Infection, Genetics and Evolution 90 (2021) 104775

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Emergence of porcine circovirus 2g (PCV2g) and evidence for recombination between genotypes 2g, 2b and 2d among field isolates from non-vaccinated pigs in Mizoram, India

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ARTICLE INFO

Keywords:
India
Complete genomic analysis
Genotyping
PCVAD
PCV2g
Recombination

ABSTRACT

The molecular genetics of fourteen Porcine Circovirus 2 (PCV2) isolates from non-vaccinated pigs that died of porcine circovirus associated disease (PCVAD) between 2012 and 2019 in the Mizoram state of North East India, was studied. The PCVAD in these pigs, that had shown characteristic clinical signs and lesions associated with post-weaning multi-systemic wasting syndrome and reproductive failure was confirmed with detection of PCV2 DNA in the tissue samples. Complete viral genomes of these fourteen field isolates were sequenced following *in house* developed overlapping PCR. The multiple sequence alignment of viral capsid proteins or the open reading frame 2 (ORF2) sequences showed highly conserved residues known for antibody recognition and genotype specificity, however, variations were noticed in the amino acid residues previously known as important for in vitro replication of PCV2. The phylogenetic analyses based on the complete genome sequences enabled identification of genotype PCV2g (9/14, 64.29%) for the first time in India along with genotypes PCV2d (3/14, 21.43%) and PCV2b (2/14, 14.29%). Further, recombination analyses showed evidence for recombination between the genotypes 2b, 2g and 2d. This is the first report on the prevalence of genotype PCV2g and natural intergenotypic (2g-2b, 2g-2d and 2d-2g) recombinants in India. The findings indicate a non-vaccine driven, natural genotypic shift and signify the need for routine PCV2 surveillance and genotyping. Our analyses also provide a solid ground for future studies to understand the consequences of multiple PCV2 genotypes within a pig population with respect to vaccination, diagnostics and emergence of new genotypes.

1. Introduction

Porcine circovirus 2 (PCV2) is the primary causative agent of Porcine circovirus-associated disease (PCVAD), which represents a group of porcine disease conditions that includes post weaning multi-systemic wasting syndrome (PMWS), porcine dermatitis and nephropathy syndrome (PDNS), porcine respiratory disease complex (PRDC), reproductive failure, enteritis and more recently, a novel per acute syndrome described as acute pulmonary edema (APE) (Chae, 2005; Cino-Ozuna et al., 2011; Opriessnig et al., 2007; Ramamoorthy and Meng, 2009). Today, PCVAD causes significant economic loss to the pig industry worldwide and hence PCV2 is considered as an economically important pathogen of swine (Opriessnig et al., 2011).

PCV2 belongs to the family *Circoviridae*, which encompasses a group of small non-enveloped, isometric virus containing a covalently closed, circular, single-stranded DNA genome (Tischer et al., 1982). Within the genus circovirus, PCV2 is closely related to non-pathogenic porcine circovirus type 1 (PCV1). The genome of porcine circoviruses is approximately of 1.7 kb, ambisense and codes for at least six open reading frames (ORFs). The ORF1 is oriented in the sense direction relative to the origin in the PCV2 genome and codes for the replicase proteins, with two splice variants (Rep and Rep). Rep is translated from the entire ORF1 transcript, whereas, Rep is derived by alternative splicing of the ORF1 transcript (Mankertz and Hillenbrand, 2001). The C-terminal 68 amino acids (aa) of Rep are derived from a different reading frame. The ORF2 is oriented in the antisense direction and codes for the 233 or 234 aa virus capsid protein (CP). CP is involved in the formation of the homopolymer capsid and is likely involved in translocating the viral genome into the nucleus during virus replication (Liu et al., 2001; Nawagitgul et al., 2000). The capsid protein is the primary

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https://doi.org/10.1016/j.meegid.2021.104775
Received 12 October 2020; Received in revised form 10 February 2021; Accepted 16 February 2021
Available online 20 February 2021

target for the host immune system, highly immunogenic and hence under high selection pressure. The greatest genomic difference between PCV1 and PCV2 are found within ORF3 (Liu et al., 2005). The ORF4 was discovered as overlapping with ORF3 and has been implicated in regulating the viral apoptosis (He et al., 2013; Lv et al., 2016). The ORF5 has been shown to overlap completely with ORF1 and found to play a role in enhancing viral replication by inhibiting IFN signaling (Lv et al., 2015; Choi et al., 2018). Recently, the function of ORF6 has been implicated in caspases regulation and was found to influence multiple cytokines expression during the PCV2 infection in cell culture (Li et al., 2018).

Based on complete genome analysis(Grau-Roma et al., 2008) or the degree of genetic variation in ORF2 (Fenaux et al., 2000; Hamel et al., 2000; Larochelle et al., 2002; Mankertz et al., 2000), PCV2 strains are classified into at least eight genotypes, PCV2a to PCV2h, with varying prevalence and clinical impact (Franzo and Segalés, 2018; Wang et al., 2009). The genotype shift from PCV2a to PCV2b was first observed in Canada with the outbreaks of severe PCVAD in 2005 (Carman et al., 2008) and subsequently reported in other countries, including US (Cheung et al., 2007; Horlen et al., 2007), China (Wang et al., 2009), Thailand (Jantafong et al., 2011), Korea (Guo et al., 2010), Denmark (Dupont et al., 2008), and Switzerland (Wiederkehr et al., 2009). PCV2c was identified in archived swine serum samples collected in 1980, 1987 and 1990 from Denmark and has not been detected in other locations since that time (Dupont et al., 2008). Since 2012, a second genotype shift is being observed from PCV2b to PCV2d as the most prevalent genotype in North America, China, South Korea and Uruguay (Franzo and Segalés, 2018; Karuppannan and Opriessnig, 2017).The prevalence of different genotypes within a population is of concern for two reasons, firstly, there is no report so far on 100% cross-protective immunity between the PCV2 genotypes and secondly, because of the threats from disease outbreaks due to fast evolution of PCV2 with emergence of recombinants. Genetic recombination events can play an important role in evolution of PCV2 and has further complicated the current classification of PCV2 genotypes (Anoopraj et al., 2015; Cai et al., 2012; Hesse et al., 2008; Lefebvre et al., 2009; Ma et al., 2007). Several reports have confirmed that PCV2a/2b chimeric viruses are a relatively common occurrence (Cheung, 2009; Hesse et al., 2008; Kim et al., 2009; Lefebvre et al., 2009; Ma et al., 2007).

In India, 40% of the total pig population is distributed within eight North Eastern (NE) states comprising of Assam, Arunachal Pradesh, Mizoram, Meghalaya, Manipur, Nagaland, Tripura and Sikkim. Studies on pig population in Meghalaya revealed prevalence of PCV2d and also highlighted the recombination events between PCV2a & PCV2b (Mukherjee et al., 2018). Although there is no report of PCV2c prevalence in India, inter-genotypic recombination events between PCV2c & PCV2d has also been reported and suggested a novel cluster of PCV2 isolates in the same pig population (Mukherjee et al., 2019). The present study was envisaged to document the molecular epidemiological status of PCV2 between 2012 and 2019 among the pig population in Mizoram, a land locked state of NER India that shares international borders with Myanmar in the south-east and Bangladesh in the west. We analyzed fourteen complete genome sequences of PCV2 isolates from pigs died of PCVAD and report here the prevalent PCV2 genotypes and the recombinants in Mizoram.

