319	50 (15.7%)	43	3	31	6	3

Table 2. Information on PCV2 PCR-positive isolates collected in Belgium from 2009 to 2018.

No.	Isolates (cases)	Collection year	Sample	PCV2 titers ($log_{10}TCID_{50}/g$)	Accession No.	Genotype
1	09V003	2009	Mixed organs	4.7	MK005834	2b
2	09V005	2009	Lung	2.0	MK005831	2a
3	09V019	2009	Spleen	4.5	MK005835	2b
4	09v026	2009	Lymph node	≥5.5	MK005832	2a
5	09V042	2009	Lung + lymph node	≥5.5	MK005836	2b
6	09V089	2009	Organs foetus	Positive[a]	MK005833	2a
7	09V117-1	2009	Lung	4.3	MK005837	2b
8	09V117-2	2009	Lung	≥5.5	MK005838	2b
9	09V118	2009	Spleen	4.7	MK005839	2b
10	09V141	2009	Lung	5.0	MK005840	2b
11	09V153	2009	Lymph node	≥5.5	MK005841	2b
12	09V178	2009	Lymph node	3.0	MK005842	2b
13	09V257	2009	Spleen	2.0	MK005843	2b
14	09V408	2009	Lung	3.2	MK005844	2b
15	09V410	2009	Kidney	2.0	MK005845	2b
16	09V448	2009	Lymph node	5.5	MK005865	2d-1
17	10V005	2010	Lymph node	4.7	MK005846	2b
18	10V008	2010	Lung	2.5	MK005847	2b
19	10V009	2010	Spleen + lymph node	4.7	MK005866	2d-1
20	10V059	2010	Lymph node	2.5	MK005848	2b
21	10V139	2010	Lung	3.0	MK005849	2b
22	10V147	2010	Lymph node	4.5	MK005850	2b
23	10V149	2010	Lung + lymph node	5.5	MK005867	2d-1
24	10V183	2010	Lymph node + heart	2.0	MK005851	2b
25	10V232	2010	Lymph node	≥5.5	MK005852	2b
26	10V513	2010	Lung	2.5	MK005868	2d-1
27	10V532	2010	Spleen + lung	2.0	MK005869	2d-1
28	10V542	2010	Lymph node	2.0	MK005870	2d-1
29	11V012	2011	Lymph Node	5.3	MK005853	2b
30	11V152	2011	Spleen	1.7	MK005854	2b
31	11V300	2011	Lung	≥5.5	MK005855	2b
32	11V519	2011	Lung	Positive[a]	MK005856	2b
33	12V001	2012	Organs foetus	2.8	MK005857	2b
34	12V042-1	2012	Mixed organs	4.5	MK005858	2b
35	12V042-2	2012	Mixed organs	4.5	MK005859	2b
36	12V042-3	2012	Mixed organs	4.5	MK005860	2b
37	13V067	2013	Lung	≥5.5	MK005861	2b
38	13V068	2013	Spleen	5.0	MK005862	2b
39	13V069	2013	Lymph node	≥5.5	MK005863	2b
40	13V071	2013	Spleen	5.0	MK005864	2b
41	18v047	2018	Serum	Negative	MH287045	2d-2
42	18v126	2018	Serum	Negative	MK005871	2d-2
43	18v152	2018	Serum	Negative	MK005872	2d-2

[a]Positive means that the sample was positive for virus isolation, but the corresponding virus titer was not recorded.

isolates to genotype 2d. PCV2c, PCV2e, and PCV2f genotypes were not detected in the sample set.

The genotype distribution of the forty-three Belgian PCV2 isolates from 2009 till 2018 is listed in Table 1. All three samples of PCV2a genotype were collected in 2009. Two of them clustered with an isolate in Canada (GenBank no. AF109398), while one of them clustered with an isolate from France in 2003 (GenBank no. AY322004). PCV2b was the most common genotype found from 2009 to 2013, with relatively limited genetic diversity. PCV2d formed an independent cluster which was further subdivided into two separate subclades: PCV2d-1 and PCV2d-2. Interestingly, PCV2d-1 isolates were found in 2009 (1/6) and in 2010 (5/6), while in 2018 all PCV2d sequences clustered in the PCV2d-2 clade (3/3). Furthermore, one strain 10V009 from the PCV2d-1 clade clustered with the VC2002-K2, the first isolated Belgian PCV2d-1 strain (Meerts et al. 2004; Saha et al. 2012b; Xiao, Halbur, and Opriessnig 2015).

