GIULIANA LORETO SARAIVA

MOLECULAR EVOLUTION AND RETROSPECTIVE DETECTION OF Porcine circovirus 3 IN PIG PRODUCTION

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Medicina Veterinária, para obtenção do título de Doctor Scientiae.

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SUMÁRIO

ABSTRACT

SARAIVA, Giuliana Loreto, D.Sc., Universidade Federal de Viçosa, June, 2018. **Molecular evolution and retrospective detection of Porcine circovirus 3 in pig production.** Adviser: Márcia Rogéria de Almeida Lamêgo. Co-adviser: Pedro Marcus Pereira Vidigal.

The pork industry has increased significantly over the last decades due to the increased consumption of this source of animal protein. The pressure for better efficiency and production has led to an increased production and intensification of international trade and genetic resources. Concurrently with the growth of the pork industry, the diseases present in pig farming have never been so diversified and the number of reports of emerging pathogens has increased significantly over the past 30 years. Considering this information, the present study aimed to conduct a retrospective study of detection and analyze the molecular evolution of an emerging virus in the pork industry, the Porcine circovirus 3 (PCV3). For the retrospective study of PCV3 detection in Brazil, laboratory-stored DNA samples were used for the detection of the virus by the conventional PCR technique. After sequencing the positive samples, phylogenetic and molecular analyzes were performed to understand the epidemiology of PCV3 in Brazil. In addition, phylogenetic analyzes and comparative genomics were conducted to find a possible evolutionary origin of PCV3. The results of this study provided important information about PCV3, which would serve as a basis for the development of control and prevention measures.

RESUMO

SARAIVA, Giuliana Loreto, D.Sc., Universidade Federal de Viçosa, junho de 2018. **Molecular evolution and retrospective detection of Porcine circovirus 3 in pig production.** Orientadora: Márcia Rogéria de Almeida Lamêgo. Coorientador: Pedro Marcus Pereira Vidigal.

A produção de suínos aumentou significativamente ao longo das últimas décadas devido ao maior consumo dessa fonte de proteína animal. A pressão para uma maior eficiência e um maior volume de produção levou ao aumento da produção e da intensificação do comércio internacional de suínos e recursos genéticos. Concomitantemente ao crescimento da indústria suína, as enfermidades presentes na suinocultura nunca foram tão diversificadas e o número de relatos de patógenos emergentes aumentou, significantemente, durante os últimos 30 anos. Considerando essas informações, o presente estudo teve como objetivo realizar um estudo retrospectivo de detecção e analisar a evolução molecular de um vírus emergente na suinocultura mundial e brasileira, o Porcine circovirus 3 (PCV3). Para o estudo retrospectivo de detecção do PCV3 no Brasil, amostras de DNA estocadas em laboratório foram utilizadas para a pesquisa do vírus por meio da técnica de PCR convencional. Após sequenciamento das amostras positivas, análises filogenéticas e comparações moleculares foram realizadas para entender a epidemiologia do PCV3 no Brasil. Além disso, análises filogenéticas e genômica comparativa foram conduzidas para encontrar uma possível origem evolucionária do PCV3 na suinocultura mundial. Os resultados desse estudo forneceram informações importantes sobre o PCV3, que servirão de base para a elaboração de medidas de controle e prevenção.

1. GENERAL INTRODUCTION

The world pig production holds the first place in the world meat market, but it is in third place in relation to the international trade of its commodities. This scenario may change as the international trade in the swine production grew significantly from 2015 to 2016, with a record growth rate of 4.4%. On the other hand, the world production of pork meat decreased in 2016 registering the second year of stagnation (FAO, 2016).

This deceleration in pork meat production was due to a 2.5% decline in Chinese production, together with a reduction in the number of piglets in Japan, South Korea, United States, Mexico and Canada. This reduction in the number of piglets was due to several outbreaks that occurred between 2013 and 2014, caused by the Porcine epidemic diarrhea virus (PEDv) (FAO, 2016). With the recovery of the effects of PEDv and in response to an increase in demand, a global growth of 1% in pork production was recorded in 2017. Countries such as United States, China, Brazil and the Russian Federation lead this global growth (FAO, 2017).

According to data from the Brazilian Animal Protein Association (ABPA), in 2017, Brazil was considered the fourth largest producer and exporter of pork meat. In order to maintain the Brazilian pig production, herds need to be protected from classical and emerging diseases. Infections of an endemic or epidemic nature, even if they do not lead to high mortality, can cause economic losses in productivity and, consequently, profitability by directly interfering with the parameters used to measure the performance of the activity, such as daily weight gain , feed conversion and reproductive performance (GASTARDELO; MELZ, 2014).

The relevance of emerging viral diseases in pig farming is high, due to the higher mutation rates and, therefore, a greater risk of immunological escape; to the immunosuppression triggered by some viruses and to the multifactorial nature of the viruses, leading to more severe diseases related to secondary pathogens. Therefore, the scientific community must be prepared as well as the animal health industry, veterinarians, producers and consumers, joining efforts to encourage global, collaborative and action-oriented strategies aimed to minimize the negative impacts of the spread of these diseases.

In this context, this study identified in the scientific literature an emerging virus, the Porcine circovirus 3 (PCV3). This virus was considered of high risk of dissemination based on the previous experience of our research group with the Porcine circovirus 2 (PCV2). Because it is a newly identified virus, it is evident the need for new studies that can clarify the epidemiology and genetic variability of PCV3 in Brazil.

2. LITERATURE REVIEW

2.1. Emerging and reemerging diseases in pig production

Emerging and reemerging diseases can be defined as those whose incidence increases after introduction into a new population or into an already existing population as a result of epidemiological changes (WOOLHOUSE, 2002). This concept may also include diseases linked to pathogens that have spread to an area that it has not yet been reported or due to infectious agents that have significantly changed their clinical-pathological presentation (JONES et al., 2008).

Despite improvements in global health, outbreaks of infectious diseases continue to occur and will likely continue to emerge in the future. This situation is especially important when dealing with herds, since loss of production and competitiveness can cause significant economic disadvantages, not only for producers but also for the whole country.

The number of reports of emerging diseases in pig farms has increased significantly during the last 30 years (SEGALES, MATEU, 2012). Most of the emerging diseases described in swine are of infectious origin and the maintenance of an infectious agent in a population can be favored by environmental factors such as: i) intensive production with high animal density; ii) significant increase in international trade; iii) inadequate prevention and control measures (SEGALÉS, 2015).

The environment provided to the virus through intensified production and international trade of commodities, such as trade of live animals, can influence viral evolution and dispersion, and thus the emergence of diseases in the world pig production (DREW, 2011). Another environmental factor that can influence the evolution of viruses is generated by control and prevention methods. The control and prevention strategy most commonly used in the pig production is the systematic vaccination of animals, but if the vaccine does not have a broad spectrum of protection against variants of a virus, it may select the variants that have mutations which alter its pathogenicity and promote the maintenance of the virus in the environment (SEGALES; MATEU, 2012).

Current solutions to the challenge of the emergence of viral diseases in pig production favor the development of prophylactic vaccines to reduce the susceptibility of pigs along with sanitary barriers in farms to reduce the risk of spread of an infectious agent. The prevention of the entry of a pathogen into a farm or country is a very difficult task, but it is possible to reduce the risk of such event. An effective approach to the challenge of the emergence of viral diseases in pig production would be to study more about the environmental factors that drive the evolution and dispersion of these pathogens and thus to develop more efficient intervention methods.

In the last two decades, important viral diseases have emerged in the world pig production, such as Porcine circovirus 2 (PCV2), Porcine reproductive and respiratory syndrome virus (PRRSV) and Porcine epidemic diarrhea virus (PEDv). Other viruses such as Torque teno virus (TTSuV), Porcine bocavirus (PBoV) and Porcine sapovirus (Porcine SaV) are mainly of subclinical manifestation in swine. Although some emerging viruses have limited clinical implications in pigs such as Swine hepatitis E virus (Swine HEV), they may pose a challenge to human health because they have zoonotic potential (MENG, 2012). In 2016, a new member of the Circoviridae family, called Porcine circovirus 3 (PCV3) was identified in the United States (PALINSKI et al., 2017).

2.2. Porcine circovirus 3 (PCV3)

The Circoviridae family consists of the genus Circovirus whose species are currently known to infect birds, mammals and fishes. Viruses classified in the Circoviridae family are non-enveloped and have circular genomes with single-stranded DNA (RAMAMOORTHY; MENG, 2009).

Mammalian circovirus includes species that infect pigs, such as Porcine circovirus (PCVs) 1 and 2 (PCV1 and PCV2) (MANKERTZ et al., 2004) and, currently, the Porcine circovirus 3 (PCV3) (PALINSKI et al., 2017). Porcine circovirus 1 (PCV1) was identified in the 1970s as a contaminating agent in pig kidney cells of the PK-15 lineage and it was not associated with clinical disease (TISCHER et al., 1986; TISCHER; RASCH; TOCHTERMANN, 1974). In contrast, PCV2 is associated with a variety of different syndromes in pigs called

Porcine Circovirus Associated Diseases (PCVAD) (OPRIESSNIG; MENG; HALBUR, 2007). Since its identification and characterization in the 1990s, PCV2 has reached a worldwide distribution and PCVAD has become an endemic syndrome in most pig producing countries (GRAU-ROMA; FRAILE; SEGALES, 2011; MADEC et al., 2008; RAMAMOORTHY; MENG, 2009).

All three PCVs are typical virus of the Circoviridae family, presenting circular DNA genomes. The size of the PCV1 genome is about 1.760 bp, the size of the PCV2 genome varies, depending on the genotype, between 1.767 bp (PCV2b, 2c and 2d) and 1.777 bp (PCV2e) (FUX et al., 2018). The PCV3 genome has approximately 2.000 nucleotides, which is converted into double-stranded DNA during replication, and has three Opens Reading Frames (ORFs), two of which are similar to replicase (REP) and capsid (CAP) proteins (PALINSKI et al., 2017).

ORF1 is the largest and encodes a 296 amino acid protein with 48% identity to PCV2 REP. ORF2 is located in the opposite orientation to the REP, encoding a 214 amino acid protein with 35% identity to the PCV2 CAP. Finally, ORF3 is oriented in the same sense as ORF1 and encodes a protein of 231 amino acids with 39% identity with a protein of unknown function of Murine Herpesvirus M169 (PALINSKI et al., 2017; PHAN et al., 2017) .

2.2.1. Epidemiology

In 2016, PCV3 was identified in the United States in sows that died acutely with clinical signs of Porcine Dermatitis and Nephropathy syndrome (PDNS) and in stillbirths from these sows. In this same study, other samples from pigs with respiratory problems were tested and PCV3 was also found (PALINSKI et al., 2017). Another study associated PCV3 with cases of acute myocarditis and multi-systemic inflammation in pigs in the United States (PHAN et al., 2017). Although these clinical manifestations are often associated with PCV2 infection, no PCV2 genomes were detected in the samples of both studies. In 2017, 356 samples from three Chinese farms with reproductive problems and with high neonatal mortality were collected and PCV3 was the only pathogen found in these samples, excluding other viruses such as Porcine pseudorabies virus (PRV), Porcine circovirus 2 (PCV2), Porcine reproductive

and respiratory syndrome virus (PRRSV), Porcine epidemic diarrhea virus (PEDV), Porcine transmissible gastroenteritis virus (TGEV), Porcine rotavirus (RV) and Classical swine fever virus (CSFV). To better understand the epidemiology of PCV3 in China, the same study collected 222 samples from 35 farms located in 11 Chinese provinces and found PCV3 in 68% of the farms and 35% of the tissue samples from sows and stillborn piglets, including tissues such as brain, lung, lymph node, tonsils, semen and serum (KU et al., 2017). Other studies have detected and analyzed the prevalence of PCV3 in different regions of China (CHEN et al., 2017, FU et al., 2018, LIU et al., 2018, SHEN et al., 2017, ZHAI et al., 2017; ZHANG et al., 2014; ZHENG et al., 2017) as well as in Thailand (KEDKOVID et al., 2018).

A retrospective PCV3 prevalence study in China analyzed 200 clinical swine samples collected between 1990 and 1999 and the results showed that 6.5% of the collected pig samples were positive for PCV3, with the first cases occurring in 1996 (SUN et al., 2018). Unexpectedly, PCV3 was also detected in serum samples from dogs collected between 2015 and 2016 in China, which shared 96.3% to 98.1% of PCV3 identity detected in pigs, suggesting that transmission of PCV3 between species is possible (ZHANG et al., 2018). In addition, high susceptibility of wild boars to PCV3 infection was reported in a study in Italy (FRANZO et al., 2018).

PCV3 has been reported in several European countries such as Poland, Germany, United Kingdom, Italy, Sweden, Denmark and Spain (FACCINI et al., 2017; FRANZO et al., 2018; FUX et al., 2018; STADEJEK et al., 2017).