2. Materials and methods

2.1. Study area and sample collection

Although porcine circovirus 2 associated diseases (PCVAD) is endemic, vaccination against PCV2 in pig population of Mizoram state of India is not practiced yet. Occurrence of PCVAD in this non-vaccinated pig population was studied. Detailed post mortem examination was carried out in total 103 pigs that died with clinical history of PMWS and in aborted or stillborn fetuses from 54 cases of reproductive failure between the period 2012 and 2019. The pathological lesions were recorded and representative tissue samples comprising of lungs, spleen, tonsil and lymph nodes (inguinal and mesenteric lymph nodes) were collected, preserved in 10% buffered formalin for histopathological examination and also stored at −80 °C for molecular diagnostic confirmation and isolation of PCV2.

2.2. PCR detection of PCV2 and full genome sequencing

Total DNA was extracted from representative tissue samples (Tonsil, spleen, lungs, mesenteric and inguinal lymph nodes) by using the Phenol-Chloroform-Isoamyl alcohol method and quantified in Biophotometerplus™(Eppendorf). Samples were tested by PCR for detection of PCV2, using the primer set PCV-LF: 5′-TAGGTTAGGGCTGTGGCCTT-3′ and PCV-LR: 5′-CCGCACCTTCGGATATACCG-3′ to amplify a 263 bp fragment of ORF-2 gene (Larochelle et al., 1999). The whole genome sequencing from 5th passage harvest of fourteen PCV2 field isolates from pigs that died of PCVAD were carried out by amplification of the full genome using three different sets of *in house* designed primers (Table 1). The PCR products were visualized in agarose gel and the size of the amplicons was determined from the Gene Ruler™ 100 bp DNA Ladder (Fermentas Life Sciences, Canada).

The amplified 764 bp, 689 bp and 664 bp fragments were purified from agarose gel by using Thermo Scientific GeneJet Gel Extraction kit and cloned into pTZ57R/T vector using InsT/Aclone™ PCR product cloning kit (Fermentas Life Sciences, Canada). The recombinant plasmids containing gene fragments were subjected to DNA sequencing at DNA sequencing facility, south campus, Delhi University, New Delhi, India. The nucleotide sequences derived from the clones of the three overlapping fragments were then analyzed and aligned to get the full-length genome of the PCV2. A total of fourteen full genome sequences of PCV2 from field outbreaks of PCVAD in Mizoram were obtained (Table 2).

2.3. Isolation of PCV2 from field outbreaks

Isolations of PCV2 from fourteen field cases from different PCVAD outbreaks were carried out in PK 15 cells (ATCC, Cat.No. CCL-33, Lot no −58808810). Tissue inoculums from pooled tissue samples (lungs, spleen, lymph nodes) from PCR positive cases were prepared in cell culture media containing antibiotics to inoculate PK-15 cell line for isolation of PCV2 (Wellenberg et al., 2000). The flask containing confluent monolayer (about 80% of cells) was used for propagation of virus. Following72 hours of incubation at 37 °C, the cell culture supernatant was harvested from the flask by alternate freezing and thawing for 3 times. The cell suspension was clarified by centrifugation at 2500 rpm for 5 min. This virus containing fluid was collected and stored at −80 °C. Each of the samples was given minimum of five passages in the cell culture. The viral propagation was confirmed by using PCR on the harvested media from each passage.

2.4. Phylogenetic and recombination analyses of porcine circovirus 2 field isolates

The phylogenetic analysis of fourteen field isolates of PCV2 was performed along with 44 reference strains representing each of the known genotypes of PCV2 (PCV2a to PCV2h) and 2 reference PCV1 strains, as the outgroup (details of reference strains are indicated in supplementary Table S1). Each virus strain/isolate is indicated by respective GenBank ID throughout this study report. The phylogenetic tree of complete genome was generated by MEGA7 (Kumar et al., 2016). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1) and the reliability of the clustering pattern was confirmed by considering 1000 bootstraps replicates. Tree topology

Table 1
List of primers used for PCV2 detection and full genome amplification.

Sl. No.	Code	Oligo nucleotide sequence	Product size	Annealing temp.	Purpose
1	PCV-LF PCV-LR	5′-TAGGTTAGGGCTGTGGCCTT-3′ 5′-CCGCACCTTCGGATATACCG-3′	263 bp	60 °C	Screening of tissue samples for PCV2 (Larochelle et al., 1999)
2	TKPCV21-F TKPCV21-R	5′AGAATGGAAGAAGCGGACCC 3′ 5′ CGGGGTCTGATTGCTGGTAA 3′	764 bp	59 °C	Full genome sequencing of field isolates (*in house* designed)
3	TKPCV22-F TKPCV22-R	5′- GCAGACCCGGAAACCACATA −3′ 5′ CTCCCGCCATACCATAACCC 3′	689 bp	56 °C	
4	TKPCV23-F TKPCV23-R	5′ AGTAGCGGGAGTGGTAGGAG 3′ 5′ CCCGTATTTTCTTGCGCTCG 3′	664 bp	60 °C	

Table 2
Details of field isolates of PCV2 from Mizoram, India.

Serial No.	Field isolates (GenBank accession numbers)	PCV2 genotype / recombinant	Major & minor parents identified from whole genome RDP analysis and confirmed by SimPlot analysis	Year of isolation	Clinical form of PCVAD
1	KX009480	2b	–	2013	PMWS
2	KX009481	2b	–	2012	Reproductive failure (stillborn)
3	KX009482	2d	–	2015	PMWS
4	MG957151	2g-2d recombinant	MH379265 (2g) - KX009482 (2d)	2017	PMWS
5	MG940984	2g-2d recombinant	MH379265 (2g) - KX009482 (2d)	2017	PMWS
6	MH379262	2g-2d recombinant	MH379265 (2g) - KX009482 (2d)	2017	PMWS
7	MH379263	2g	–	2017	Reproductive failure (stillborn)
8	MH379264	2g	–	2017	Reproductive failure (stillborn)
9	MH379265	2g	–	2017	Reproductive failure (stillborn)
10	MN735207	2g-2b recombinant	HQ202972.1(2g) - KX009481(2b)	2018	PMWS
11	MN735208	2d-2g recombinant	MN735213 (2d) - MH379263 (2g)	2018	PMWS
12	MN735209	2g-2b recombinant	HQ202972.1(2g) - KX009481(2b)	2018	PMWS
13	MN735206	2d	–	2018	PMWS
14	MN735213	2d	–	2019	Acute pulmonary oedema (APE)

less than 60% bootstrap values were collapsed to avoid less statistically significant lengths on branches and tree was rooted by the PCV1 sequences.