The length of the ORF2 of PCV2a and PCV2b was 702 bp, while that of PCV2d was 705 bp resulting in an additional amino acid, lysine, at the 3′-terminus of the capsid protein. Pairwise-sequence comparisons revealed that the complete ORF2 sequences of forty-three PCV2 isolates shared 89.7–100 per cent nucleotide sequence and 88.5–100 per cent amino acid sequence identities. For each genotype, the homology of nucleotide and amino acid sequences were as follows: for the PCV2a isolates ($n = 3$): 94.9–99.9 per cent and 95.4–100 per cent; for the PCV2b isolates ($n = 31$): 95.2–100 per cent and 94–100 per cent, and for the PCV2d isolates ($n = 9$): 97.7–100 per cent and 97.0–100 per cent (Table 3). The PCV2d sequences exhibited the lowest variability among the three Belgian PCV2 genotypes. Despite this highest homology, the Belgian PCV2d genotype could be

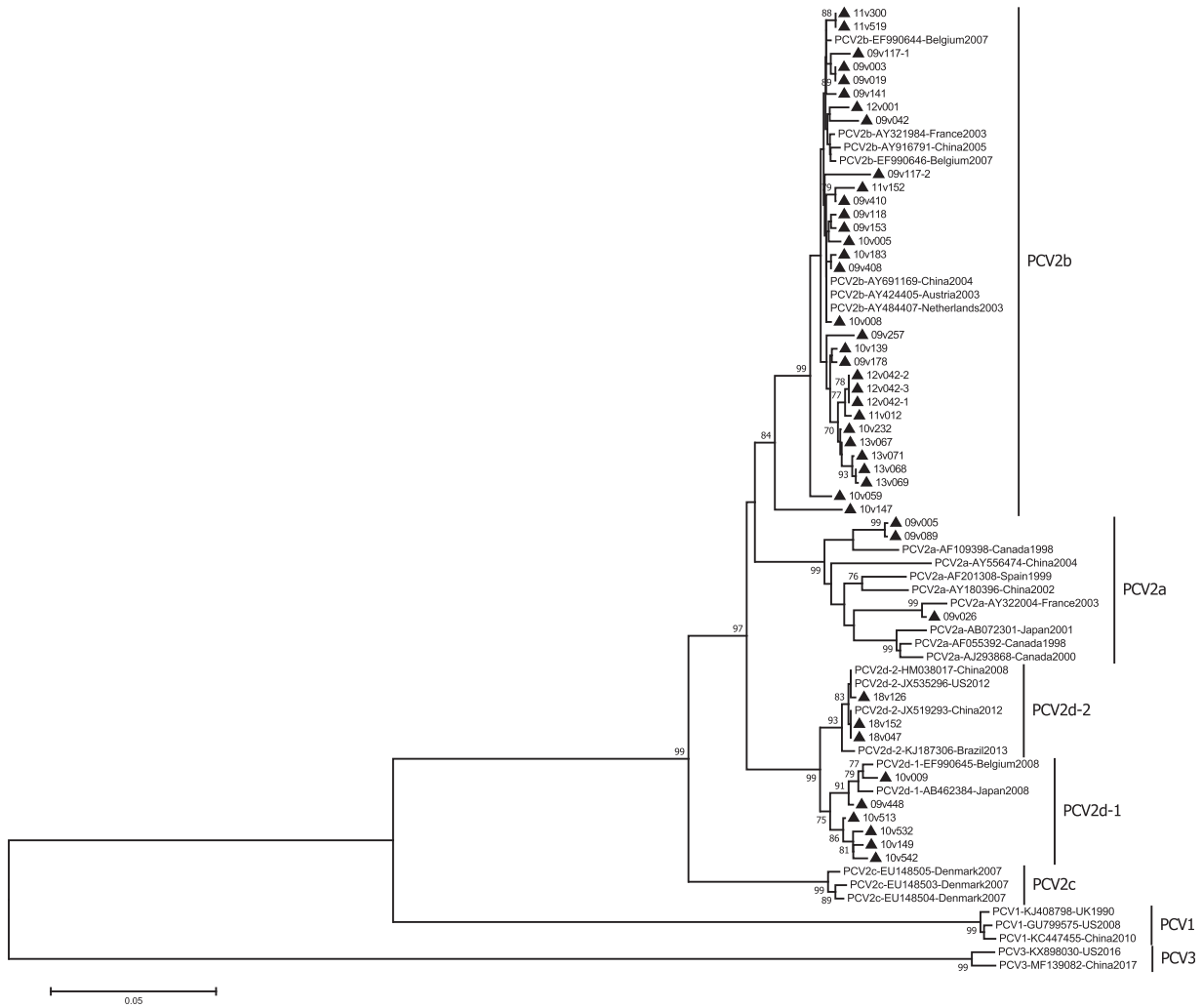

Figure 1. Phylogenetic analysis of PCV2 isolates based on ORF2 sequences obtained in this study (▲) together with the reference sequences from GenBank. The neighbour-joining tree was constructed by molecular evolutionary genetics analysis (MEGA) software version 7.0. The PCV1 and PCV3 sequences provided an outgroup to root the tree.

separated into two subtypes. The ORF2 gene of 6 PCV2d-1 isolates demonstrated 97.9–99.4 per cent nucleotide sequence and 97.0–99.1 per cent amino acid sequence identities whereas that of three PCV2d-2 sequences had 97.7–100 per cent and 97.0–100 per cent identities.