In Poland, the analysis of pig serum samples indicated that PCV3 is highly prevalent in the country and PCV3 infection is more prevalent in weaned piglets (STADEJEK et al., 2017). Similar results were found in a Korean study in which PCV3 was more prevalent in weaned piglets than in adult pigs (KWON et al., 2017).

In Germany, 1.060 sera from weaned piglets from 53 farms with respiratory clinical signs were analyzed for PCV3 detection by real-time PCR. This study demonstrated a high prevalence of PCV3 in German pig farms (75%) and revealed through phylogenetic analyzes two groups of clearly separated PCV3 strains which were described as PCV3a and PCV3b genotypes. In

addition, the study also raises the hypothesis that PCV3 is not a recent virus in Germany (FUX et al., 2018).

Results from a retrospective study in the United Kingdom showed that PCV3 was detected in 20% of fecal samples collected between 2002 and 2017, as well as in 5% of tissue samples collected between 2001 and 2004 (COLLINS; MCKILLEN; GORDON, 2017). In Italy, PCV3 was detected in the tissues of stillborn piglets from two farms (FACCINI et al., 2017). DNA from 49 lymph node samples collected between 1993 and 2007 in Sweden was tested for PCV3 using conventional PCR. In this study, 20.4% of the samples were positive, and one of these samples was collected in 1993 (YE et al., 2018). In Spain, 11.4% of serum samples from pigs collected between 1996 and 2017 were positive for PCV3, with the first case reported in 1996 (KLAUMANN et al., 2018).

Two Brazilian genomes of PCV3 were described, validating the hypothesis that this virus is circulating in Brazil. The genomes were recovered from serum samples from sows, which had just been delivered litters with variable numbers of stillborn piglets. No sequence of PCV3 was detected in sows from the same farms that did not have stillborn piglets (TOCHETTO et al., 2017).

2.2.2. Pathogenesis and clinical manifestations

PCV3 detection was associated with different diseases and clinical manifestations in pigs from different ages. Porcine Dermatitis and Nephropathy syndrome (PDNS) was described associated with PCV3 (PALINSKI et al., 2017). It was also associated with reproductive disorders (FU et al., 2018; KU et al., 2017; LIU et al., 2018; PALINSKI et al., 2017; PHAN et al., 2017; TOCHETTO et al., 2017), cardiac and multi-systemic inflammation (PHAN et al., 2017), respiratory diseases (KEDKOVID et al., 2018; SHEN et al., 2017), congenital tremors (CHEN et al., 2017) and diarrhea in weaned piglets (ZHAI et al., 2017).

Palinski et al. (2017) found PCV3 in a farm with PDNS clinical cases, along with a 10.2% increase in sows' mortality and 1.19 more stillborn per litter. Tests for PCV2, PRRSV and Swine influenza virus (SIV) were

negative, based on immunohistochemistry and real-time PCR, while the heart, lung and lymph node samples were positive for PCV3 by real-time PCR and immunofluorescence assay.

Another investigation of the epidemiological characteristics and evolutionary dynamics of PCV3 in southern China found that 53% of PCV3 cases occurred between 2015 and 2017 were detected in stillbirths and in 15% of these cases PCV3 was the only pathogen found, correlating the occurrence of PCV3 to reproductive disorders (FU et al., 2018). A case-control study investigated the presence of PCV3 in pigs with different clinical presentations and found that PCV3 may have an association with respiratory disease and diarrhea in weaned piglets (ZHAI et al., 2017). The transmission of PCV3 has not yet been well characterized, but viral excretion of PCV3 in colostrum has been reported (KEDKOVID et al., 2018).

Wen et al. (2017) found that the prevalence of PCV3 in healthy pigs was lower (19.14%) than that found in diseased pigs (37.95%), suggesting that a larger portion of pigs may be infected with PCV3 showing clinical signs of infection. However, high prevalence of PCV3 in pigs without specific clinical signs was reported in farms in Poland (STADEJEK et al., 2017). Similar results were found in Shandong province in China and Sweden (YE et al., 2018; ZHENG et al., 2017). Although PCV3 was detected in association with various clinical syndromes in swine, its role as a possible swine pathogen is still unclear, and studies are needed to clarify the pathogenesis of this virus. These studies need to focus on the isolation and propagation of PCV3 in vitro in order to obtain the isolate for use in animal experiments.

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4. ARTICLE I

EVOLUTIONARY ANALYSIS OF PORCINE CIRCOVIRUS 3 (PCV3) INDICATES AN ANCIENT ORIGIN FOR ITS CURRENT STRAINS AND A WORLDWIDE DISPERSION

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Evolutionary analysis of Porcine circovirus 3 (PCV3) indicates an ancient origin for its current strains and a worldwide dispersion

Abstract

Porcine circovirus 3 (PCV3) is an emerging virus that was identified in the United States in 2016. Since its first identification, PCV3 has been identified in Brazil, China, United States, Poland and Republic of Korea. In this study, we used molecular phylogenetic analysis of available sequences to address questions surrounding the emergence of PCV3 in porcine world industry. Our data indicates that PCV3 did not emerge through recombination events among currently known circoviruses and that its speciation is not a recent evolutionary event. The most common recent ancestor (tMRCA) analysis suggests that PCV3 lineages have emerged over the past 50 years. PCV3 is not genetically closely related with other Porcine circovirus and it has been evolving undetected for some time in swine and probably in bovine population. We also found groups of genetically related isolates of PCV3 originated from different countries that may be associated with dispersal routes, suggesting that PCV3 has already been circulating in pig-producing countries for some time before its first detection.

Keywords: PCV3; Porcine circovirus; phylogeny; recombination; dispersal route.

Introduction

Porcine circoviruses (PCV) are non-enveloped, single-stranded circular DNA virus that can be separated into two main species: Porcine circovirus 1 (PCV1) and Porcine circovirus 2 (PCV2) [1,2]. PCV1 was initially identified in the 1970s as being a contaminating agent in pig kidney cells and it was not associated with clinical disease [3,4]. In contrast, PCV2 is associated with a vast range of syndromes in pigs called porcine circovirus associated diseases (PCVAD) [5]. Since its first identification in the 1990s, PCV2 has been detected worldwide and PCVAD has been recognized as an enzootic syndrome in most swine-producing countries and is considered as the main cause of losses on pig farms [6–9].

In 2016, a novel circovirus called Porcine circovirus 3 (PCV3) was identified in the United States in sows that died acutely with clinical signs of the porcine dermatitis and nephropathy syndrome (PDNS) [10]. Another study linked this emerging virus to clinical cases of acute myocarditis and multi-systemic inflammation also in pigs [11]. PCV3 has spread widely in China and was detected at different tissues, such as brain, lung, lymph node and tonsil [12–14]. In Poland, analysis of serum samples indicated that PCV3 is highly prevalent and the results showed that the PCV3 infection is more frequent in pigs that are 9 weeks of age and older [15]. In contrast, growing and finisher pig groups have been found to have relatively lower prevalence than the weaned group in South Korea. This study also highlighted that PCV3 is widely distributed in Korean pig populations [16]. Moreover, two Brazilian PCV3 genomes have been deposited in GenBank, confirming that this virus is also circulating in Brazil [17]. Interestingly, PCV3 has a high genetic identity with the NW2 sub-genomic molecule, which was first identified in 2008 from pork meat market in the United States [18]. After its first identification, closely related sequences to NW2 were identified in pork (PorkNW2) and beef (SFBeef) [19,20].

The genome of PCV3 has a single-stranded circular DNA with 2,000 nucleotides, which is converted into double-stranded DNA during replication, and has three opens reading frames (ORFs). The ORF1 is the largest ORF and encodes a 296-aa protein that is 95% identical to the

replication associated protein (Rep) of PorkNW2/USA/2009 circovirus (GenBank accession no. ADU77001; 221 aa), 55 % identical to the Rep of bat circoviruses (GenBank accession no. AIF76248) and 48% identical to Rep of PCV2 (GenBank accession no. AAC35309). The ORF2 encodes a 214-aa protein that is 87% identical to the capsid protein (Cap) of PorkNW2/USA/2009 (110 aa) and 23% to 35% identical to the Cap of bat circoviruses and PCV2, respectively. The ORF3 encodes a 231-aa protein that is 94% identical to an ORF identified in PorkNW2/USA/2009 and 39% identical to murid herpesvirus M169 [10,11].

In this study, we used molecular phylogenetic analyses to address the following questions to fill the first gaps about the emergence of PCV3 in pork world industry: i) Has PCV3 emerged through recent processes of recombination? ii) When the most recent common ancestor of current PCV3 strains has emerged? iii) Would it be possible to reconstruct dispersal routes of PCV3 from the first sets of available sequences?

Materials and Methods

Selecting data for analysis

Complete genomes and ORF2 sequences of PCV3 isolates were downloaded from the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) and were organized into two datasets (**Online Resource 1**). A third dataset was organized containing genome sequences of PCV3 isolates and reference sequences of different circovirus species, including Porcine circovirus [PCV1, PCV1/2 (a recombinant between PCV1 and PCV2) and PCV2 (PCV2a, PCV2b, PCV2c and PCV2d)] and other mammalian (bat, canine, chimpanzee, human and mink), avian and fish circoviruses (**Online Resource 1**). The sequences of PorkNW2 and SFBeef were also added to this third dataset. The sequences of all datasets were aligned by MAFFT version 7.310 [21], using a global pairwise alignment with 100,000 iterations of parameters refinement to improve the alignment.

Recombination analysis

In order to test whether PCV3 has emerged through processes of recombination between known circoviruses, the aligned genomes of Dataset III (**Online Resource 1**) (including positions with gaps) were analyzed using the Recombination Detection Program version 3.44 [22]. In this software, the methods RDP [23], GENECONV [24], Chimaera [25], MaxChi [26], 3Seq [27], and Bootscan/Siscan [28] were selected to identify phylogenetic evidence for recombination and predict recombination breakpoints (P-value < 0.05 and 1,000 permutations). Furthermore, a similarity plot analysis was conducted in the SimPlot version 3.5.1 [29], using the two-parameter (Kimura) distance model with a sliding window of 200 bp and step size of 20 bp.

Phylogenetic tree

Phylogenetic tree was inferred by Bayesian Inference (BI) from the circovirus genomes (Dataset III, **Online Resource 1**). To expedite the construction of the phylogenetic tree, the alignment was trimmed using Gblocks Server version 0.91 [30] (considering parameters for a less stringent selection) and the model of nucleotide substitution GTR+I+G was chosen using the program jModelTest version 2.1.10 [31]. The phylogenetic tree was calculated by MrBayes version 3.1.2 [32] using the Bayesian Markov Chain Monte Carlo (MCMC) method in four runs with 5,000,000 generations and a sampling frequency of 1,000. The parameter convergence was analyzed in Tracer version 1.6 (http://tree.bio.ed.ac.uk/software/tracer) and 1% of the generated trees was burnt to produce the consensus tree.

Comparative genomics

Comparative genomics was performed with the circovirus genomes (Dataset III, **Online Resource 1)** using the BLAST Ring Image Generator (BRIG) version 0.95 [33] for answering further questions about the genetic relationship of PCV3 with other circovirus, especially with PCV2. BRIG was used to align the PCV3 reference sequence (GenBank accession number NC_031753) to the circovirus genomes of third dataset using BLAST version

2.6.0 [34] (e-value threshold: 0.00001), making a plot with obtained alignments as concentric rings.

Time to the most recent common ancestor (tMRCA) analysis

To estimate the time to the most recent common ancestor (tMRCA) of PCV3 lineages, the Dataset I (**Online Resource 1**) with complete PCV3 genomes was analyzed using BEAST version 2.4.7 [35]. The HKY+I nucleotide substitution model was chosen using the program jModelTest [31] and the tMRCA was estimated using a Bayesian MCMC analysis. In this analysis, four molecular clock models (strict clock, relaxed clock exponential, relaxed clock log normal and random local clock) were tested with a coalescent Bayesian skyline prior. For each molecular clock assumption, the priors of substitution rates were predicted considering a log normal distribution (see **Online Resource 3**). MCMC runs were performed with 20,000,000 and the convergence of the parameters was analyzed using Tracer version 1.6 to calculate the Effective Sample Size (ESS) statistics. The marginal likelihoods obtained in each test were compared by Akaike Information Criterion for MCMC samples (AICM) [36] with 1,000 bootstrap replicates. The test with the lower AICM corresponds to the best-fit clock model. In tMRCA analysis, the best-fit clock model was selected and the prior of MRCA time was predicted considering a Laplace distribution (see **Online Resource 3**). Then, a MCMC run was performed with 20,000,000, the convergence of the parameters was analyzed using Tracer, and 10% of the trees generated was burnt to produce a consensus time tree using TreeAnnotator [35].