The field isolates were tested for recombination, a commonly observed event in PCV2, by Recombination Detection Programme, RDP v.4.100 software (Martin et al., 2010) initially using different integral statistical methods such as RDP, GENECONV, Maxchi, Chimaera, Siscan, Bootscan and Phylpro with *p*-value ranging from 1.279×10^{-19} to 1.936×10^{-03} for the complete genome along with Bonferroni correction with highest acceptable *P*-value of 0.05 for circular sequences. This was then followed by a more stringent analysis through selection of 'more than five statistical methods' in the RDP that showed six isolates as recombinants, each with recombinant scores of more than 0.65 (Supplementary Table S2). The potential break point positions were detected by Simplot version 3.5.1 and marked in the whole genome [Fig. 3(i-vi)]. The other parameters that were considered were, window size of 200 bp (20 bp step size), Gapstrip of On, Kimura (2-parameter) and T/t: 2.0, in the Simplot (Lole et al., 1999). For further confirmation, the recombinant isolates were individually taken as query sequence along with their respective major and minor parents with PCV1 as the out group and the potential break points were obtained by bootscan analysis [supplementary Fig. S1(i-vi)].

3. Results

3.1. Clinical and pathological findings and diagnosis by PCR

The affected piglets had clinical history of coughing, recurrent diarrhea and clinically showed wasting, anemia with visible pale mucus membranes, prominent rib cage and palpable enlarged inguinal lymph nodes. Post mortem examination of dead pigs revealed edematous and enlarged lymph nodes (inguinal lymph nodes, mesenteric lymph nodes), mild to moderate ascites, hydro pericardium, hemorrhagic gastritis (particularly at the body of the stomach) and thickening of wall in small intestine. Lungs were edematous, non-collapsing with rib imprint on the surface. Liver was yellowish in colour and surface of the kidney revealed white foci with mild congestion [Fig. 1A(i-iii)]. The sows affected with reproductive failure apparently looked normal but either aborted at the late gestation, or furrowed still born or mummified fetuses. Aborted fetuses were of varying sizes and showed swollen, oedematous inguinal lymph nodes, some of them showed ascites and hydrothorax with congested, non collapsing lungs [Fig. 1B(i-vi)].

Histopathological examination of tissue samples collected from PMWS affected piglets showed severe lymphoid depletion with infiltration of histiocytes in lymph nodes and spleen. Lung revealed severe interstitial pneumonia with air spaces compressed and filled with infiltrating mononuclear cells. Mononuclear cell infiltration and tubular degeneration was observed in kidney [Fig. 1A(iv-vi)]. Centrilobular degeneration, vacuolation, karyomegaly of hepatocytes, lymphohistiocytic inflammatory infiltration in the portal zone and disorganization of hepatic cords were observed in liver. Tissue sections from lungs of aborted fetuses showed dilated interlobular septae due to edema with few inflammatory cells in alveolar walls. Basophilic circular intranuclear inclusion bodies are observed in alveolar macrophages and also in reticulo-endothelial cells of spleen [Fig. 1B(v-vi)].

A total of 56 cases of PMWS (out of total 103 cases) and 28 cases of reproductive problem in sows (out of 54 cases) screened based on characteristic clinical signs and pathological lesions, tested positive for PCV2 DNA by PCR. Further, isolation of PCV2was attempted from fourteen different PCR positive cases in PK-15 cell line. Full genome sequences from 5th passage harvest of all these isolates were submitted to GenBank (Table 2) and their analysis is presented here.

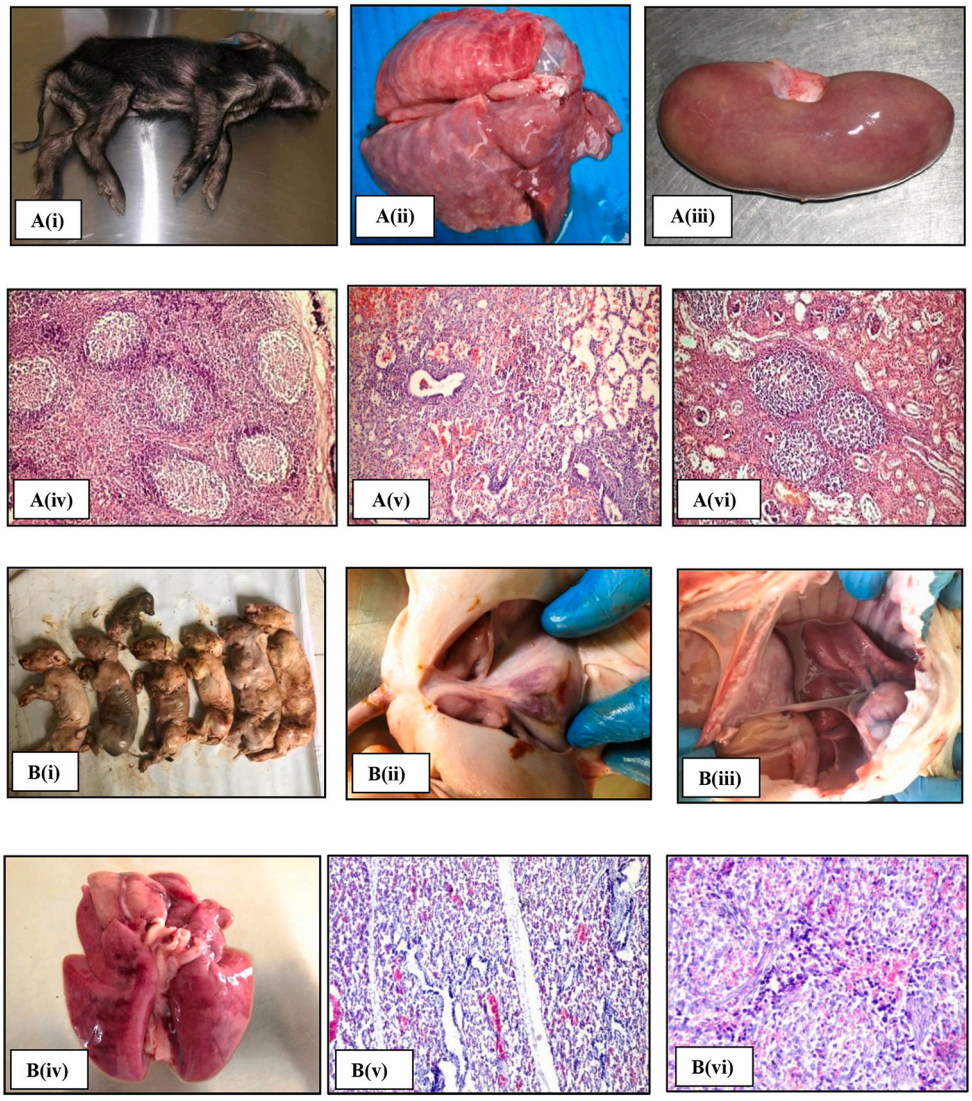

Fig. 1. A(i) Two months old Pig died of PMWS. A(ii) Non-collapsing, oedematous lungs with imprints of ribs on the dorsal surface. A(iii) Kidney showing white spots on surface with areas of congestion. A(iv) Lymphoid depletion in inguinal lymph nodes H & E. 10×. A(v) Congestion, mononuclear cell infiltration in alveolar spaces of lungs, H & E. 10×. A(vi) Mononuclear cell infiltration and tubular degeneration in kidney, H & E. 10×. B(i) PCV2 associated reproductive problem, Aborted fetuses B(ii) Swollen oedematous inguinal lymph nodes in aborted foetus B(iii) Hydrothorax in the aborted foetus B(iv) Non-collapsing congested lungs of aborted foetus B(v) Oedema of interlobular septae, hyperplasia of bronchiolar epithelium and congestion in lungs of aborted foetus. H & E. 10×. B (vi) Congestion, lymphoid depletion and hyperplastic reticuloendothelial cells with round, basophilic intranuclear inclusions in tissue section from spleen of aborted foetus, H & E. 40×.