3.4 Selection pressure analysis

Selection pressure on the capsid protein of Belgian PCV2 isolates was estimated. In the difference between non-synonymous and synonymous substitution rates (dN-dS), the cap gene was negative (−1.575), indicating that the capsid of Belgian PCV2 had evolved under purifying selection. This is in accordance with previous studies where purifying selection was identified in the cap gene (Olvera, Cortey, and Segalés 2007; Xiao, Halbur, and Opriessnig 2015). However, three codons (positions 59, 131, and 191) were identified under positive selection through the MEME ($P < 0.1$) and FUBAR approaches (Post. Pr. > 0.9) (Table 4). Position 59 is a critical amino acid that is part of the neutralizing epitope (Huang et al. 2011); position 131 is important for determining neutralization activity (Saha et al. 2012a); and the mutation at position 191 may affect the growth ability of PCV2 both *in vitro* and *in vivo* (Fenaux et al. 2004a).

3.5 Amino acid analysis of PCV2 cap sequences

The amino acid alignment of the capsid protein encoded by the ORF2 gene was further conducted for the forty-three PCV2 strains in this study (Supplementary Fig. S1). Amino acid comparisons among the forty-three PCV2 isolates and twenty-one reference strains revealed that a greater diversity was found in three major regions (53–91, 121–136, and 169–217) and at three major positions (151, 232, and 234). Moreover, certain amino acids only appear in strains of one but not the other two genotypes. For instance, ten amino acid residues (75N/K, 76L, 86T, 88K, 89I, 91I, 123I/V, 136Q, 190S/T, and 232N/K, indicated in red) were only found in clusters of PCV2a isolates; fifteen (11K/R, 29F/L, 82K/P, 85H/G, 89L/R, 112P/T, 141S/Y, 169A/S, 188H/Q, 190A, 206V, 210D/G/E, 215G/I/V, 217L/M, and 222T/R, indicated in orange) in PCV2b isolates, and twelve (10G/R, 53I, 59A/K/R, 68N, 130I/V, 134N, 169W/R/G, 188P/Q, 200P/T, 207H/Y, 215I, and 216A/T, indicated in green) in the clusters of PCV2d (Supplementary Table S2). Among these variable amino acids,

Table 3. Per cent of nucleotide and amino acid sequence similarity of ORF2 among reference strains and PCV2 isolates in our study by pairwise comparison.

		Nucleotide (nt) and amino acid (aa) identities of strains (%)							
		Type C (AF109398) (PCV2a)	Isolates in our study (PCV2a)	1206 (EF990644) (PCV2b)	Isolates in our study (PCV2b)	VC2002-K2 (EF990645) (PCV2d-1)	Isolates in our study (PCV2d-1)	BDH (HM038017) (PCV2d-2)	PCR isolates in our study (PCV2d-2)
Type C (AF109398) (PCV2a)	nt	100	95.2–97.5	93.5	91.9–94	92.3	91.2–92.3	92.1	92.1
	aa	100	93.1–96.2	91.9	88.8–93.8	89.4	88.1–89.4	88.8	88.8
Isolates in our study (PCV2a)	nt	–	94.9–99.9	92.6–93.3	91.5–95.2	90.0–93.7	89.7–94.2	90.2–93.4	90.0–93.6
	aa	–	95.7–100	90.6–91.9	89.3–94.9	88.9–90.2	87.2–90.6	87.6–89.7	87.6–90.2
1206 (EF990644) (PCV2b)	nt	–	–	100	98.3–99.6	93.6	93.4–94.6	94.3	94.2–94.3
	aa	–	–	100	95.3–99.6	93.2	93.2–94.4	93.6	93.6
Isolates in our study (PCV2b)	nt	–	–	–	95.2–100	92.3–94.7	92.6–95.4	93.2–95.4	93.0–95.4
	aa	–	–	–	94.0–100	91.5–94.9	91.0–96.2	91.9–95.3	91.9–95.3
VC2002-K2 (EF990645) (PCV2d-1)	nt	–	–	–	–	100	97.9–99.3	98	97.9
	aa	–	–	–	–	100	97.4–99.1	98.3	97.9–98.3
Isolates in our study (PCV2d-1)	nt	–	–	–	–	–	97.9–99.4	97.9–98.4	97.7–98.3
	aa	–	–	–	–	–	97.0–99.1	97.4–99.1	97.0–100
BDH (HM038017) (PCV2d-2)	nt	–	–	–	–	–	–	100	99.9
	aa	–	–	–	–	–	–	100	99.6–100
PCR isolates in our study (PCV2d-2)	nt	–	–	–	–	–	–	–	97.7–100
	aa	–	–	–	–	–	–	–	97.0–100

Table 4. Selection pressure analysis of cap protein of PCV2 using SLAC, FEL, MEME, and FUBAR methods.

Codon	SLAC		FEL		MEME		FUBAR	
	dN–dS	*P*-value	dN–dS	*P*-value	β^+	*P*-value	dN–dS	Post. Pr.
59	3.95	0.348	−0.272	0.898	184.84	**0.00**	14.118	**0.913**
131	6.22	0.151	2.393	0.159	3.47	**0.09**	6.623	**0.973**
191	6.74	**0.0869**	4.695	**0.098**	7.04	**0.02**	5.287	**0.984**

Codon with *P*-value < 0.1 in SLAC, FEL, and MEME methods, or with Post. Pr. ≥ 0.9 in FUBAR method (showed in bold), was considered under positive selection. Condon: positively selected sites (AA), β^+: the MLE of the unconstrained β non-synonymous rate, and Post. Pr.: posterior probability.

some mutations were limited to a few strains, such as 11K/R among PCV2b isolates, with K present in one out of thirty-one PCV2b isolates and R in the remaining thirty strains. These mutations seldom reflect the whole picture of PCV2 genotypic evolution, and thus are of little importance.