Reconstruction of dispersal routes

The possible dispersal routes of PCV3 isolates in pork world industry were predicted following the methodology described by Vidigal et al. [9]. The alignment of complete genomes (Dataset I, **Online Resource 1**) was fully selected and the alignment of ORF2 (Dataset II, **Online Resource 1**) was trimmed by excluding positions with gaps. The infinite sites model of genome evolution [37] was considered and the violations to this model were removed in

the trimmed alignments. Then, the sequences of each dataset were grouped into haplotypes (groups of sequences with 100% identity after trimming) using DnaSP version 5 [38]. The haplotype networks were constructed using the program Network version 5 (http://www.fluxus-engineering.com) and the Median Joining algorithm (MJ) [39]. To establish an economic context for haplotype network analysis, the recent statistics of live pigs trading (commodity code 0103) were downloaded from the United Nations Commodity Trade Statistics Database DESA/UNSD, UN Comtrade (http://comtrade.un.org/).

Results and Discussion

The first step to understand the evolutionary origin of PCV3 is to clarify if NW2 is a defective PCV3 or a replicative intermediate. The nucleotide sequence of PorkNW2 (HQ738638) has 1,202 nt and it was compared to 32 genomes of PCV3 with 2,000 nt. The alignment of PorkNW2 and PCV3 showed 1,202 aligned positions, presenting 12 SNPs, 2 indels, and a segment of 800 nt that was deleted. The identity ranged from 97.83% for PCV3-China/GD2016 strain (KY418606) and 99.17% for PCV3/CN/Fujian strain 5/2016 (KY075986). In addition, the alignment of SFBeef and PCV3 showed 859 aligned positions with only 4 SNPs and 1 indel, but the deleted segment was longer comparing with the PorkNW2, with 1,142 nt (**Online Resource 2**). Therefore, these observations suggest that PorkNW2 and SFBeef are actually defective PCV3 strains or replicative intermediates. The segment deleted in the PorkNW2 cover the major part of the capsid protein and the generation of such truncated genomes may be related to that viruses are passaged at high multiplicity of infection, leading to defective interfering viral particles retaining only essential elements required for genome replication [40].

Considering that, SFBeef sequences (or PCV3-like sequences) were found in bovine samples, we suggest that PCV3 is capable of infecting bovine cells. Whether PCV3 simply passes through the bovine cells or is capable of causing disease remains unknown. PCV2 has been reported to be present in cattle with respiratory disease and in bovine fetuses [41], but this finding has not been subsequently confirmed [42]. PCV2 has also been detected in calves with a fatal hemorrhagic disease syndrome [43]. Another

study investigated the susceptibility and immune response of calves to experimental PCV2 inoculation and the results revealed that host susceptibility of PCV2 is not restricted to pigs [44]. It would have been interesting to find out if PCV3 was also present in these PCV2-positive samples.

A previous study performed an analysis of identity and it was suggested that PCV3 could be the result of recombination events with bat circoviruses [12]. We performed a similar analysis, but with a different dataset, which included not only PCV3 isolates and bat circovirus but also other circoviruses. Further, we conducted another recombination analysis in the RDP software. The analysis implemented in the Simplot package showed three main picks of nucleotide identity with PCV3, but those points of identity did not include only bat circoviruses, but also the isolates of PCV2 and other circoviruses of mammals, fish and birds (**Figure 1**). The analysis performed with RDP didn´t find any recombination signal involving PCV3. The results of both analyses indicate that PCV3 has not emerged through recombination processes between current known circoviruses.

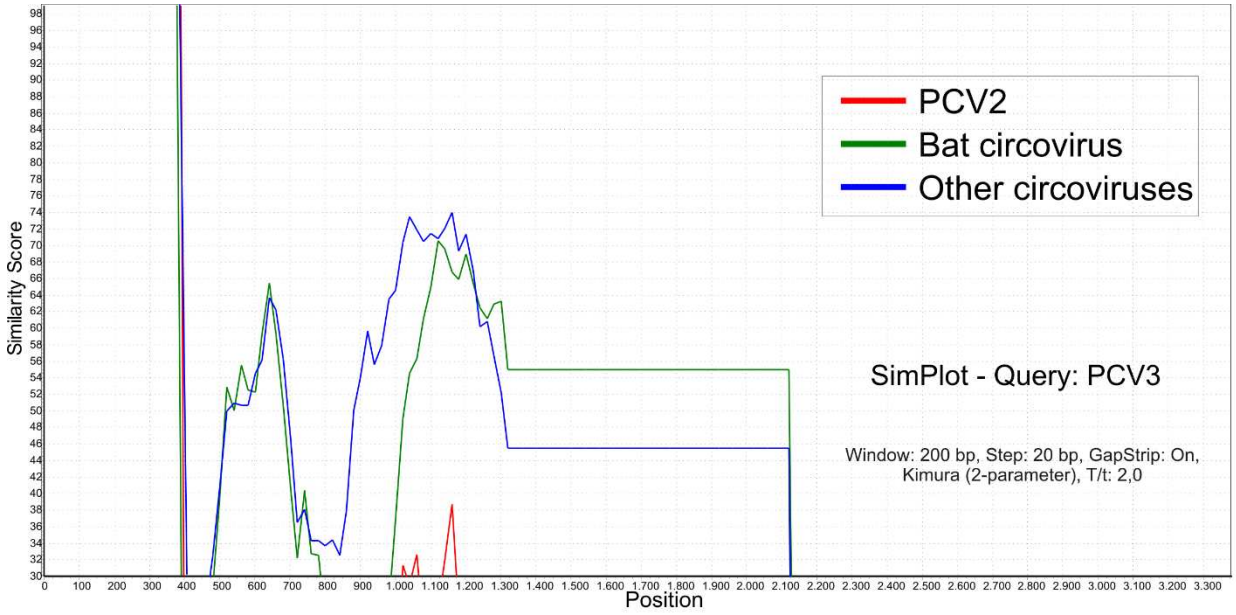

Fig. 1 Similarity plot of the whole genomes of 65 different circoviruses, including unique sequences of PCV1, PCV1/2, PorkNW2, SFBeef, mink, canine, human and chimpanzee CVs as well as 32 PCV3, 5 PCV2, 6 bat CV, 13 avian CV and 2 fish CV. The vertical and horizontal axes indicate the nucleotide similarity (%) and the nucleotide position (bp) of the alignment, respectively.

In the phylogenetic tree (**Figure 2**), the circovirus genomes were clustered in distinct monophyletic clades according to their respective hosts. The phylogenetic tree shows that PCV3 has a distinct origin from other porcine circoviruses and it shares an ancient common ancestor with the bat circoviruses.

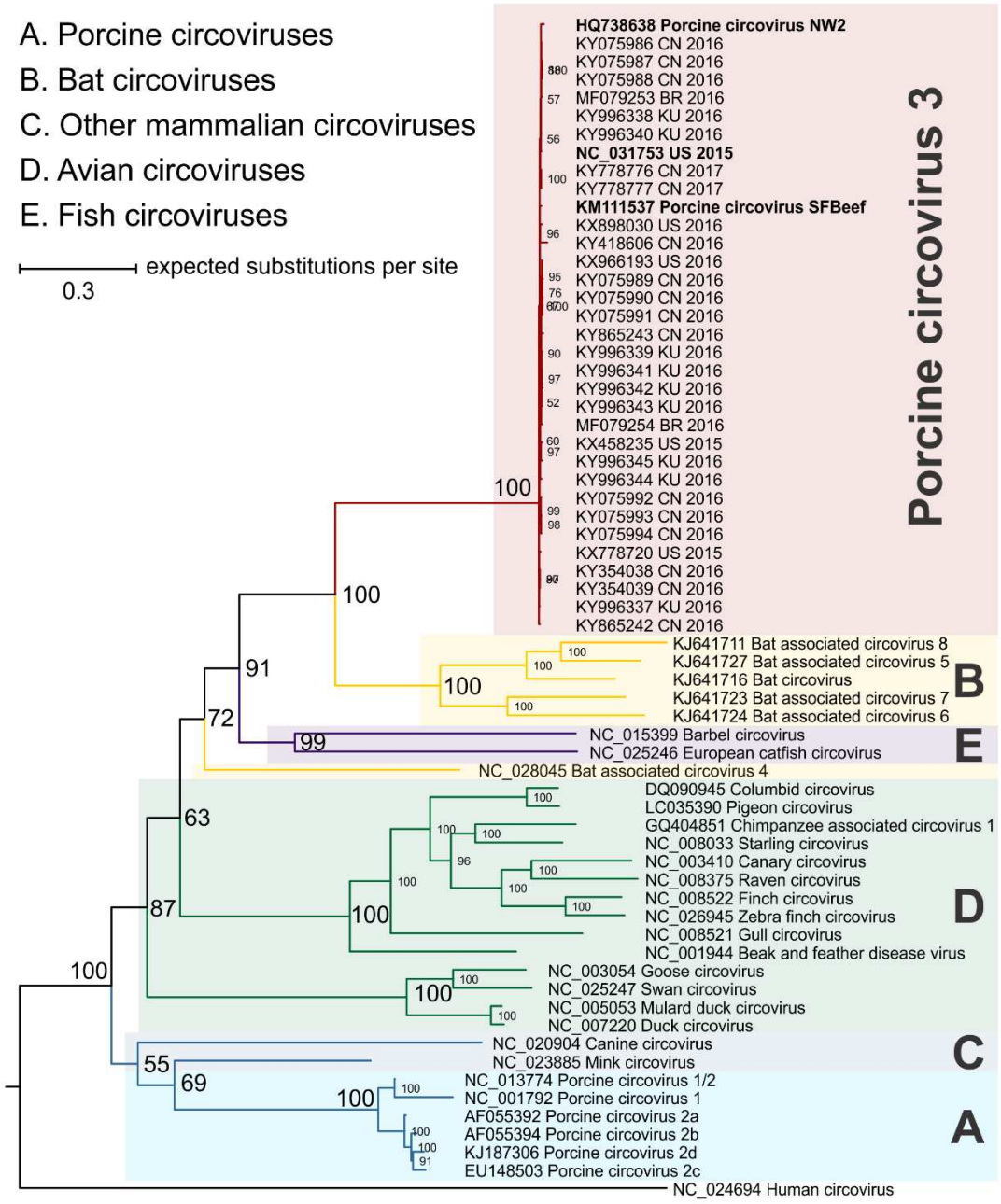

Fig. 2 Evolutionary relationships between circoviruses. The midpoint rooted majority-rule consensus tree was obtained by Bayesian Inference (BI) analysis of complete genomes of 65 circoviruses. The posterior probability values (PP) (expressed as percentages) calculated using the best trees found by MrBayes are shown beside each node.

Comparative genomics showed that the conservation between PCV3 and other circovirus genomes, including PCV2 and Bat associated circovirus, is restricted to the ORF1 that encodes the replication associated protein. No significant alignment was found in the ORF2 that encodes the Cap protein (**Figure 3**).

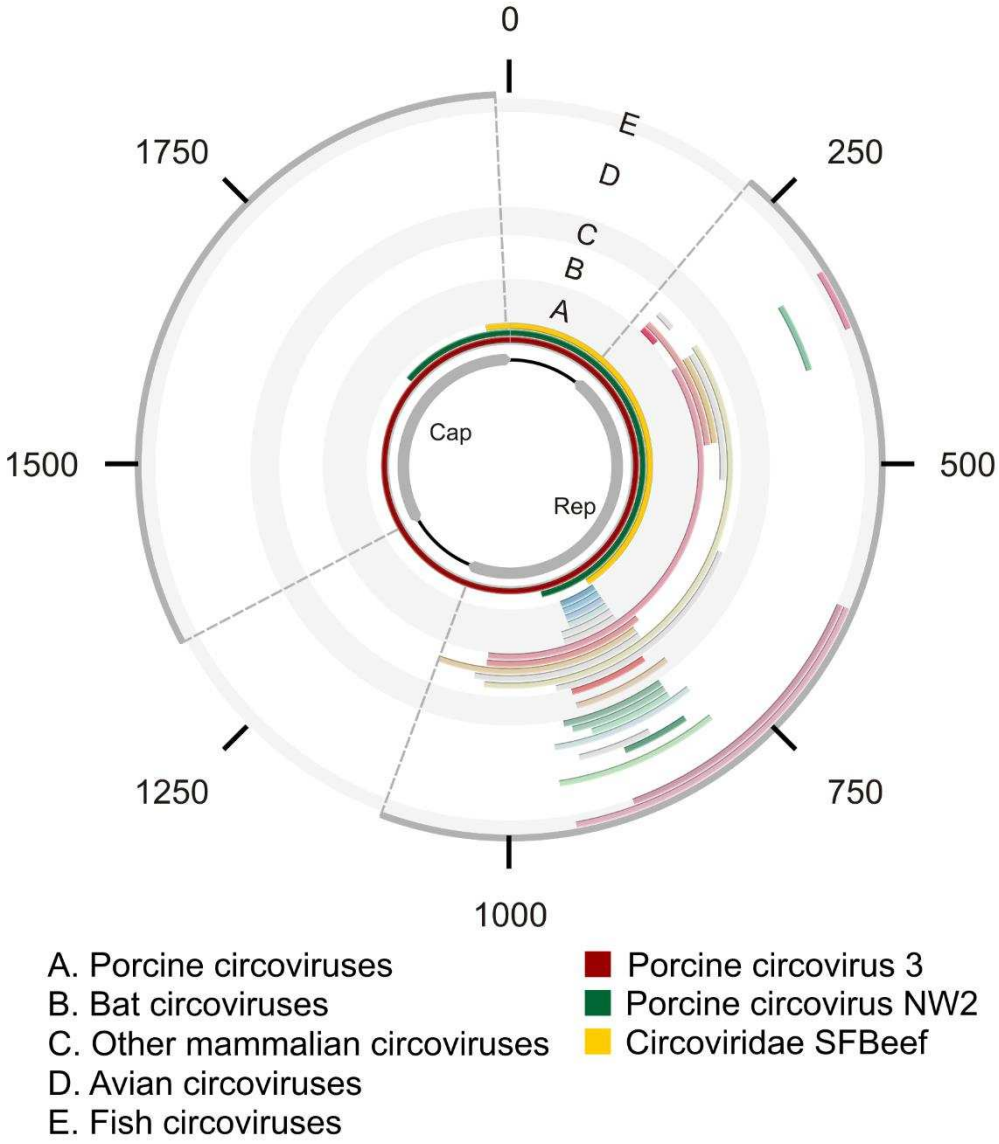

Fig. 3 Comparative genomics between PCV3 and other circovirus genomes. The analysis was performed with the 65 circovirus genomes using the BLAST Ring Image Generator (BRIG). BRIG displayed an image showing similarity between a PCV3 reference sequence (GenBank accession number KT869077) and other sequences of the dataset as concentric rings.