3.2. Phylogenetic and recombination analyses

The phylogenetic tree was generated by MEGA7 based on the complete genome of the fourteen field isolates and representative PCV2 strains (of all eight known genotypes) and two representative PCV1 strains. The PCV1 strains formed a separate group in the phylogenetic tree. Phylogenetic analyses based on the whole genome sequences revealed the following: nine isolates- MH379263, MH379264, MH379265, MH735207, MH735209, MH379262, MG957151, MG940984 and MN735208 clustered with strains previously classified as genotype PCV2g, while three isolates- MN735206, KX009482 and MN735213 grouped together with genotype PCV2d strains and two isolates- KX009480 and KX009481 clustered with PCV2b strains (Fig. 2).

Recombination analysis based on the whole genome revealed six isolates as inter-genotypic recombinants (6/14, 42.87%) which is summarized in Table 2 and as Simplot showing the potential break points (Fig. 3). The recombinants are identified as '2g-2d' recombinants (MG957151, MG940984 and MH379262), '2g-2b' recombinants (MN735207 and MN735209) and '2d-2g' recombinant (MN735208) based on their major and minor parents as identified from RDP analysis and verified by Simplot analysis.

3.3. Multiple sequence alignment of ORF2 proteins

The multiple amino acid sequence alignment of ORF2 of fourteen field isolates indicated genotype specific conservation of amino acid residues in the four previously known general antibody recognizing domains, A, B, C and D in the capsid protein with few exceptions. Genotype 2d isolate, MN735213, showed variations in domains A, B and C (Fig. 4). The amino acid residues between 86 and 89 are known to distinguish genotypes; in this study, the 2b and 2d isolates/recombinants carried their respective genotype specific signatures, ^{86}SNPR^{89} and ^{86}SNPL^{89} while the 2g isolates had the same motif as that of 2d isolates. The following variations were also observed: (i) At position 59, where except for 2 isolates of genotype 2d (that had alanine similar to genotype 2a) and one strain of genotype 2d (that had lysine as previously reported for genotype 2d), the rest of the isolates (2b and 2g) had arginine (R) as previously reported for genotype 2b, (ii) at position 89, isolates belonging to genotype 2b, had arginine (R), whereas the genotypes 2d and 2g contained leucine (L) and (iii) at amino acid residue 169, genotype specific conservation (Serine in 2b and Arginine in 2g, 2d) was observed except in two isolates (MN735208 and MN735213), which both had glycine (G) residue. Mutations are also observed at amino acid position 206, where lysine (K) was seen replaced either by isoleucine (I) or arginine (R) in all the fourteen field isolates. The amino acid motif ^{173}YFQP^{175} and

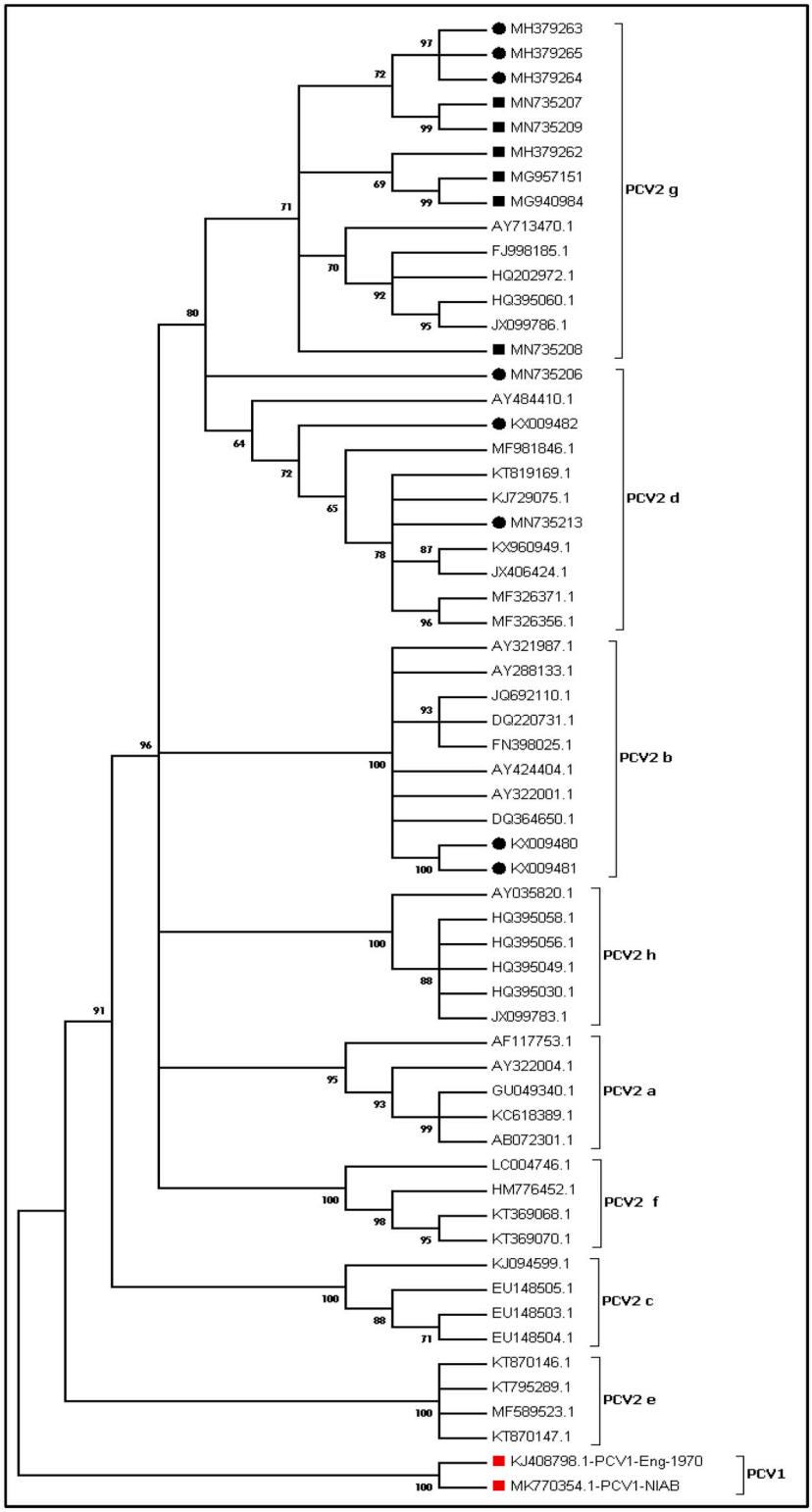

Fig. 2. PCV2 whole genome phylogenetic tree analysis performed by MEGA 7 software. PCV2 isolates from this study are represented by black dots/squares and the PCV2 recombinants identified in this study are indicated by black squares while the red squares indicate the out group PCV1 strains.