Other mutations were present in all or at least the majority of the strains of the genotype, such as 57V/I among PCV2b isolates, with V present in two out of thirty-one PCV2b isolates and one in the other twenty-nine strains. These mutations may represent the general evolutionary trace of that genotype, and is thereby of great importance. Such mutations were selected and presented in Table 5. Amino acids at these important mutation sites were mapped on the corresponding 3D structure of the cap protein of PCV2a (strain Fh17), PCV2b (strain 1206), and PCV2d (strain K2) (Fig. 2). Different colour codes were used for different amino acid sites, and the same colour was used for the same amino acid site across the three different genotypes. On the inner side of the cap protein where the cap protein interacts with the viral genome, three mutations at positions 53 (F-F-I), 121 (S-S-T), and 215 (V-V-I) were found on the β-sheet structure across the three genotypes (PCV2a–PCV2b–PCV2d). These mutations are conserved and less likely to induce any changes, since F, I, and V belong to the hydrophobic amino acids, and S and T to the polar amino acids. Because the amino acids of the inner part of the cap protein that interact with the viral genome are conserved, it became clear that the inner β-sheets are essential in the PCV2 morphogenesis and stability of the structure. Interestingly, most of the amino acid mutations were found on the outside of the cap protein where the cap protein interacts with the environment. On the upper part of the cap protein, mutations were found in amino acids at positions 59, 206, and 63 that were very close to each other and formed a large surface area (A/K/S for PCV2a, R/I/R for PCV2b, and A/K/R for PCV2d). It is obvious that this area became more basic with the evolution from PCV2a to PCV2b and PCV2d, due to the formation of a cluster of basic polar amino acids (K + R). A single amino acid at position 169 (S-S-R) followed this trend as well. This indicated that this area of the PCV2 capsid was evolving toward more positive charges which may lead to a more efficient binding to the negatively charged glycosaminoglycan (GAG) receptors. Another large surface area where mutations were found was formed by amino acids at positions 57, 68, and 134, which is V/A/T for PCV2a, I/A/T for PCV2b, and V/N/N for PCV2d. Compared with PCV2a and PCV2b, this area of PCV2d increased its polarity by adding a second polar amino acid (NN) instead of a non-polar amino acid (A, I, or V). In this way, a better environment is created for receptor binding by hydrogen bonds. Similarly, a single mutation of the amino acid at position 131 (I-T-T) also shifted from a hydrophobic amino acid (I) to a polar one (T), which fitted in this hypothesis as well. Together, this suggested that, with mutations of amino acids at the upper part of the cap protein, PCV2 has evolved for a more efficient binding with GAGs.

Table 5. Genotype-specific amino acid residues in the cap protein of forty-three Belgian PCV2 isolates (PCV2a: three isolates; PCV2b: thirty-one isolates; PCV2d-1: six isolates; and PCV2d-2: three PCR isolates) and twenty-one reference strains (PCV2a: eight strains; PCV2b: seven strains; PCV2d-1: two strains; and PCV2d-2: four strains).

Position	PCV2a		PCV2b		PCV2d-1		PCV2d-2	
	Isolates in our study	Reference strains[a]	Isolates in our study	Reference strains	Isolates in our study	Reference strains	Isolates in our study	Reference strains
53	F	F	F	F	I	I	I	I
57	V	V	2V/29I	3V/4I	V	V	V	V
59	R	2R/6A	R	R	1A/1R/4K	A	K	K
63	1S/2T	1S/3R/4T	1T/9R/21K	3K/4R	R	R	R	R
68	A	A	A	A	N	N	N	N
77	D	1N/7D	1D/30N	N	N	N	N	N
80	V	1L/7V	1V/30L	L	L	L	L	L
86	T	T	S	S	S	S	S	S
88	K	K	P	P	P	P	P	P
89	I	I	1L/30R	R	L	L	L	L
90	S	S	1T/30S	S	T	T	T	T
91	I	1V/7I	V	V	V	V	V	V
121	S	3S/5T	1T/30S	S	T	T	T	T
131	1I/2P	1I/3T/4P	12P/19T	T	T	T	T	T
134	T	1P/7T	T	T	N	N	N	N
151	1P/2T	1T/7P	T	1P/6T	1T/5P	P	T	T
169	1S/2G	S	1A/30S	S	1W/5R	R	1R/2G	2R/2G
190	1S/2T	S	A	A	T	T	T	T
206	K	K	1V/30I	I	I	1K/1I	I	I
210	D	D	1D/2G/28E	E	2D/4E	D	D	D
215	V	V	1G/1I/29V	V	I	I	I	I
232	K	1N/6K	N	N	N	N	N	N
234	–	–	–	–	K	K	K	K

PCV2a reference sequences: AY556474, AF109398, AY180396, AF201308, AY322004, AJ293868, AF055392, AB072301; PCV2b reference sequences: EF990646, EF990644, AY916791, AY691169, AY484407, AY424405, AY321984; PCV2d reference sequences: EF990645, AB462384 (PCV2d-1); KJ187306, JX535296, JX519293, HM038017. –, Dashes indicate amino acid residues at this position are absent among these PCV2 genotypes.