Rep is a less polymorphic protein comparing to the Cap protein and thereat it would be expected that ORF1 shows a higher conservation among circoviruses. Although the ORF1 of PCV3 presented conserved segments with

different circoviruses, they are genetically distant and accumulate many mutations between them, indicating that the divergence of these viruses is not a recent event. Akaike Information Criterion for MCMC samples (AICM) suggested that the strict molecular clock was the best-fit model for PCV3 genomes (**Online Resource 3**), and the estimated mean substitution rate was 1.22E-4 [7.74E-5, 1.72E-4] substitution/site/year. This estimative aggress with what would be expected of ssDNA viruses, which are evolving at ~ 1E-4 sub/site/year [45].

The time to the most recent common ancestor (tMRCA) analysis predicted an origin for most of current PCV3 strains between 1946 and 1987 [95% High Posterior Density (HPD) interval] (**Online Resource 3**). The mean MRCA time calculated was 1967, suggesting that the PCV3 lineages diverged around 50 years ago (**Figure 4** and **Online Resource 3**).

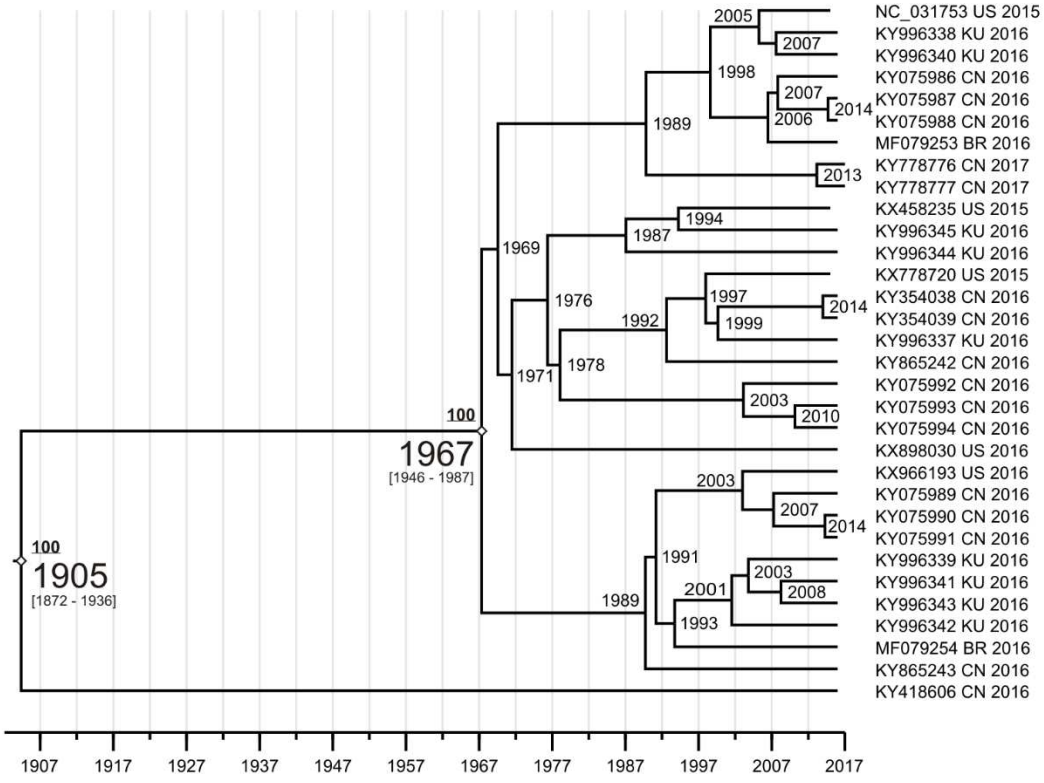

Fig. 4 The time to the most recent common ancestor (tMRCA) analysis of PCV3 genomes. The majority-rule consensus trees of PCV3 genomes were obtained by a coalescent Bayesian skyline analysis with a strict molecular clock model assumption using BEAST. The mean MRCA time calculated by BEAST are shown beside each node. The numbers between brackets beside the main nodes correspond to the 95% High Posterior Density (HPD) interval calculated by BEAST. The bold and underlined numbers correspond to the posterior probability values (expressed as percentages) calculated using the best trees found by BEAST.

The GD2016 (KY418606) strain stood out for being genetically different and tMRCA analysis indicates that it diverged from current PCV3 strains around 1905 (between 1872 and 1936). Although the MRCA time was calculated with a large HPD interval and the time of origin of PCV3 could be questioned, phylogenetic analysis and comparative genomics indicated that the PCV3 origin is not a recent evolutionary event as it was predicted by the tMRCA analysis. In order to increase the accuracy of the date of PCV3 origin, it will be necessary to conduct retrospective studies aiming the sequencing of ancient viral isolates of PCV3.

Haplotype networks have allowed a more feasible discussion of epizootiology of Porcine circovirus [9], adding information to the phylogenetic analysis. In haplotype networks, the central haplotypes are the possible ancestors and it is possible reconstruct reticulations that are not suitable to a phylogenetic tree representation [9,46]. These reticulations reflect evolutionary constraints such as homoplasy, parallel or convergent evolution [46]. The haplotype networks of PCV3 presented some reticulations that interfere on prediction of its genealogy (**Figure 5**). In addition, the higher number of sequences from China together with few countries with available sequences of PCV3 isolates could prompt us to misinterpret the origins of PCV3 in the pork industry worldwide.

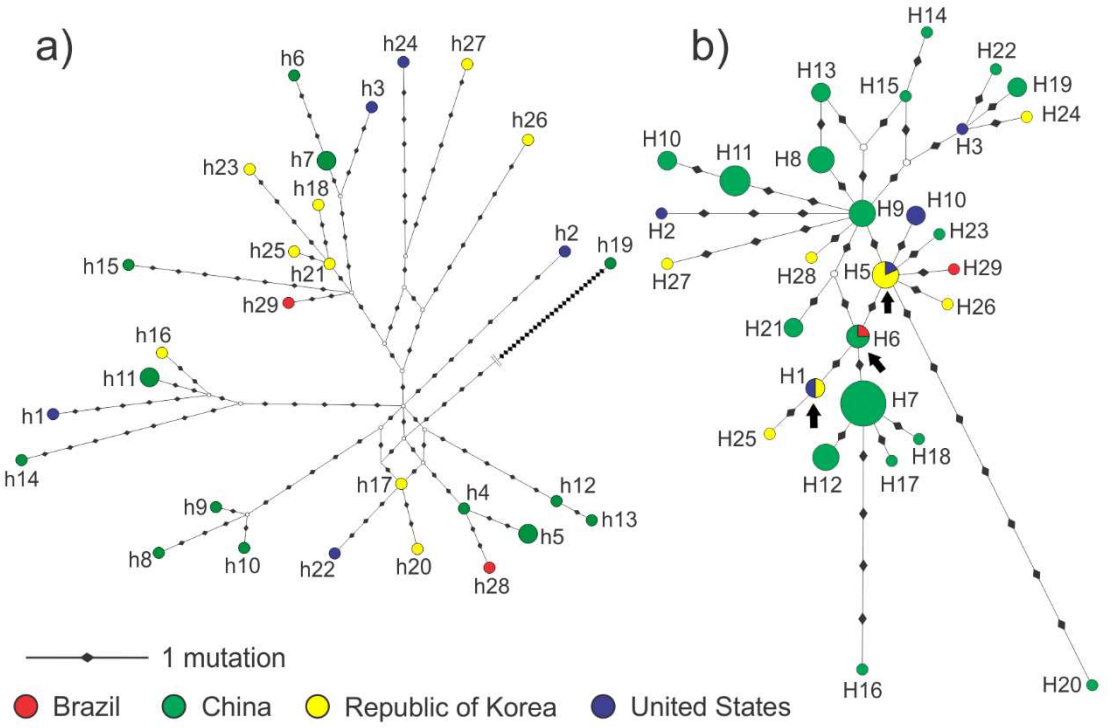

Fig. 5 Haplotype networks of PCV3. (a) Haplotype network of the 32 complete genomes of PCV3. (b) Haplotype network of the 65 sequences of ORF2. The haplotypes were colored according to the country of origin. The size of the circumferences is proportional to the haplotype frequencies. The black arrows indicate the haplotypes associated to the possible routes of dispersion of PCV3.

Most of the haplotypes were separated by one or few mutations, except the strain GD2016 which was separated by 29 mutations in genome network (h19) and 7 mutations in ORF2 network (H20) (**Figure 5** and **Online Resource 1**). In addition, the haplotype network of ORF2 showed three haplotypes clustering isolates of PCV3 from United States and Republic of Korea (H1 and H5); also from China and Brazil (H6) (**Figure 5**). These haplotypes correspond to groups of viral isolates that share identical ORF2 sequences. The haplotype H5 also stood out as a possible ancestor of haplotypes H10 (United States), H23 (China), H29 (Brazil), and H26 (Republic of Korea). In the haplotype network of genomes, haplotype h4 (China) is separated by two mutations of h28 (Brazil), and h17 (Republic of Korea) is separated by three mutations of h22 (United States). All these haplotypes correspond to key viral isolates that are available to understand the dispersal routes of PCV3 and an indicative that this virus may be highly spreading globally.

Analyzing the recent economic statistics of live pig trading available at UN Comtrade database, it is possible to identify the trade flows among Brazil, China, United States and Republic of Korea that could be related with the dispersal routes of PCV3. In the last ten years (2006 to 2016), the analysis of reported trades of live pigs shows mainly export flows from the USA to these countries. However, all these countries have reported exports to the "world" without specifying a commercial partner. Brazil reported exports of 35,135 of live pigs to the world, China exported 13.81 million, Republic of Korea exported 238 and USA exported 684.11 thousand. Considering this intense movement of live pigs among producing countries, it would be necessary establish effective sanitary barriers to control the dispersion of PCV3.

Our data suggests that the most recent common ancestor of the current PCV3 strains might have emerged over the past 50 years. PCV3 has a different origin of other porcine circoviruses and it was not possible to detect signal of recombination between PCV3 and current known circoviruses. The results also suggest that PorkNW2 and SFBeef are actually the defectives PCV3 or replicative intermediates and it has probably been evolving for some time in swine and possibly in bovine population. PCV3 may be highly spreading globally, considering that we found groups of genetically related isolates originated from different countries that have important roles in exports of live pigs.

Statement of author contributions

All authors contributed to this work and agreed to its publication.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical statement: This article does not contain any studies with human participants or animals performed by any of the authors.

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Online Resource 1. Information of each nucleotide sequence that were downloaded from GenBank database and organized into three datasets. The sequences are identified by the GenBank accession codes and the information about strain, host, country and year of isolation are listed. The sequences of PCV3 are also identified according to their respective haplotype.