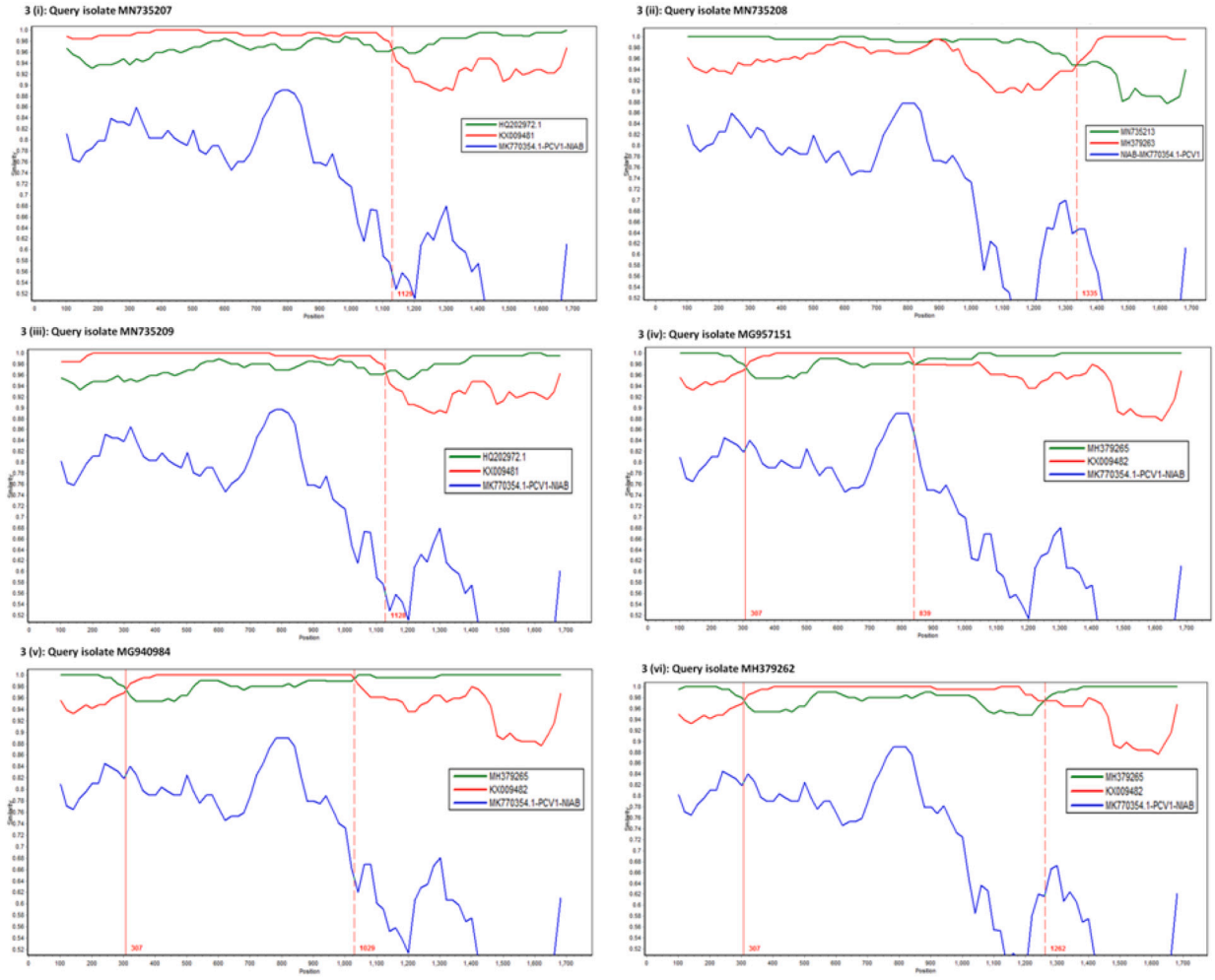

Fig. 3. (i to vi): Simplot recombination analysis based on full genome with PCV1 as the outgroup. Potential breakpoint was observed at (i) 1129 position in the similarity plot, for isolate MN735207 with HQ202972.1 and KX009481 as parents, (ii) 1335 position in the similarity plot for isolate MN735208 with MN735213 and MH379263 as parents, (iii) 1128 position in similarity plot for isolate MN735209 with HQ202972.1 and KX009481 as parents, (iv) 307 and 839 positions in similarity plot for isolate MG957151 with MH379265 and KX009482 as parents, (v) 307 and 1029 positions in similarity plot for isolate MG940984 with MH379265 and KX009482 as parents and (vi) 309 and 1261 positions in similarity plot for isolate MH379262 with MH379265 and KX009482 as parents.

residue ^{179}K previously reported as important for antibody recognition, were conserved in all the fourteen field isolates.

4. Discussion

The major aim of this study was to understand the genetic variations of circulating PCV2 in pigs that died of clinical PCVAD in Mizoram state of India, between 2012 and 2019. The complete genome of fourteen field isolates that were found positive for PCV2 DNA were sequenced and analyzed. Our results confirm the prevalence of genotype PCV2g for the first time in India, along with genotypes PCV2d and PCV2b. The average p-distance between the PCV2 genotypes derived in this study (data not shown) suggests the need for fine tuning of the current genotype-defining cut off of 0.035 or 3.5% in order to clearly differentiate the genotypes.

Further, six inter-genotypic recombinants were identified within the fourteen isolates based on complete genome. Recombination events along with mutations are considered to be important for genetic variability and PCV2 evolution. Such natural recombination events have been reported previously to occur easily during PCV2 coinfections (Ma et al., 2007). Our findings from the viral recombination analysis based on complete PCV2 genome revealed breakpoints in both ORF1 & ORF2 genes indicating the need for considering the complete genome sequence for evolutionary studies of PCV2. We also report here for the first time recombinations between genotypes 2b, 2g and 2d.

The two genotypic shifts (PCV2a to PCV2b followed by PCV2b to PCV2d) recorded so far worldwide are attributed to the global use of PCV2 vaccine (Franzo and Segalés, 2018). It is now established that prevalence of 2a genotype has been reduced with extensive use of PCV2a-derived vaccines and genotypes PCV2b and PCV2d have evolved by both natural and vaccination-induced selection (Weissenbacher-Lang et al., 2020). It is important to note here that the genetic diversity of PCV2 field isolates observed in this study were from pig population with no history of vaccination against PCV2. Unlike the earlier report from neighbouring state Meghalaya (Mukherjee et al., 2019), our findings from the phylogenetic tree analysis revealed predominance of PCV2g [64.29% of field isolates (9/14)] in NER, India, which is clearly not vaccine driven but a natural genetic shift.