[a]One reference strain PCV2a-AF109398-Canada1998 has only a partial cap sequence (positon 1-168) deposited on GenBank.

On the lower part of the cap protein, there were three large surface areas which were formed by groups of mutated amino acids among three genotypes. The first one (V/D/S for PCV2a, I/N/A for PCV2b, and L/N/T for PCV2d) was formed by amino acids at positions 80, 77, and 190; the second one (V/S/I for PCV2a, L/S/V for PCV2b, and L/T/V for PCV2d) was formed by amino acids at positions 80, 90, and 91; and the last one (T/K/I for PCV2a, S/P/R for PCV2b, and S/P/L for PCV2d) was formed by amino acids at positions 86, 88, and 89. Mutations in the former two areas were generally conserved, since the mutated amino acids were replaced by ones with similar biochemical properties; mutations in the last area resulted in a loss of the basic positively charged polar amino acid. This means that there were less changes in the lower part of the capsid protein. On the 'tail' part of the cap protein, an amino acid at position 232 changed from a basic polar K for PCV2a to a polar neutral N for both PCV2b/d. For PCV2d, there was an extra basic polar amino acid K at position 234, while this amino acid K at position 234 was absent for PCV2a/b. Moreover, the surface of the basic polar amino acid K at position 234 was very close to that of the polar amino acid N at position 232 for PCV2d. It was inferred that the polar amino acid N at position 232 could extend further from the capsid surface, thus projecting the neighbouring positively charged K to the outer environment to search for/enhance the initial, non-specific interaction of the virus with negatively charged heparan/chondroitin sulphate on the cell membrane during PCV2 infection.

In conclusion, the amino acid motifs were multi-variate in the cap sequences of these forty-three Belgian PCV2 isolates. From an evolutionary point of view, most amino acid mutations were found on the outer surface of the cap protein, with the upper and tail parts of the cap protein consisting of more basic amino acid clusters and the lower part being more conserved but somewhat less basic; the amino acids at the inner part were conserved to interact with the viral genome and maintain the stability of the PCV2 virion.

4. Discussion

In Belgium, the first retrospective serological study was performed on archived samples from nursery pigs, fattening pigs, and young sows by IPMA, showing that the presence of PCV2 dates back to 1985 (Labarque et al. 2000); another detailed serological survey of PCV2 antibodies in domestic and feral pig population later in Belgium showed the evidence of PCV2 infections in 1969 (Sanchez, Nauwynck, and Pensaert 2001a). Apart from these serological studies, little genetic information of PCV2 strains in Belgium was available. In the present study, we examined the molecular epidemiology and evolution of PCV2 strains circulating in Belgium, by evaluating the frequency of the virus detection, the genotype distribution, and their heterogeneity from archived diagnostic samples from 2009 till 2018. This study is the first report on the molecular characterization of Belgian PCV2 strains.