Dataset I: PCV3 GENOMES

Haplotype	GenBank accession	Strain	Country	Host	Year
h1	KX778720	PCV3-US/MO2015	United States	pig	2015
h2	KX898030	PCV3-US/MN2016	United States	pig	2016
h3	KX966193	PCV3-US/SD2016	United States	pig	2016
h4	KY075986	PCV3/CN/Fujian-5/2016	China	pig	2016
h5	KY075987	PCV3/CN/Fujian-12/2016	China	pig	2016
h5	KY075988	PCV3/CN/Henan-13/2016	China	pig	2016
h6	KY075989	PCV3/CN/Jiangxi-62/2016	China	pig	2016
h7	KY075990	PCV3/CN/Chongqing-147/2016	China	pig	2016
h7	KY075991	PCV3/CN/Chongqing-148/2016	China	pig	2016
h8	KY075992	PCV3/CN/Chongqing-150/2016	China	pig	2016
h9	KY075993	PCV3/CN/Chongqing-155/2016	China	pig	2016
h10	KY075994	PCV3/CN/Chongqing-156/2016	China	pig	2016
h11	KY354038	CN/Hubei-610/2016	China	pig	2016
h11	KY354039	CN/Hubei-618/2016	China	pig	2016
h12	KY778776	PCV3/CN/Shandong-1/201703	China	pig	2017
h13	KY778777	PCV3/CN/Shandong-2/201703	China	pig	2017
h14	KY865242	CHN_Shanghai_0706_2016	China	pig	2016
h15	KY865243	CHN_Shanghai_0708_2016	China	pig	2016
h16	KY996337	PCV3/KU-1601	Korea	pig	2016
h17	KY996338	PCV3/KU-1602	Korea	pig	2016
h18	KY996339	PCV3/KU-1603	Korea	pig	2016
h19	KY418606	PCV3-China/GD2016	China	pig	2016
h20	KY996340	PCV3/KU-1604	Korea	pig	2016
h21	KY996341	PCV3/KU-1605	Korea	pig	2016

h22	NC_31753	29160	United States	pig	2015
h23	KY996342	PCV3/KU-1606	Korea	pig	2016
h24	KX458235	2164	United States	pig	2015
h25	KY996343	PCV3/KU-1607	Korea	pig	2016
h26	KY996344	PCV3/KU-1608	Korea	pig	2016
h27	KY996345	PCV3/KU-1609	Korea	pig	2016
h28	MF079253	PCV3-BR/RS/6	Brazil	pig	2016
h29	MF079254	PCV3-BR/RS/8	Brazil	pig	2016

Dataset II: PCV3 ORF2

Haplotype	GenBank accession	Strain	Country	Host	Year
H1	KY996338	PCV3/KU-1602	Korea	pig	2016
H1	NC_031753	29160	United States	pig	2015
H2	KX458235	2164	United States	pig	2015
H3	KX778720	PCV3-US/MO2015	United States	pig	2015
H4	KX898030	PCV3-US/MN2016	United States	pig	2016
H5	KX966193	PCV3-US/SD2016	United States	pig	2016
H5	KY996339	PCV3/KU-1603	Korea	pig	2016
H5	KY996341	PCV3/KU-1605	Korea	pig	2016
H5	KY996343	PCV3/KU-1607	Korea	pig	2016
H6	KY075986	PCV3/CN/Fujian-5/2016	China	pig	2016
H6	KY075996	PCV3/CN/Fujian-5/201608	China	pig	2016
H6	MF079253	PCV3-BR/RS/6	Brazil	pig	2016
H7	KY075987	PCV3/CN/Fujian-12/2016	China	pig	2016
H7	KY075988	PCV3/CN/Henan-13/2016	China	pig	2016
H7	KY075995	PCV3/CN/Anhui-2/201608	China	pig	2016
H7	KY075998	PCV3/CN/Fujian-12/201608	China	pig	2016
H7	KY075999	PCV3/CN/Henan-13/201608	China	pig	2016
H7	KY076001	PCV3/CN/Henan-21/201609	China	pig	2016
H7	KY076012	PCV3/CN/Henan-65/201608	China	pig	2016
H7	KY076014	PCV3/CN/Jiangxi-69/201609	China	pig	2016
H7	KY076015	PCV3/CN/Jiangxi-72/201607	China	pig	2016
H7	KY076016	PCV3/CN/Jiangxi-95/201607	China	pig	2016
H7	KY076017	PCV3/CN/Henen-99/201607	China	pig	2016
H7	KY076018	PCV3/CN/Henen-101/201607	China	pig	2016
H7	KY076019	PCV3/CN/Henan-105/201607	China	pig	2016
H7	KY076021	PCV3/CN/Hubei-118/201607	China	pig	2016
H8	KY075989	PCV3/CN/Jiangxi-62/2016	China	pig	2016
H8	KY076005	PCV3/CN/Liaoning-32/201607	China	pig	2016
H8	KY076011	PCV3/CN/Jiangxi-62/201607	China	pig	2016
H8	KY076020	PCV3/CN/Henan-111/201607	China	pig	2016
H9	KY075990	PCV3/CN/Chongqing-147/2016	China	pig	2016
H9	KY075991	PCV3/CN/Chongqing-148/2016	China	pig	2016
H9	KY076022	PCV3/CN/Chongqing-147/201608	China	pig	2016
H9	KY076023	PCV3/CN/Chongqing-148/201608	China	pig	2016
H10	KY075992	PCV3/CN/Chongqing-150/2016	China	pig	2016
H10	KY076024	PCV3/CN/Chongqing-150/201608	China	pig	2016
H11	KY075993	PCV3/CN/Chongqing-155/2016	China	pig	2016
H11	KY075994	PCV3/CN/Chongqing-156/2016	China	pig	2016
H11	KY076025	PCV3/CN/Chongqing-155/201608	China	pig	2016
H11	KY076026	PCV3/CN/Chongqing-156/201608	China	pig	2016
H11	KY076027	PCV3/CN/Jiangxi-332/201605	China	pig	2016

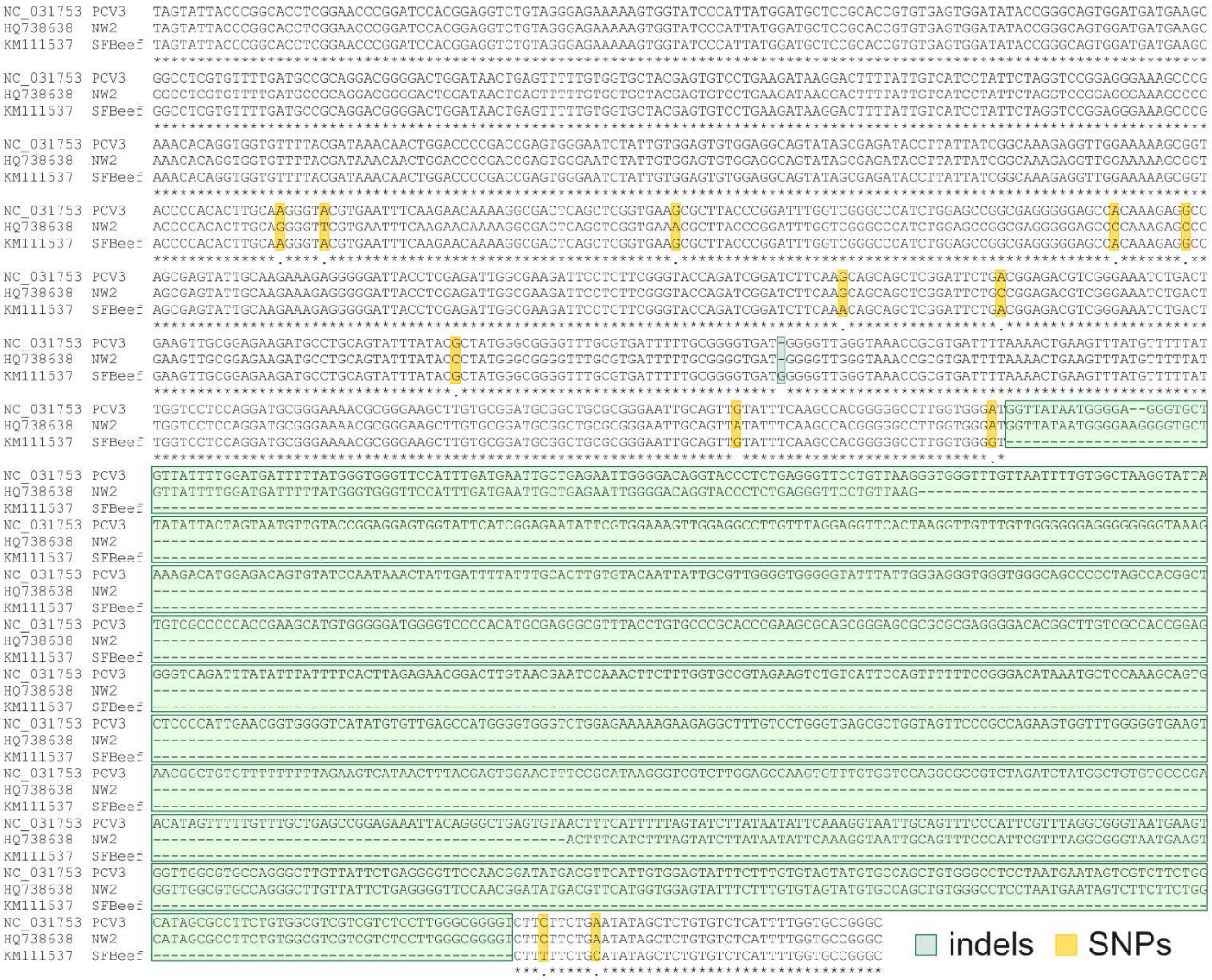
H12	KY075997	PCV3/CN/Fujian-8/201608	China	pig	2016
H12	KY076006	PCV3/CN/Liaoning-36/201607	China	pig	2016
H12	KY076007	PCV3/CN/Liaoning-40/201607	China	pig	2016
H12	KY076008	PCV3/CN/Liaoning-48/201607	China	pig	2016
H13	KY076000	PCV3/CN/Henan-19/201609	China	pig	2016
H13	KY076004	PCV3/CN/Hubei-29/201607	China	pig	2016
H14	KY076002	PCV3/CN/Jiangxi-24/201607	China	pig	2016
H15	KY076003	PCV3/CN/Jiangxi-27/201607	China	pig	2016
H16	KY076009	PCV3/CN/Liaoning-56/201607	China	pig	2016
H17	KY076010	PCV3/CN/Jiangxi-60/201607	China	pig	2016
H18	KY076013	PCV3/CN/Jiangxi-67/201609	China	pig	2016
H19	KY354038	CN/Hubei-610/2016	China	pig	2016
H19	KY354039	CN/Hubei-618/2016	China	pig	2016
H20	KY418606	PCV3-China/GD2016	China	pig	2016
H21	KY778776	PCV3/CN/Shandong-1/201703	China	pig	2017
H21	KY778777	PCV3/CN/Shandong-2/201703	China	pig	2017
H22	KY865242	CHN_Shanghai_0706_2016	China	pig	2016
H23	KY865243	CHN_Shanghai_0708_2016	China	pig	2016
H24	KY996337	PCV3/KU-1601	Korea	pig	2016
H25	KY996340	PCV3/KU-1604	Korea	pig	2016
H26	KY996342	PCV3/KU-1606	Korea	pig	2016
H27	KY996344	PCV3/KU-1608	Korea	pig	2016
H28	KY996345	PCV3/KU-1609	Korea	pig	2016
H29	MF079254	PCV3-BR/RS/8	Brazil	pig	2016

Dataset III: CIRCOVIRUSES GENOMES

GenBank accession	Species/virus name | strain	Host	Year
NC_015399	Barbel circovirus	Barbus barbus (barbel)	2008
NC_028045	Bat associated circovirus 4	Tadarida brasiliensis (bat)	2013
KJ641727	Bat associated circovirus 5 | BtPa-CV-1/NX2013	Plecotus auritus (bat)	2013
KJ641724	Bat associated circovirus 6 | BtRa-CV/JS2013	Rhinolophus affinis (bat)	2013
KJ641723	Bat associated circovirus 7 | BtRs-CV/HuB2013	Rhinolophus sinicus (bat)	2013
KJ641711	Bat associated circovirus 8 | BtMr-CV/GD2012	Myotis ricketti (bat)	2012
KJ641716	Bat circovirus | BtPspp-CV/GD2012	Pipistrellus sp. (bat)	2012
NC_001944	Beak and feather disease virus	-	1998
NC_003410	Canary circovirus	Serinus canaria (canary)	2000
NC_020904	Canine circovirus | UCD1-1698	Canis lupus familiaris (dog)	2011
GQ404851	Chimpanzee associated circovirus 1 | Chimp17	Chimpanzee stool	2002
KM111537	Circoviridae SFBeef	bovine meat	2014
HQ738638	Circoviridae SFPorkNW2	pig meat	2009
DQ090945	Columbid circovirus | zj1	Pigeon	2005
NC_007220	Duck circovirus | 33753-52	Pekin duck	2005
NC_025246	European catfish circovirus | H5	Silurus glanis (wels catfish)	2011
NC_008522	Finch circovirus	Finch	2006
NC_003054	Goose circovirus	Anser sp. (bird)	1997
NC_008521	Gull circovirus	Gull	2006
NC_024694	Human circovirus | VS6600022	Homo sapiens	2009
NC_023885	Mink circovirus | MiCV-DL13	mink	2013
NC_005053	Mulard duck circovirus	Mulard duck	2002
LC035390	Pigeon circovirus | PiCV/Japan/2/2010	Columba livia	2010
NC_001792	Porcine circovirus 1 | PCVgp1	Sus scrofa (pig)	1998
NC_013774	Porcine circovirus 1/2a | FMV08-1114252	Sus scrofa (pig)	2008
AF055392	Porcine circovirus 2 (PCV2a)	Sus scrofa (pig)	1998
AF055394	Porcine circovirus 2 (PCV2b)	Sus scrofa (pig)	1998
EU148503	Porcine circovirus 2 (PCV2c)	Sus scrofa (pig)	1980
KJ187306	Porcine circovirus 2 (PCV2d) | UFV1	Sus scrofa (pig)	2013
NC_31753	Porcine circovirus 3 (PCV3)	Sus scrofa (pig)	2015
NC_008375	Raven circovirus | 4-1131	Corvus coronoides (raven)	2005
KX458235	PCV3 | 2164	Sus scrofa (pig)	2015