The genetic variability of PCV2 has been potentially linked to different phenomena with remarkable practical implications, on virulence (Guo et al., 2012), pathogenesis (Fenaux et al., 2004; Opriessnig et al., 2007), cross protection (Xiao et al., 2012), vaccine escape (Franzo et al., 2016b), differential epidemiological fitness (Franzo et al., 2016a), etc. Recombination events involving the ORF2 also influence the antigenicity and virulence of PCV2 as capsid protein is highly immunogenic (He et al., 2019). Highly specific signature motif that distinguishes

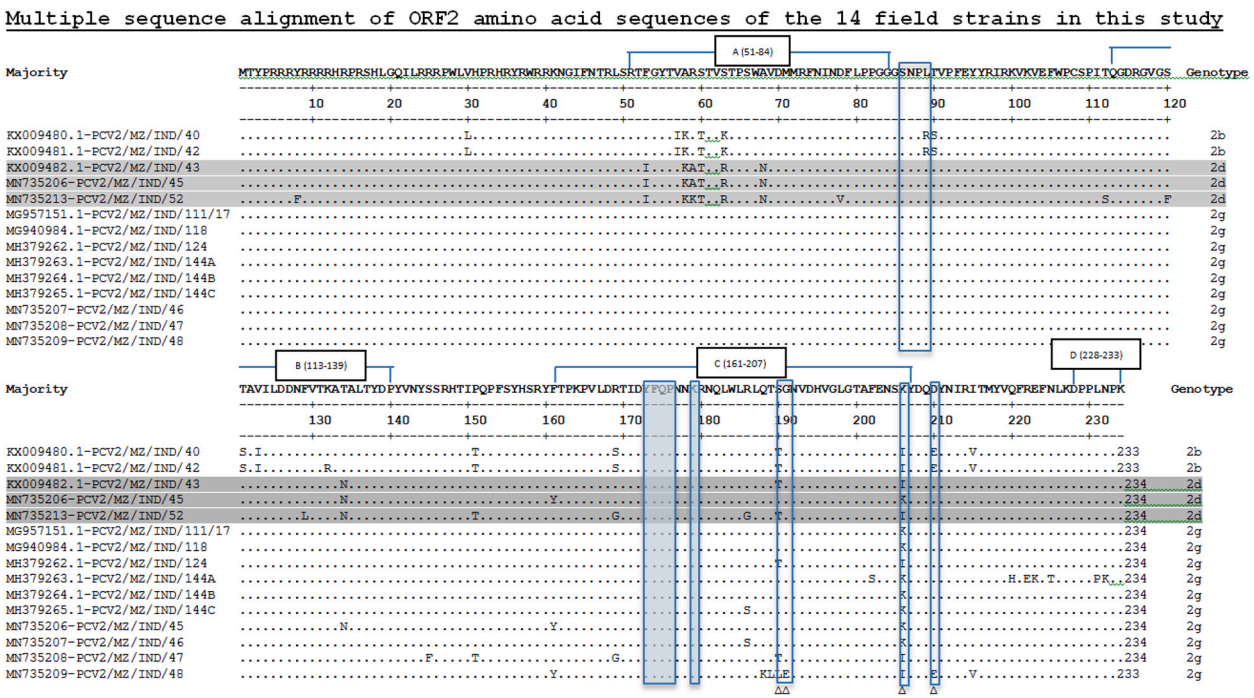

Fig. 4. Multiple sequence alignment of ORF2 amino acid sequences of the 14 field isolates. Dots represent residues that match the consensus sequence exactly. The four general antibody recognition regions are labeled as A (51–84), B (113–139), C (161–207) and D (228–233). The amino acids residues between 86 and 89 known to distinguish genotypes 2a and 2b are boxed. Amino acid residues 173 to 175 and 179 known to be important for antibody recognition are boxed and shaded. Amino acid residues 190–191, 206 and 210 that are known to be important for PCV2 replication in vitro are marked with Δ and boxed.

genotypes was found conserved in all the field isolates as previously described (Kleymann et al., 2020). Further, the antibody recognition residues at position 173 to 175 and 179, the putative heparin sulphate binding receptor domain (residues 98 to 103) and the decoy epitope region (170 to 180), known for generation of non-protective antibodies, were all highly conserved among all the fourteen isolates. However, variations were noted within the general antibody recognition region A and in amino acid residues 59 and 206 which influence the vaccine-induced antibody binding abilities (Sahaa et al., 2012). It has been reported earlier that amino acids at positions 190, 191, 206 and 210 are important for in vitro replication of PCV2 (Song et al., 2020). We have observed mutations particularly at amino acid position 206, in all the fourteen field isolates, while positions 190–191 were found conserved in all isolates except in the isolate MN735209 (2g-2b recombinant). This isolate clusters within genotype 2g and resembled genotype 2b at position 210 reiterating the fact that it is a recombinant of genotype 2g and 2b. These variations warrant further studies to understand their significance. Despite these amino acid differences, we did not observe any genotype specific clinical manifestations or pathology in the affected pigs in this study. However, whether these genotype differences could contribute to varied immune responses during vaccination, natural infection, and concurrent coinfections with other pathogens or aid or deter the cross protection between genotypes remains to be explored (Trible and Rowland, 2012).

This study also emphasizes the need for routine surveillance, monitoring the PCV2 evolution and genotyping of circulating PCV2 specifically in NE India, considering the economic importance of piggery farming as well as the shared international borders unique to this region.

Declarations of interest

None.

Ethical approval

This article does not contain any studies with live animals. Thus, Ethical Statement is not applicable.

Data availability statement

The data that supports the findings of this study are available in the Supplementary Material of this article.

CRediT authorship contribution statement

T. K. Rajkhowa: Conceptualization of the research work, Formal analysis, Funding acquisition, Methodology, Project administration as the principal investigator, Resources, Supervision, Writing and editing the original draft. **P. Lalnunthanga:** Investigation and Data curation. **P. L. Rao:** Phylogenetic analyses of PCV2, analysis of PCV2 recombinants and helped in drafting the bioinformatics work in the manuscript. **M. Subbiah:** Designed and analysed the bioinformatics data, and contributed in drafting the final manuscript along with other authors. **B. Lalrohlua:** Investigation and Data curation.

Acknowledgments

This work was supported by the Department of Biotechnology, Government of India under grant no. BT/PR17175/NER/95/471/2015. The authors thank the Dean, CVSc& AH, CAU, Selesih, Aizawl, Mizoram for providing necessary facilities to carry out the investigation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2021.104775.

References

Anoopraj, R., Rajkhowa, T.K., Cherian, S., Arya, R.S., Tomar, N., Gupta, A., Ray, P.K., Somvanshi, R., Saikumar, G., 2015. Genetic characterisation and phylogenetic analysis of PCV2 isolates from India: indications for emergence of natural inter-genotypic recombinants. Infect. Genet. Evol. 31, 25–32. https://doi.org/10.1016/j.meegid.2015.01.006.

Cai, L., Ni, J., Xia, Y., Zi, Z., Ning, K., Qiu, P., Li, X., Wang, B., Liu, Q., Hu, D., Yu, X., Zhou, Z., Zhai, X., Han, X., Tian, K., 2012. Identification of an emerging recombinant cluster in porcine circovirus type 2. Virus Res. 165 (1), 95–102. https://doi.org/10.1016/J.Virus Res.2012.01.008.

Carman, S., Cai, H.Y., DeLay, J., Youssef, S.A., McEwen, B.J., Gagnon, C.A., Tremblay, D., Hazlett, M., Lusis, P., Fairles, J., Alexander, H.S., van Dreumel, T., 2008. The emergence of a new strain of porcine circovirus-2 in Ontario and Quebec swine and its association with severe porcine circovirus associated disease – 2004–2006. Can. J. Vet. Res. 72 (3), 259–268.

Chae, C., 2005. A review of porcine circovirus 2- associated syndromes and diseases. Vet. J. 169, 326–336.