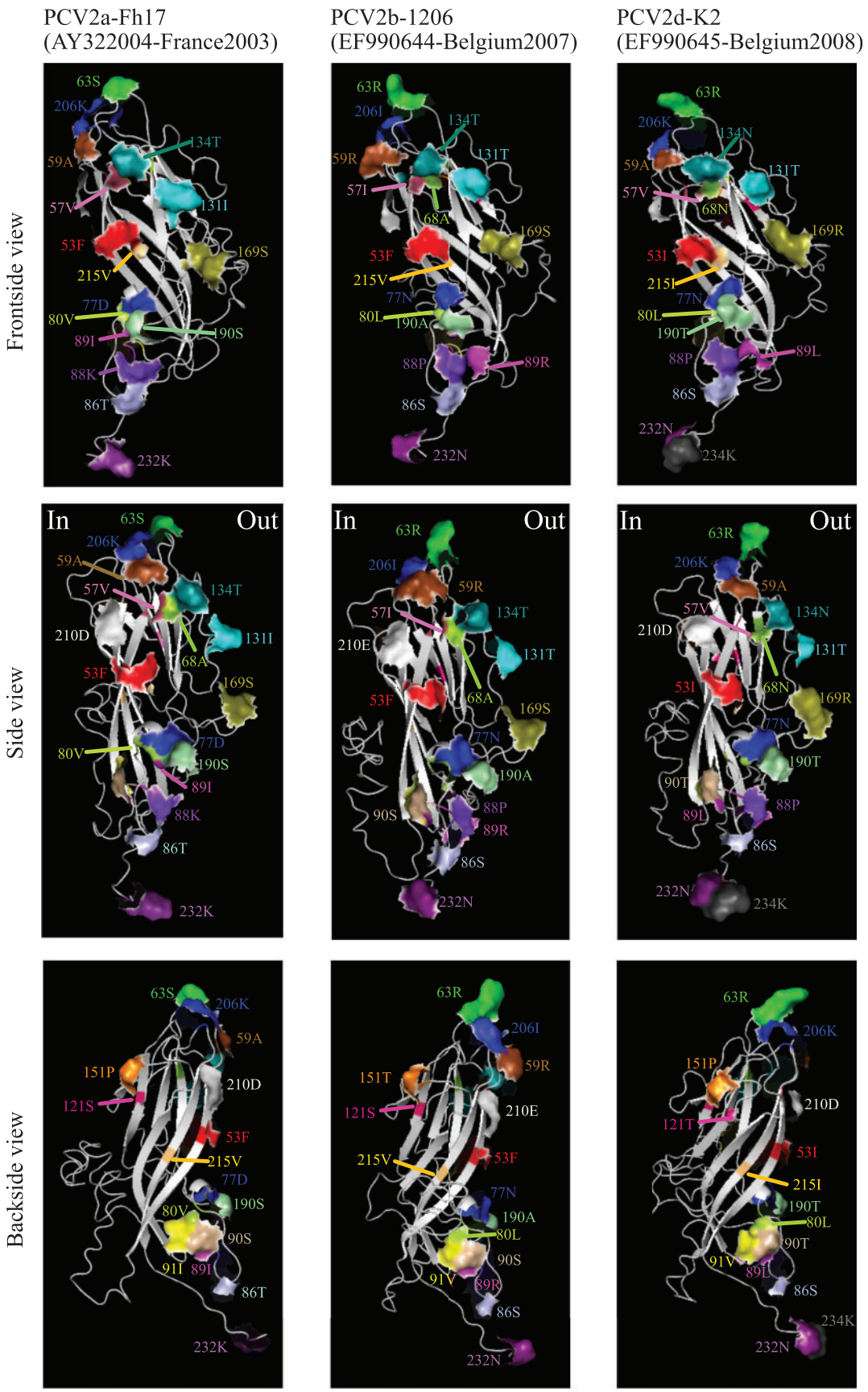

Figure 2. Three-dimensional mapping of critical amino acids presented in Table 5 on capsid of PCV2a, PCV2b, and PCV2d. The 3D structures of capsid were generated using I-TASSER server and displayed with PyMOL.

Despite the early presence of PCV2 in 1969, Belgian farms were rarely affected by PMWS-related problems. In three Belgian farms with a history of PMWS, the percentage of PMWS-positive piglets was very low (2%) compared to those described in the UK (up to 22%), USA (up to 15%), France (11%), and Spain (up to 30%) (Kiupel et al. 1998; Gresham et al. 2000; Madec et al. 2000; Segales and Domingo 2002; Meerts et al. 2004). Until now, only four Belgian PCV2 strains were isolated from PMWS-affected piglets. Strains 1206 and VC2002-K39 were identified as PCV2b, strain VC2002-K2 as PCV2d-1 and strain 4D4 as a recombinant strain of VC2002-K2 and VC2002-K39 (Lefebvre et al. 2009). The successful application of vaccination, improved biosafety measures, and better hygiene may explain the decreased number of samples that were sent in our laboratory for PCV2 diagnosis from 2014 to 2018. Another reason could be that PCV2 diagnosis became a routine in a number of laboratories across Belgium during that period, resulting samples being sent to other laboratories.

In this study, three PCV2 genotypes (PCV2a, PCV2b, and PCV2d) were identified in Belgium. PCV2b was the predominant genotype from 2009 till 2013. The three isolates from 2018 belonged to genotype PCV2d. Samples from 2014 to 2017 were negative for virus isolation and thus were not submitted for further PCR analysis. Only three cases of PCV2a were detected exclusively in 2009 in our study in Belgium, while on a worldwide scale PCV2a was the most dominant genotype in pig population before 2003 (Xiao, Halbur, and Opriessnig 2015). During the period of 2003–4, a global shift occurred from genotype 2a to 2b. This agrees with the predominance of PCV2b from 2009 till 2013 in Belgium. In 2018, PCV2d, especially PCV2d-2, was the only circulating subtype in Belgium in our study. The emergence and spread of PCV2d-2, which appears to be replacing PCV2b, has been suggested in several studies (Xiao, Halbur, and Opriessnig 2015; Franzo et al. 2016). PCV2d-1 was only found in 2009 (1/6) and 2010 (5/6) in Belgium. This is in line with the global trend in the identification of PCV2d-1 between 1999 and 2011 and confirms the proposal that PCV2d-1 is a possible ancestor of PCV2d-2 (Xiao, Halbur, and Opriessnig 2015). The emergence of PCV2d-1 concurred with the spread of PCV2b, indicating that PCV2b and PCV2d bifurcated early and evolved independently in the pig population (Xiao, Halbur, and Opriessnig 2015). These findings generally indicate a genotype shift from PCV2a to PCV2b and from PCV2d-1 to PCV2d-2. The limited timeframe of our study and the absence of PCV2 genetic data between 2014 and 2017, made it difficult to determine the exact genetic changes in Belgium during that period.