KY865242	PCV3 | CHN_Shanghai_0706_2016	Sus scrofa (pig)	2016
KY865243	PCV3 | CHN_Shanghai_0708_2016	Sus scrofa (pig)	2016
KY075990	PCV3 | CN/Chongqing-147/2016	Sus scrofa (pig)	2016
KY075991	PCV3 | CN/Chongqing-148/2016	Sus scrofa (pig)	2016
KY075992	PCV3 | CN/Chongqing-150/2016	Sus scrofa (pig)	2016
KY075993	PCV3 | CN/Chongqing-155/2016	Sus scrofa (pig)	2016
KY075994	PCV3 | CN/Chongqing-156/2016	Sus scrofa (pig)	2016
KY075987	PCV3 | CN/Fujian-12/2016	Sus scrofa (pig)	2016
KY075986	PCV3 | CN/Fujian-5/2016	Sus scrofa (pig)	2016
KY075988	PCV3 | CN/Henan-13/2016	Sus scrofa (pig)	2016
KY354038	PCV3 | CN/Hubei-610/2016	Sus scrofa (pig)	2016
KY354039	PCV3 | CN/Hubei-618/2016	Sus scrofa (pig)	2016
KY075989	PCV3 | CN/Jiangxi-62/2016	Sus scrofa (pig)	2016
KY778776	PCV3 | CN/Shandong-1/201703	Sus scrofa (pig)	2017
KY778777	PCV3 | CN/Shandong-2/201703	Sus scrofa (pig)	2017
KY996337	PCV3 | KU-1601	Sus scrofa (pig)	2016
KY996338	PCV3 | KU-1602	Sus scrofa (pig)	2016
MF079253	PCV3| BR/RS/6	Sus scrofa (pig)	2016
MF079254	PCV3| BR/RS/8	Sus scrofa (pig)	2016
KY418606	PCV3| China/GD2016	Sus scrofa (pig)	2016
KY996339	PCV3| KU-1603	Sus scrofa (pig)	2016
KY996340	PCV3| KU-1604	Sus scrofa (pig)	2016
KY996341	PCV3| KU-1605	Sus scrofa (pig)	2016
KY996342	PCV3| KU-1606	Sus scrofa (pig)	2016
KY996343	PCV3| KU-1607	Sus scrofa (pig)	2016
KY996344	PCV3| KU-1608	Sus scrofa (pig)	2016
KY996345	PCV3| KU-1609	Sus scrofa (pig)	2016
KX898030	PCV3| US/MN2016	Sus scrofa (pig)	2016
KX778720	PCV3| US/MO2015	Sus scrofa (pig)	2015
KX966193	PCV3| US/SD2016	Sus scrofa (pig)	2016
NC_008033	Starling_circovirus	European Starling	2005
NC_025247	Swan circovirus | H51	Cygnus olor (mute swan)	2006
NC_026945	Zebra finch circovirus 8454V25-1	Taeniopygia guttate (zebra finch)	2010

Online Resource 2. Genome sequences alignment of PorkNW2, SFBeef and PCV3 strains.



Online Resource 3. Most common recent ancestor (MRCA) analysis of PCV3 lineages.

Figure S1. Priors selected for estimate the substitution rates. Initial parameter corresponds to the starting point of simulation and its respective bounds. M and S parameters correspond to the mean and standard deviation of Log Normal distribution, respectively.

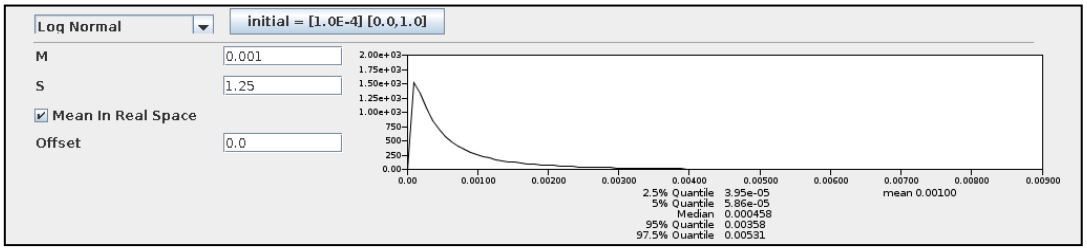


Table S1. Statistical parameters estimated by BEAST to test the best-fit molecular clock model.

Molecular clock	**Marginal Likelihood** [a]	**AICM** [b]	**Difference** [b]
<u>Strict</u>	-4075.0077 (-4083.5234, 4066.8498)	8187.471 +/- 0.045	<u>0.00</u>
Lognormal	-4074.7250 (-4083.6283, 4066.6405)	8189.248 +/- 0.088	-1.777
Random	-4074.7529 (-4083.5676, 4066.2870)	8189.497 +/- 0.095	-2.026
Exponential	-4071.1607 (-4081.9145, 4061.4925)	8197.766 +/- 0.1100	-10.295

a. The numbers in parentheses correspond to the 95% lower and upper bounds of the highest-probability density intervals. **b.** The highest -$\log_{10}$ Akaike Information Criterion for MCMC samples (AICM) corresponds to the best-fit clock model (<u>underlined</u>). The $\log_{10}$ AICM difference (± standard error) is compared with the clock model with strongest support.

Figure S2. Priors selected for estimate the time to the most common recent ancestor (tMRCA). Mu parameter of Laplace Distribution was calculated based on TreeHeight estimated for the best-fit molecular clock model.

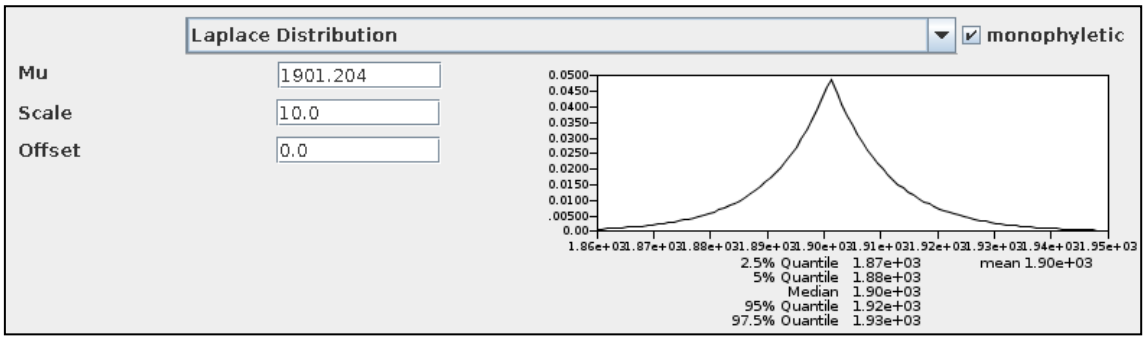
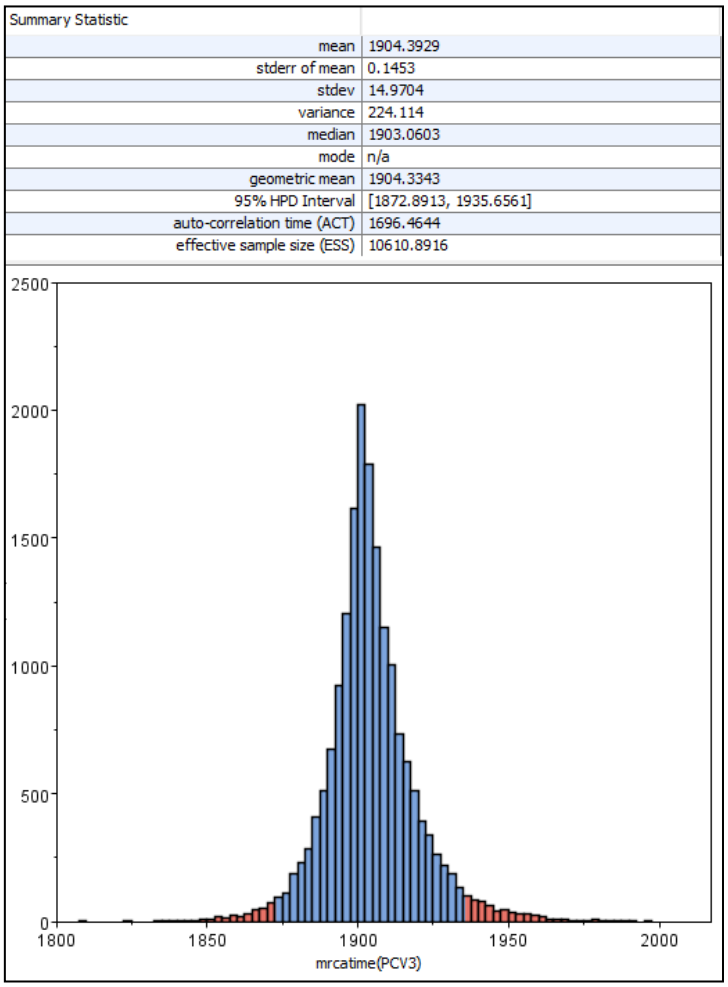


Figure S3. Summary statistic of the time to the most common recent ancestor (tMRCA) of all PCV3 lineages calculated by BEAST under a strict molecular clock assumption.



5. ARTICLE II

RETROSPECTIVE DETECTION AND GENETIC CHARACTERIZATION OF PORCINE CIRCOVIRUS 3 (PCV3) STRAINS IDENTIFIED BETWEEN 2006 AND 2007 IN BRAZIL

Manuscript in preparation

Retrospective detection and genetic characterization of Porcine circovirus 3 (PCV3) strains identified between 2006 and 2007 in Brazil.

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Summary

Porcine circovirus 3 (PCV3) is an emerging virus that was first identified in the United States in 2016. Since its first detection, PCV3 has already been found in America, Asia and Europe. Although PCV3 has already been described in Brazil, knowledge of its detection and molecular characteristics before 2016 is limited as well as its distribution in the main swine producing regions of Brazil. In this study, 67 porcine clinical samples collected from nine states of Brazil between 2006 and 2007 were analyzed for PCV3 infection by PCR. Results showed that 47.8% of the samples were PCV3 positive, including all nine states. Of the PCV3-positive samples, 37.5% were also positive for PCV2. Interestingly, no clinical signs were noticed in singular PCV3 infection. Moreover, the positive PCV3 rate in healthy pigs was higher (29.8%) than that found in sick pigs (17.9%), suggesting that most pigs could live with PCV3 infection without any clinical sign in the analyzed cases. Nucleotide sequence analysis showed that PCV3 strains obtained in this study shared 94.44% to 99.83% sequence identity at the ORF2 gene level with available strains from different countries. Moreover, PCV3 sequences collected in 2006 and 2007 shared 97.94% to 99.62% identity with the Brazilian strains obtained in 2016, indicating a genetic stability of PCV3 Brazilian strains over the past 10 years. PCV3 has not shown a differentiated independent molecular evolution in Brazil and multiple events of introduction may have occurred. Phylogenetic analysis showed at least two clusters that can be potentially defined, both including strains collected in America, Asia, Europe and Brazil. Two Brazilian strains were clustered on the phylogenetic tree with the strains obtained from wild boars, suggesting that it may play an important epidemiological role as a reservoir of PCV3 for commercial swine in Brazil.