Cheung, A.K., 2009. Homologous recombination within the capsid gene of porcine circovirus type 2 subgroup viruses via natural co-infection. Arch. Virol. 154 (3), 531–534. https://doi.org/10.1007/s00705-009-0329-5.

Cheung, A.K., Lager, K.M., Kohutyuk, O.I., Vincent, A.L., Henry, S.C., Baker, R.B., Rowland, R.R., Dunham, A.G., 2007. Detection of two porcine circovirus type 2 genotypic groups in United States swine herds. Arch. Virol. 152 (5), 1035–1044. https://doi.org/10.1007/s00705-006-0909-6.

Choi, C.Y., Choi, Y.C., Park, I.B., Lee, C.H., Kang, S.J., Chun, T., 2018. The ORF5 protein of porcine circovirus type 2 enhances viral replication by dampening type I interferon expression in porcine epithelial cells. Vet. Microbiol. 226, 50–58. https://doi.org/10.1016/j.vetmic.2018.10.005.

Cino-Ozuna, A.G., Henry, S., Hesse, R., Nietfeld, J.C., Bai, J., Scott, H.M., Rowland, R.R. R., 2011. Characterization of a new disease syndrome associated with porcine circovirus type 2 (PCV2) in previously vaccinated herds. J. Clin. Microbiol. 49 (5), 2012–2016.

Dupont, K., Nielsen, E.O., Baekbo, P., Larsen, L.E., 2008. Genomic analysis of PCV2 isolates from Danish archives and a current PMWS case–control study supports a shift in genotypes with time. Vet. Microbiol. 128 (1–2), 56–64. https://doi.org/10.1016/j.vetmic.2007.09.016.

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with post weaning multi-systemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. 38 (7), 2494–2503.

Fenaux, M., Opriessnig, T., Halbur, P.G., Elvinger, F., Meng, X.J., 2004. Two amino acid mutations in the capsid protein of type 2 porcine circovirus (PCV2) enhanced PCV2 replication in vitro and attenuated the virus in vivo. J. Virol. 78 (24), 13440–13446.

Franzo, G., Segalés, J., 2018. Porcine circovirus 2 (PCV-2) genotype update and proposal of a new genotyping methodology. PLoS One 13 (12). https://doi.org/10.1371/journal.pone.02085 e0208585.

Franzo, G., Cortey, M., Segale's, J., Hughes, J., Drigo, M., 2016a. Phylodynamic analysis of porcine circovirus type 2 reveals global waves of emerging genotypes and the circulation of recombinant forms. Mol. Phylogenet. Evol. 100, 269–280. https://doi.org/10.1016/j.ympev.2016a.04.028.

Franzo, G., Tucciarone, C.M., Cecchinato, M., Drigo, M., 2016b. Porcine circovirus type 2 (PCV2) evolution before and after the vaccination introduction: A large scale epidemiological study. Sci. Rep. 6, 39458. https://doi.org/10.1038/srep39458 (PMID: 27991573).

Grau-Roma, L., Crisci, E., Sibila, M., López-Soria, S., Nofrarias, M., Cortey, M., Fraile, L., Olvera, A., Segalés, J., 2008. A proposal on porcine circovirus type 2 (PCV2) genotype definitions and their relation with post weaning multi-systemic wasting syndrome (PMWS) occurrence. Vet. Microbiol. 128 (1–2), 23–35.

Guo, L.J., Lu, Y.H., Wei, Y.W., Huang, L.P., Liu, C.M., 2010. Porcine circovirus type 2 (PCV2): genetic variation and newly emerging genotypes in China. Virol. J. 7, 273. https://doi.org/10.1186/1743-422X-7-273.

Guo, L., Fu, Y., Wang, Y., Lu, Y., Wei, Y., Tang, Q., et al., 2012. A porcine circovirus type 2 (PCV2) mutant with 234 amino acids in Capsid protein showed more virulence in vivo, compared with classical PCV2a/b strain. PLoS One 7 (7). https://doi.org/10.1371/journal.pone.0041463 e41463. (PMID: 22829951).

Hamel, A.L., Lin, L.L., Sachvie, C., Grudeski, E., Nayar, G.P., 2000. PCR detection and characterization of type-2 porcine circovirus. Can. J. Vet. Res. 64 (1), 44–52.

He, J., Cao, J., Zhou, N., Jin, Y., Wu, J., Zhoua, J., 2013. Identification and functional analysis of the novel ORF4 protein encoded by porcine circovirus type 2. J. Virol. 87 (3), 1420–1429. https://doi.org/10.1128/JVI.01443-12.

He, W., Zhao, J., Xing, G., Li, G., Wang, R., Wang, Z., Zhang, C., Franzo, G., Su, S., Zhou, J., 2019. Genetic analysis and evolutionary changes of Porcine circovirus 2. Mol. Phylogenet. Evol. 139, 106520. https://doi.org/10.1016/j.ympev.2019.106520.

Hesse, R., Kerrigan, M., Rowland, R.R., 2008. Evidence for recombination between PCV2a and PCV2b in the field. Virus Res. 132, 201–207.

Horlen, K.P., Schneider, P., Anderson, J., Nietfeld, J.C., Henry, S.C., Tokach, L.M., Rowland, R.R.R., 2007. A cluster of farms experiencing severe porcine circovirus associated disease: clinical features and association with the PCV2b genotype. J. Swine Health Prod. 15 (5), 270–278.

Jantafong, T., Boonsoongnern, A., Poolperm, P., Urairong, K., Leckcharoensuk, C., Leckcharoensuk, P., 2011. Genetic characterization of porcine circovirus type2 in piglets from PMWS affected and negative farms in Thailand. Virol. J. 8, 88.

Karuppannan, A.K., Opriessnig, T., 2017. Porcine circovirus type 2 (PCV2) vaccines in the context of current molecular epidemiology. Viruses. 9 (5), 99. https://doi.org/10.3390/v9050099.

Kim, H.K., Luo, Y., Moon, H.J., Park, S.J., Keum, H.O., Rho, S., Park, B.K., 2009. Phylogenetic and recombination analysis of genomic sequences of PCV2 isolated in Korea. Virus Genes 39 (3), 352–358.

Kleymann, A., Soto, E., Illanes, O., Malik, Y.S., Fuentealba, C., Ghosh, S., 2020. High rates of detection and complete genomic analysis of porcine circovirus 2 (PCV2) in the Lesser Antilles island of St. Kitts: identification of PCV2b-PCV2d recombinants. Transbound. Emerg. Dis. 00, 1–8. https://doi.org/10.1111/tbed.13583.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874.

Larochelle, R., Antaya, M., Morin, M., Magar, R., 1999. Typing of porcine circovirus in clinical specimens by multiplex PCR. J. Virol. Methods 80 (1), 69–75.

Larochelle, R., Magar, R., D'Allaire, S., 2002. Genetic characterization and phylogenetic analysis of porcine circovirus type 2 (PCV2) strains from cases presenting various clinical conditions. Virus Res. 90 (1–2), 101–112.

Lefebvre, D.J., Van Doorsselaere, J., Delputte, P.L., Nauwynck, H.J., 2009. Recombination of two porcine circovirus type 2 strains. Arch. Virol. 154 (5), 875–879.