Selection pressure plays a critical role in evolutionary biology by shaping genetic variation. The high mutation rate of PCV2, which is closer to that of a single-stranded RNA virus, can enhance the frequency of selection, as well as the rapid emergence of novel PCV2 genotypes. In our study, we identified three codons (positions 59, 131, and 191) in the capsid protein of Belgian PCV2 under positive selection pressure. Positions 59 and 191 are crucial in neutralizing activities, while the position 131 is related to the growth ability of PCV2. Broad vaccination, which was used to control numerous PCVADs, might contribute to the alteration of antigenic properties. Research on the role of vaccination in PCV2 evolution is extremely challenging because of the difficulty in recruiting a large number of farms where vaccination has not been applied despite PCV2 circulation. However, it is interesting to note that the first genotype shift from PCV2a to PCV2b during the period of 2003–4 predates the vaccination against PCV2 using PCV2a-type commercial vaccines (first registration in 2007; Circovac®, Merial). This genotype shift/evolution is probably mainly driven by infection immunity which is present on all farms. Although the ongoing genotype shift from PCV2b to PCV2d occurs in the background of extensive vaccination with the PCV2a-type vaccines, PCV2 infections still occur and induce a strong population immunity. Vaccination did not reduce the variability of PCV2 strains in vaccinated herds (Reiner, Hofmeister, and Willems 2015). As a result, it is likely that population immunity against circulating wild-type PCV2 viruses is the main driving force behind the genotypic evolution.

Since the PCV2 capsid protein is immunogenic, mutations on the capsid protein may explain the direction of PCV2 genotypic evolution. Mapping genotype-specific amino acids on the 3D structure of the cap protein of different genotypes is of great interest. It helps to identify the real locations of amino acids on the cap protein, thus revealing the possible effect of amino acid mutations on the structure of the virion and on the interaction of virions with the environment (e.g. receptors). In the current study, the amino acids on the inner part of the cap protein (where the cap protein interacts with the viral genome) were conserved to maintain the stability of PCV virion, and most amino acid mutations were found on the surface of the cap protein (where the cap protein interacts with the environment). Mutations on the upper part of the cap protein consisted of more basic amino acid clusters and the lower part more conserved but somewhat less basic (Fig. 2). The tail was extended with one amino acid and remained basic. It is likely that the capsid protein has mutated to escape from the host immunity, evolving to bind more strongly to its GAG receptors. This may explain the grouping of basic amino acids in an area composed of the 'head' of one capsomere and the 'tail' of the neighbouring capsomere and the longer extended basic 'tail' (Fig. 3). It appears that PCV2d has formed an extruding part to the cap protein, which improves the efficiency of binding of the virus to the target cell.

Comparison of the complete ORF2 sequences of forty-three Belgian PCV2 isolates revealed 89.7–100 per cent nucleotide homology and 88.5–100 per cent amino acid sequence identity. Amino acid comparisons showed that a greater diversity was found in the regions 53–91, 121–136, and 169–217 and at the positions 151, 232, and 234. Similar non-conserved regions have been demonstrated in another report (Wei et al. 2013). These regions contain immunorelevant epitopes (residues 65–87, 113–139, and 193–207) identified using pepscan analysis (Mahe et al. 2000). Frequent mutations in these regions may change the antigenic features of PCV2 and help the virus to evade the immune response, thus promoting virus propagation under field conditions. Specifically, position 59 is a critical amino acid that is part of the neutralizing epitope (Huang et al. 2011); positions 131 and 190 are important for determining neutralization activity (Saha et al. 2012a); region 163–180 is suggested as a decoy epitope resulting in the production of non-neutralizing antibodies against PCV2 (Trible et al. 2011, 2012). Position 233 is critical in maintaining effective binding between the epitope and antibody, which is conserved among all genotypes in our study (Shang et al. 2009). Interestingly, a pair of signature motifs in the ORF2 (aa position 86–91) of PCV2 which was described to distinguish viruses of genotype 2a (peptide sequence 86TNKISI91) from strains of genotype 2b (86SNPRSV91), can also distinguish viruses of genotype 2d (86SNPLTV91) in our study (Cheung et al. 2007; Wei et al. 2013). Thus, amino acids in region 86–91 can serve as a 'genetic marker' to distinguish these three genotypes.

In this study, only the tissue samples from which virus was isolated were subjected to further PCR analysis (except for the serum samples in 2018). We believe that virus isolation is the 'gold standard' method to demonstrate that PCV2 is related to