Keywords: Brazil; detection; PCV3; phylogenetic

Introduction

Porcine circoviruses (PCVs) are non-enveloped and single-stranded circular DNA virus. Until recently, PCVs could be separated into two main species: Porcine circovirus 1 (PCV1) and Porcine circovirus 2 (PCV2) (Mankertz et al., 2004). PCV1 was first discovered in 1974 and was not associated with clinical disease (Tischer, Mields, Wolff, Vagt, & Griem, 1986; Tischer, Rasch, & Tochtermann, 1974). PCV2 was first discovered in 1990s and is associated with a range of syndromes in pigs called Porcine Circovirus Associated Diseases (PCVAD), which can cause significant economic losses (Grau-Roma, Fraile, & Segales, 2011; Madec, Rose, Grasland, Cariolet, & Jestin, 2008; Opriessnig, Meng, & Halbur, 2007; Ramamoorthy & Meng, 2009). In 2016, a novel circovirus named Porcine circovirus 3 (PCV3) was identified in the United States in sows that died acutely with clinical signs of the porcine dermatitis and nephropathy syndrome (PDNS) and were PCV2 negative (Palinski et al., 2017).

Since then, PCV3 was also reported in the Americas, Asia and Europe and its detection was associated with different clinical presentations in pigs. Reproductive problems were correlated with PCV3 (Fu et al., 2018; Ku et al., 2017; Liu et al., 2018; Palinski et al., 2017; Phan et al., 2017; Tochetto et al., 2017) as well as cardiac and multisystemic inflammation (Phan et al., 2017), respiratory diseases (Kedkovid, Woonwong, Arunorat, & Sirisereewan, 2018; Shen et al., 2017; Zhai et al., 2017), congenital tremors in neonatal pigs (Chen et al., 2017) and diarrhea in weaned piglets (Zhai et al., 2017). However, high prevalence of PCV3 in pigs and in wild boars without specific clinical signs has been also reported (Stadejek, Wozniak, Milek, & Biernacka, 2017; Ye, Berg, Fossum, Wallgren, & Lie, 2018; Zheng et al., 2017; Franzo et al., 2018).

Retrospective studies of prevalence were performed to understand the epidemiology and molecular evolution of PCV3. In China, clinical samples of pigs collected between 1990 and 1999 were analyzed and 6.5% were positive for PCV3, with the first cases occurring in 1996 (Sun et al., 2018). Results from a retrospective study in the United Kingdom showed that PCV3 was detected in 5% of tissue samples collected between 2001 and 2004 (Collins, McKillen, & Gordon, 2017). In Sweden, 20.4% of the samples collected between 1993 and

2007 were positive, and one of these samples was collected in 1993 (Ye et al., 2018). Results of an epidemiological study in Spain confirmed that PCV3 has been circulating in the Spanish pig population since 1996 (Klaumann et al., 2018). These results are in agreement with the estimates that PCV3 have emerged over the past 50 years in the pork industry (Saraiva et al., 2018; Fu et al., 2018)

Two Brazilian genomes of PCV3 were deposited in GenBank, confirming that PCV3 is also circulating in Brazil. The genomes were obtained from pooled serum samples collected in 2016 from sows who had just delivered litters with variable numbers of stillbirths. No PCV3 sequence was detected in samples from sows which had no stillbirths on the same farms (Tochetto et al., 2017).

Although PCV3 has already been described in Brazil, knowledge of its prevalence and molecular characteristics before 2017 is limited as well as its distribution in the country. The aim of this study was to investigate the epidemiology and molecular evolution of PCV3 from Brazilian pig samples collected before its first detection to answer the following questions: i) Had PCV3 already circulating in Brazil before 2016? ii) Can PCV3 be found in the main pork producing regions of Brazil? iii) How much conserved are the sequences of Brazilian strains? iv) How significant are the events of introduction of PCV3 in Brazil?

Materials and Methods

PCV3 detection

A total of 67 tissue samples were collected from pigs with clinical signs of Porcine Circovirus Associated Diseases (PCVAD) and also in good health conditions between 2006 and 2007 from nine states located in the main swine producing regions of Brazil (Midwest, Southeast and South) (**Figure 1**). The samples had been used for epidemiological analysis of Porcine circovirus 2 (PCV2) in 2008. The DNA of these samples was extracted and has been stored at -80℃ since the PCV2 investigation.

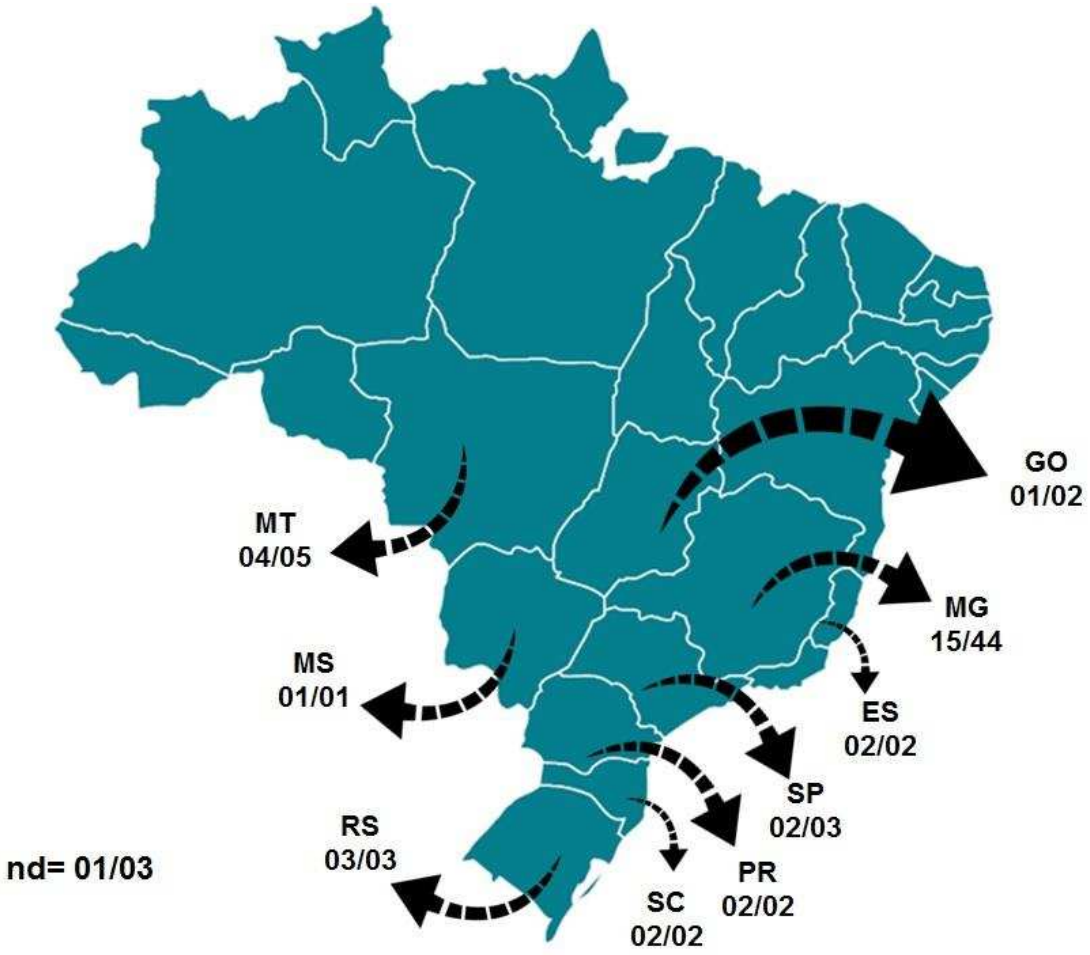

Figure 1- Distribution of the 67 tissue samples collected from pigs between 2006 and 2007 from nine states of Brazil. The number before the bar indicates the total of positive samples and the number after the bar indicates the sampling in each state. Nd = no data about sample origin.

The stored DNA samples were submitted to PCR assay using primers described previously, which amplify the region that encodes the ORF2 protein (Ku et al., 2017). ORF2 encodes the capsid protein and it has been proven to be the major target of the host immune response for PCV2 (Franzo, Cortey, Segales, Hughes, & Drigo, 2016 ;.Nawagitgul et al., 2000).

The 25 µL PCR reaction mixtures contained 12.5 µL of DreamTaq PCR Green PCR Master Mix (2x) (Thermo Fisher Scientific, Massachusetts, EUA), 2 µL of each 10 mM primer, 4 µL of DNA template and 4.5 µL of H_2O nuclease free. PCR assays were performed with the following thermal profile: 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 30 s, annealing at 57℃ f or 45 s, an extension at 72℃ for 1 min and a final step of 72℃ for 10 min.

The reactions generated 649 bp PCR products that were submitted to electrophoresis on 1% agarose gel. Positive samples were purified by a GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK) and were submitted to sequencing using the Sanger method by Myleus Biotechnology (Belo Horizonte, Brazil).

Sequence assembly and analysis

Chromatograms were analyzed using CLC Genomics Workbench version 7.5 software (Qiagen). During analysis, sequences were trimmed (quality score limit: 0.01; ambiguous nucleotide residues: 0), and consensus sequences ranging from 491 to 581 nucleotides were obtained using the Assemble Sequences tool.

A dataset of 101 PCV3 ORF2 sequences from different countries collected in the period from 1996 to 2017 was downloaded from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). The ORF2 nucleotide sequences were aligned, with a final alignment of 642 nucleotides, and translated at amino acid level to more 7 sequences obtained in the present study using the MUSCLE codon alignment tool of MEGA 7 version 7.0.21 (Kumar, Stecher, & Tamura, 2016). The amino acid sequences were then back translated to nucleotide using the same programme. MEGA 7 version 7.0.21 was also used for identity and polymorphism analyzes of the sequences.

Phylogenetic tree based on the 108 ORF2 nucleotide sequences was reconstructed using the Bayesian Markov Chain Monte Carlo (MCMC) method in four runs with 10,000,000 generations and a sampling frequency of 1,000 implemented in MrBayes version 3.1.2 (Kumar, Stecher, & Tamura, 2016). The model of nucleotide substitution HKY+G was chosen using the program jModelTest (Darriba, Taboada, Doallo, & Posada, 2012). The parameter convergence was analyzed in Tracer version 1.6 (http://tree.bio.ed.ac.uk/software/tracer) and 1% of the generated trees was burnt to produce the consensus tree.

Results

PCV3 epidemiology back in 2006 and 2007

Sixty seven clinical samples collected between 2006 and 2007 from nine states of Brazil were screened for PCV3 by PCR and 47.8% of the samples (32/67) were found positive. All nine states from the main swine producing regions of Brazil were found positive for PCV3 (Midwest region: Mato grosso, Mato Grosso do Sul and Goias states; Southeast region: Minas Gerais, São Paulo and Espírito Santo states; South region: Santa Catarina, Rio Grande do Sul and Paraná states). The total positive sample rate of PCV2 was 40.3% (27/67). Of the 32 PCV3-positive samples, 37.5% (12/32) were also positive for PCV2. The singular infection rate of PCV3 in the samples was 29.8% (20/67) (**Table 1 – Supplementary material**)

Interestingly, clinical signs were only noticed in PCV2-positive samples or in co-infected samples. Moreover, the positive PCV3 rate in healthy pigs was 29.8% (20/67) and in sick pigs was 17.9% (12/67) (**Table 1-Supplementary material**).

Sequence comparison

Alignment analyses of the amino acid sequence of the ORF2 revealed that Brazilian strains from 2006 and 2007 had up to seven amino acid (aa) substitutions compared with the recently Brazilian strains collected in 2016 (GenBank accession numbers: MF079253 and MF079254). Polymorphisms were found in the following aa positions: 24 (V24A), 27(K27R), 75 (A75S), 77 (S77G), 100 (T100S) and 104 (F104Y). At position 150, two strains (916|BR|2007 and 295|BR|2007|co-infection) have the amino acid Leucine (L) similar to strain MG079253 and the remaining strains have the amino acid Isoleucine (I), similar to strain MG079254 (**Table 2**).

At position 75, only the 916 Brazilian strain and other strain from Spain (PCV3|Spain|2014, GenBank accession number: MG807088) mutated the aa residue Serine (S) in place of Alanine (A), compared with other PCV3 strains downloaded from GenBank. At position 77 and 100, only the Brazilian

strains 1343 and 916 (1343|BR|2006|co-infection and 916|BR|2007) showed Glycine (G) instead of Serine (S) or Threonine (T) and Serine (S) instead of Treonine (T), respectively, compared to the whole dataset of ORF2 sequences.

At position 104, substitutions of the amino acid Phenylalanine (F) for Tyrosine (Y) were found in two Brazilian strains (1343|BR|2006|coinfection and 872|BR|2007) and in only four strains from China and Denmark. Interestingly, this same substitution (F104Y) is commonly found in strains that were collected in Italy from wild boar.