Li, D., Wang, J., Xu, S., Cai, S., Ao, C., Fang, L., Xiao, S., Chen, H., Jiang, Y., 2018. Identification and functional analysis of the novel ORF6 protein of porcine circovirus type 2 in vitro. Vet. Res. Commun. 42 (1), 1–10.

Liu, Q., Tikoo, S.K., Babiuk, L.A., 2001. Nuclear localization of the ORF2 protein encoded by porcine circovirus type 2. Virology. 285 (1), 91–99.

Liu, J., Chen, I., Kwang, J., 2005. Characterization of a previously unidentified viral protein in porcine circovirus type 2-infected cells and its role in virus-induced apoptosis. J. Virol. 79, 8262–8274. https://doi.org/10.1128/JVI.79.13.8262-8274.2005.

Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W., Ray, S.C., 1999. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected Seroconverters in India, with evidence of inter subtype recombination. J. Virol. 73, 152–160.

Lv, Q., Guo, K., Xu, H., Wang, T., Zhang, Y., 2015. Identification of putative ORF5 protein of porcine circovirus type 2 and functional analysis of GFP-fused ORF5 protein. PLoS One 10 (7). https://doi.org/10.1371/journal.pone.0134203 e0134203.

Lv, Q., Guo, K., Zhang, G., Zhang, Y., 2016. The ORF4 protein of porcine circovirus type 2 antagonizes apoptosis by stabilizing the concentration of ferritin heavy chain through physical interaction. J. Gen. Virol. 97, 1636–1646.

Ma, C.-M., Hon, C.-C., Lam, T.-Y., Li, V.Y.-Y., Wong, C.K.-W., de Oliveira, T., Leung, F.C.-C., 2007. Evidence for recombination in natural populations of porcine circovirus type 2 in Hong Kong and mainland China. J. Gen. Virol. 88 (Pt 6), 1733–1737.

Mankertz, A., Hillenbrand, B., 2001. Replication of porcine circovirus type 1 requires two proteins encoded by the viral rep gene. Virology. 279 (2), 429–438. https://doi.org/10.1006/viro.2000.0730.

Mankertz, A., Domingo, M., Folch, J.M., LeCann, P., Jestin, A., Segalés, J., Chmielewicz, B., Plana-Durán, J., Soike, D., 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. 66 (1), 65–77.

Martin, D.P., Lemey, P., Lott, M., Moulton, V., Posada, D., Lefeuvre, P., 2010. RDP3: a flexible and fast computer program for analyzing recombination. Bioinformatics. 26 (19), 2462–2463. https://doi.org/10.1093/bioinformatics/btq467.

Mukherjee, P., Karam, A., Barkalita, L., Borah, P., Chakrabarty, A.K., Das, S., Puro, K., Sanjukta, R., Ghatak, S., Shakuntala, I., Laha, R.G., Sen, A., Sharma, I., 2018. Porcine circovirus 2 in the north eastern region of India: disease prevalence and genetic variation among the isolates from areas of intensive pig rearing. Acta Trop. 182, 166–172.

Mukherjee, P., Karam, A., Chakrabarty, A.K., Baruah, S., Pegu, R., Das, S., Milton, A.A.P., Puro, K., Sanjukta, R., Ghatak, S., Shakuntala, I., Laha, R.G., Sen, A., 2019. Identification of a novel cluster of PCV2 isolates from Meghalaya, India, indicates possible recombination along with changes in capsid protein. Infect. Genet. Evol. 71, 7–15.

Nawagitgul, P., Morozov, I., Bolin, S.R., Harms, P.A., Sorden, S.D., Paul, P.S., 2000. Open reading frame 2 of porcine circovirus type 2 encodes a major capsid protein. J. Gen. Virol. 81 (9), 2281–2287. https://doi.org/10.1099/0022-1317-81-9-2281.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Investig. 19 (6), 591–615. https://doi.org/10.1177/104063870701900601.

Opriessnig, T., Madson, D.M., Schalk, S., Brockmeier, S., Shen, H.G., Beach, N.M., Meng, X.J., Baker, R.B., Zanella, E.L., Halbur, P.G., 2011. Porcine circovirus type 2 (PCV2) vaccination is effective in reducing disease and PCV2 shedding in semen of boars concurrently infected with PCV2 and mycoplasma hyopneumoniae. Theriogenology. 76 (2), 351–360.

Ramamoorthy, S., Meng, X.J., 2009. Porcine circoviruses: a minuscule yet mammoth paradox. Anim. Health Res. Rev. 10 (1), 1–20.

Sahaa, D., Huang, L., Bussalleu, E., Lefebvre, D.J., Fort, M., Doorsselaere, J.V., Nauwynck, H.J., 2012. Antigenic subtyping and epitopes' competition analysis of porcine circovirus type 2 using monoclonal antibodies. Vet. Microbiol. 157, 13–22. https://doi.org/10.1016/j.vetmic.2011.11.030.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Song, S., Park, G.N., Choe, S., Cha, R.M., Kim, S.Y., Hyun, B.H., Park, B.K., An, D., Jun., 2020. Genetic diversity of porcine circovirus isolated from Korean wild boars. Pathogens. 9, 457. https://doi.org/10.3390/pathogens9060457.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature. 295 (5844), 64–66.

Trible, B.R., Rowland, R.R.R., 2012. Genetic variation of porcine circovirus type 2 (PCV2) and its relevance to vaccination, pathogenesis and diagnosis. Virus Res. 164, 68–77.

Wang, F., Guo, X., Ge, X., Wang, Z., Chen, Y., Cha, Z., Yang, H., 2009. Genetic variation analysis of Chinese strains of porcine circovirus type 2. Virus Res. 145 (1), 151–156. https://doi.org/10.1016/j.virusres.2009.05.015.

Weissenbacher-Lang, C., Kristen, T., Mendel, V., Brunthaler, R., Schwarz, L., Weissenböck, H., 2020. Porcine circovirus type 2 (PCV2) genotyping in Austrian pigs in the years 2002 to 2017. BMC Vet. Res. 16, 198. https://doi.org/10.1186/s12917-020-02413-4.

Wellenberg, G.J., Pesch, S., Berndsen, F.W., Steverink, P.J.G.M., Hunneman, W., Van der Vorst, T.J.K., Peperkamp, N.H.M.T., Ohlinger, V.F., Schippers, R., Van Oirschot, J.T., de Jong, M.F., 2000. Isolation and characterization of porcine circovirus type 2 from pigs showing signs of post–weaning multi-systemic wasting syndrome in the Netherlands. Vet Q. 22, 167–172.

Wiederkehr, D.D., Sydler, T., Buergi, E., Haessig, M., Zimmermann, D., Pospischil, A., Brugnera, E., Sidler, X., 2009. A new emerging genotype subgroup within PCV- 2b dominates the PMWS epizooty in Switzerland. Vet. Microbiol. 136 (1–2), 27–35.

Xiao, C.-T., Halbur, P.G., Opriessnig, T., 2012. Complete genome sequence of a novel porcine circovirus type 2b variant present in cases of vaccine failures in the United States. J. Virol. 86 https://doi.org/10.1128/JVI.02345-12, 12469–12469. (PMID: 23087125).