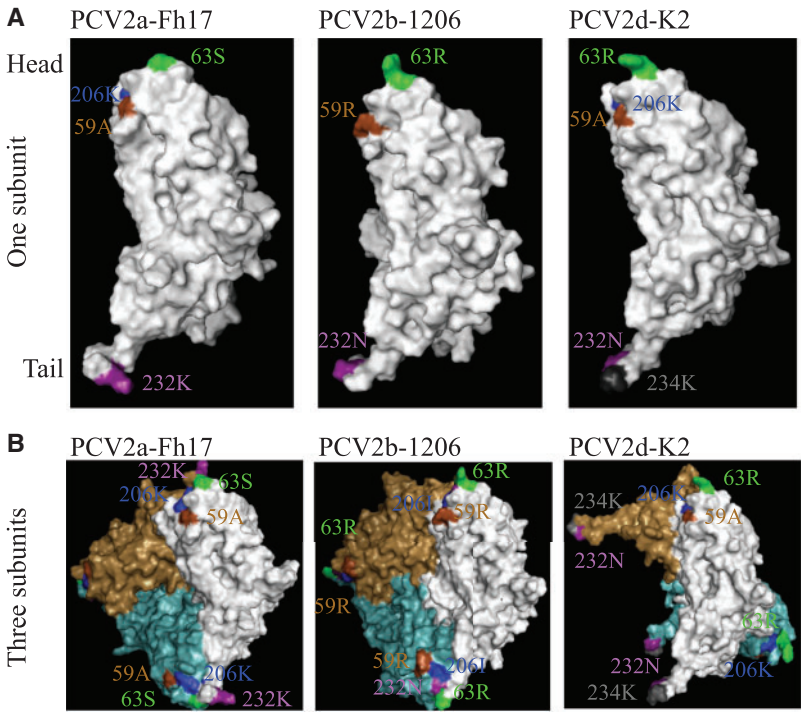

Figure 3. Hypothetical model on the evolutionary amino acid mutations on the 'head' and 'tail' of the cap protein. (A) Front side: Surface mapping of amino acid mutations on the head (positions 59, 206, and 63) and tail (positions 232 and 234) of the cap protein across three genotypes. (B) Three subunits forming a trimer, with indications of the changes mentioned in (A). For PCV2a-Fh17 and PCV2b-1206, the head of one capsomere is close to the tail of the neighbouring capsomere; while for PCV2d-K2, the head of one capsomere is apart from the tail of the neighbouring capsomere, and both the head and tail extrude further from the capsid surface. The trimer was generated by SYMMDOCK server (Schneidman et al. 2005). The 3D structures of capsid were generated using I-TASSER server and displayed with PyMOL.

disease, based on the fact that DNA of circoviruses is detected everywhere nowadays and that this DNA is not always associated with viable virus. For instance, the contaminating PCV, which was detected in a porcine-derived commercial pepsin product by PCR, was confirmed to be non-infectious as demonstrated by both *in vitro* and *in vivo* infection experiments (Fenaux et al. 2004b). The PCV DNA detected in some rotavirus vaccines by qPCR consisted of small fragments of PCV genetic material since the full length or partial amplicons of PCV could not be amplified by conventional PCR (Esona et al. 2014). PCV2 DNA was detected in water samples in Brazil, farm air in Canada, house flies in UK (Verreault et al. 2010; Blunt et al. 2011; Garcia et al. 2012). Even in soil, PCV2 genome components were demonstrated (Kim et al. 2008). In the USA, 70 per cent of the store-bought pork products contain PCV sequences. This resulted in the detection of PCV in stool samples from adults in the USA (5%) possibly due to the dietary consumption of PCV-infected pork (Li et al. 2010). Interestingly, previous work from our laboratory demonstrated that monocytic cells, known as scavenger cells and being responsible for cleaning up foreign material in the body, can indeed take up PCV2 virions and disintegrate viral capsids, but have problems with degrading/digesting PCV2 DNA (Wei et al. 2018). The difficult-to-degrade property of PCV2 genome may explain the ubiquitous presence of the viral DNA. Taken together, it is clear that the detection of PCV2 genetic material does not automatically mean that the sample contains complete, viable, and infectious PCV2 virions. This important yet overlooked information alerts us on the use of the PCR, especially the sensitive qPCR for diagnostic purposes. Normally the advisable product length of qPCR is around 150–200 bp, which means only a small part of the viral genome is amplified. Can one state that pigs are positive for PCV2 based on the detection of such small fragments of the PCV2 genome? As to the question of whether pigs should be considered as positive for PCV2 based on the detection of such small fragments of the PCV2 genome, we feel that virus should be successfully isolated, or at least the full length of PCV2 genome amplified by conventional PCR, together with observations of PCVAD clinical and pathological manifestations from pigs, before PCV2 should be considered as contributing to the clinical disease observed.

In conclusion, we demonstrated that at least three genotypes (PCV2a, PCV2b, and PCV2d) circulated in Belgium from 2009 till 2018, and that PCV2 evolved from PCV2a to PCV2b and from PCV2d-1 to PCV2d-2. Mapping PCV2 genotype-specific amino acids on the 3D structure of the capsid protein revealed that most of the mutations were on the outside of the cap protein, with a few conserved mutations on the inner side of the protein. Mutations on the upper and tail parts of the capsid protein evolved toward a more efficient binding to GAGs, while the lower part of the protein was relatively conserved. Additional epidemiological studies, including a retrospective analysis of virus circulation in Belgium before 2009, along with more continuous up-to-date surveillance, are needed to better understand the epidemic and evolutionary dynamics of PCV2 in Belgium, and to see how the situation in Belgium conforms to what is known about the global evolution of PCV2.

Authors' contributions

R.W. and H.J.N. conceived and designed the experiments; R.W. performed the experiments; R.W., J.X., and S.T. analyzed the data; R.W. wrote the article and H.J.N. revised the article.

Supplementary data

Supplementary data are available at *Virus Evolution* online.

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