Table 2 - Polymorphism analyses of the amino acid (aa) sequence of the ORF2 of Brazilian strains from 2006 and 2007 and the recently Brazilian strains collected in 2016 (MF079253 and MF079254). Polymorphisms were found in the aa residues 24, 27, 75, 77, 100 and 104

	24	**27**	**75**	**77**	**100**	**104**	**150**
MF079253/2016	A	R	A	S	T	F	L
MF079254/2016	V	K	A	S	T	F	I
*1343/2006	A	R	A	G	T	Y	I
916/2007	A	R	S	S	S	F	L
*295/2017	-	-	A	S	T	F	L
872/2017	-	-	A	S	T	Y	I
1373/2007	-	-	A	S	T	F	I
*986/2007	-	-	A	S	T	F	I
622/2007	-	-	A	S	T	F	I

* Co-infection with PCV2

Nucleotide sequence analysis showed that PCV3 strains obtained in this study shared 94.44% to 99.83% sequence identity at the ORF2 gene level with available strains from different countries collected between 1996 and 2017. The Brazilian strain 1343 showed the highest identity (99.83%) with a strain of Denmark collected in 2017 (GenBank accession number: MF805724). Other strains from China, Italy, Korea and the United States also showed high identity (99.81%) with the strains obtained in this study. Moreover, our sequences collected in 2006 and 2007 shared high identity (97.94% to 99.62%) with the Brazilian strains obtained in 2016.

Phylogenetic analysis

A phylogenetic tree based on the nucleotide sequence of the ORF2 including seven Brazilian strains obtained in this study and 101 reference sequences of PCV3 is shown in **Figure 2**.

Phylogenetic analysis of Brazilian and worldwide sequences revealed at least two clusters that can be potentially defined, both including strains collected in America, Asia and Europe. A third cluster could be defined in the phylogenetic tree (in blue) with only 4 sequences from Spain, China and Germany collected between 2014 and 2017. The phylogenetic tree also showed that all PCV3 strains were distributed without any geographical linkage (**Figure 2**).

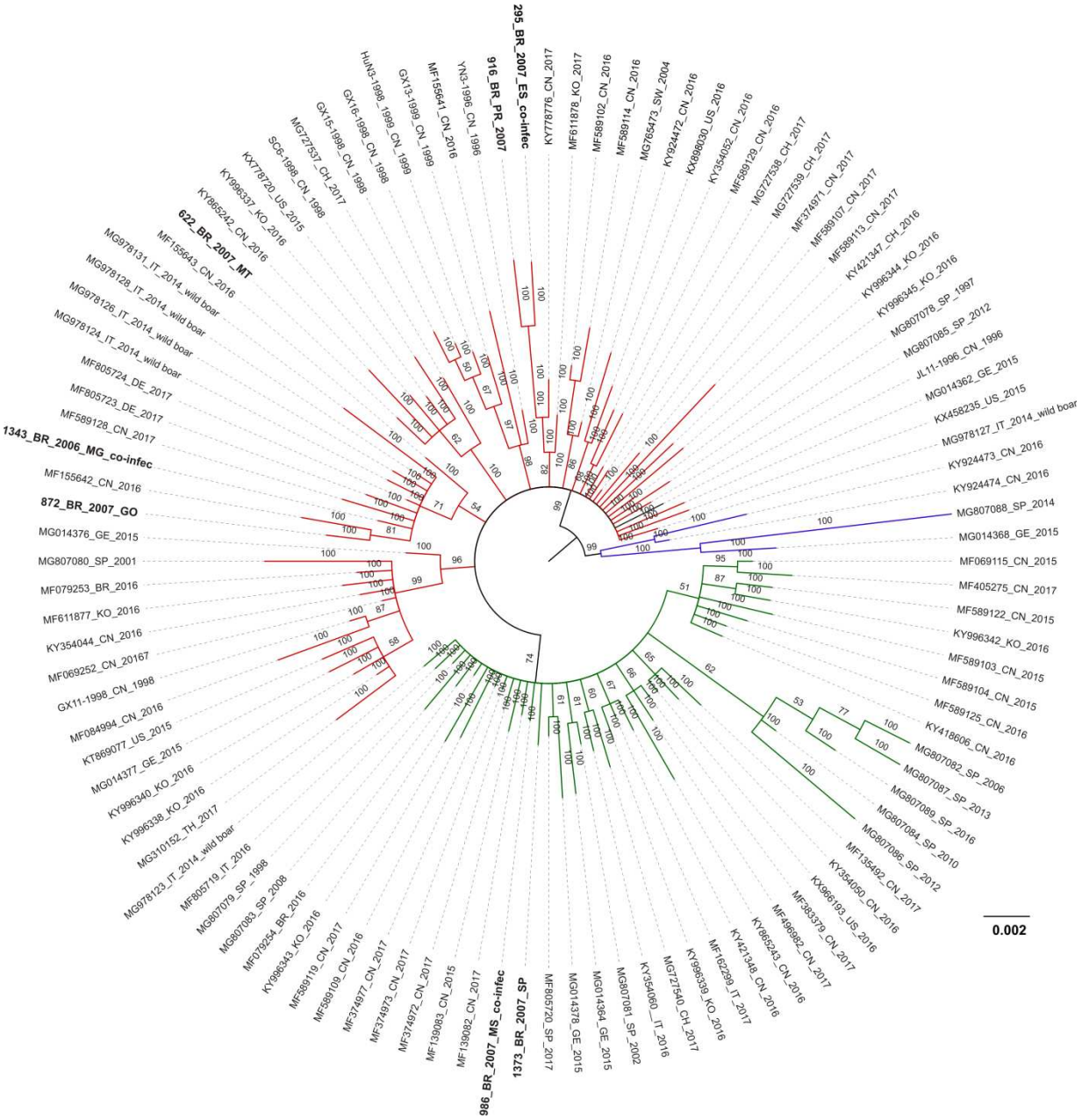

Figure 2 - Phylogenetic tree based on ORF2 of 108 PCV3 strains. The midpoint rooted majority-rule consensus tree was obtained by Bayesian Inference (BI) analysis. The scale bar indicates nucleotide substitutions per site. The posterior probability values (expressed as percentages) are shown beside each node. The sequences obtained in this study are highlighted in bold. PCV3a and PCV3b clusters are marked in green and red, respectively.

Brazilian PCV3 strains identified in the present study were clustered in the two major groups in the phylogenetic tree. Two Brazilian sequences (1373|BR|2007 and 986|BR|2007|coinfection) were genetically related to other strains described as being part of the PCV3a group, including

the Brazilian strain (MG079254). The other Brazilian sequences were genetically related to strains described as belonging to the PCV3b group, including the Brazilian strain (MG079253). Brazilian sequences showing amino acid substitution (F104Y) were clustered on the phylogenetic tree with the strains obtained from wild boars, also including other sequences from China and Denmark that also showed this substitution.

Discussion

The positive rates of PCV3 in the tissues samples were 47.8%. The prevalence of PCV3 in southern China was 21.9%, 27.8% and 31.1% in 2015, 2016 and 2017, respectively, and the total prevalence of positive samples was 26.7% (Fu et al., 2018). Studies in China have reported a positive rate of PCV3 in stillborn, tissue, semen and serum samples of 34.7% and 59.4% (Ku et al., 2017; Zheng et al., 2017). In the United States, a positive PCV3 rate of 12.5% for tissue samples and 55% for serum samples were reported (Palinski et al., 2017). In Poland, PCV3 was detected in 5.9% to 65% of sera obtained from PCV3-positive farms (Stadejek et al., 2017). In South Korea, the total prevalence of PCV3 in individual oral fluid samples was 44.2% (Kwon, Yoo, Park, & Lyoo, 2017). This wide variation in the prevalence of PCV3 may be due to the variable number of samples in each study, the epidemic situation of the region and the intrinsic management of the farms as a level of biosecurity and hygiene.

In this study, the co-infection rate of PCV3 with PCV2 was 37.5% and the singular infection rate of PCV3 in the samples was 29.8%. An investigation of the epidemiological characteristics and evolutionary dynamics of PCV3 in southern China found that 22.3% samples were co-infected with PCV2 (Fu et al., 2018). Another Chinese study, showed a co-infection rate of 45.4% of the PCV3 positive samples (Ku et al., 2017). In a screening of 265 clinical samples for co-infection of PCV2 and PCV3, 6.8% of the samples were positive (Zhang, Luo, Liang, Li, & Cui, 2018). Co-infection rate more similar to that found in our work was noticed in a survey in Shandong Province, China. The study

showed a prevalence of 39.3% and 59.4% for PCV3 and PCV2 co-infection and PCV3 infection alone, respectively (Zheng et al., 2017).

In our retrospective study, the positive PCV3 rate in healthy pigs was higher (29.8%) than that found in sick pigs (17.9%), suggesting that most pigs could live with PCV3 infection without any clinical infection sign in the analyzed cases. High positive PCV3 rate in pigs without specific clinical signs was also reported in Poland, China and Sweden (Stadejek et al., 2017; Ye et al., 2018; Zheng et al., 2017). In contrast, another survey noticed that the positive PCV3 rate in healthy pigs was lower (19.14%) than that found in sick pigs (37.95%) (Wen et al., 2018).

Interestingly, no clinical signs of Porcine Circovirus Associated Diseases (PCVAD) were observed in PCV3-positive and PCV2-negative samples. In an investigation of co-infection of Torque teno sus virus 1 (TTSuV1), Torque teno sus virus 2 (TTSuV2) and PCV2 with PCV3, no clinical signs of infection in pigs, that were both PCV3-positive and PCV2-negative, were noticed in either multiparous sows or live-born infants (Zheng et al., 2018).

Although these results were found, our study does not pretend to correlate the detection of PCV3 and its pathogenic potential. For this, requirements would have to be fulfilled as the explicit description of the clinical disease, combined with a specific histopathological picture and the quantification of the virus in association with the lesions (Fux et al., 2018). In our opinion, the presence of PCV3 and the absence of clinical signs indicate that PCV3-alone is unlikely to cause disease in pigs and other cofactors are required for the clinical manifestation of disease. More applied researches are essential to identify PCV3 pathogenic potential and to better understand the prevalence of PCV3 in Brazil, considering that the sampling used in the present study was not probabilistic.

PCV3 strains obtained in this study shared 94.44% to 99.83% sequence identity at the ORF2 gene level with available strains from different countries. One Brazilian strain (1343|BR|2006|co-infection) from the Southwest region of Brazil showed the highest identity (99.83%) with a strain of Denmark (MF805724). Trade of living pigs from Brazil to Denmark has not been reported, whereas a large number of Danish sows have been exported to other countries, including Brazil (trade statistics extracted from: https://comtrade.un.org/data/).

Therefore, a possible event of introduction of PCV3 in Brazil can be explained by the export of living pigs from Denmark to Brazil. Another possible event of introduction of PCV3 in Brazil may have occurred through the trade of living pigs with the United States, since the Brazilian strain 986 (986|BR|2007|co-infection) showed high identity (99.81%) with a United States strain collected in 2016 (KX966193). Although the export of living pigs from Brazil to the United States is rare, this route of dispersion cannot be ruled out (trade statistics extracted from: https://comtrade.un.org/data/).

Our PCV3 sequences collected in 2006 and 2007 shared high identity (97.94% to 99.62%) with the Brazilian strains obtained in 2016 (Tochetto et al., 2017), indicating the genetic stability of PCV3 Brazilian strains over the past 10 years. The Brazilian strains were intermingled amongst other PCV3 sequences available worldwide in the phylogenetic tree. This result would suggest that PCV3 has not shown a differentiated independent molecular evolution in Brazil and multiple events of introduction may have occurred.

Specific nucleotide and amino acid marker positions in ORF2 may serve for intraspecies classification and genotyping of PCV3 strains into two main groups, which might be considered as genotypes PCV3a and PCV3b (Sun et al., 2017; Fux et al., 2018; Fu et al., 2018). The amino acid marker positions in ORF2 (aa 24, 27, 77 and 150) resulted in a specific aa pattern for genotype PCV3a (V K S I) and for genotype PCV3b (A R S/T I/L) (Fux et al., 2018).

In the alignment analyses of the ORF2 aa sequences, we also found substitutions at the codons 24, 27, 77 and 150, that were used for genotyping of our strains into the groups PCV3a e PCV3b (**Figure 3**). In the phylogenetic tree, the strains 1373 and 986 were clustered in the PCV3a genotype and the strains 1343, 916, 295, 872 and 622 were clustered in the PCV3b genotype. This result revealed that different genotypes could be found in Brazil since its first detection in 2016.

Interestingly, two Brazilian sequences from the states of Minas Gerais and Goias were clustered on the phylogenetic tree with the strains obtained from wild boars in Italy (Franzo et al., 2018) and other four sequences from China and Denmark. All sequences from this clade showed an aa substitution in the position 104 (F104Y). Only one more aa substitution (codon 156) distinguishes the strain “462_Italy_wildboar_2014” (MG978131) from the

other strains of PCV3 ORF2, considering an alignment of 156 aa of the total 214 aa of the capsid protein.

The study that investigated the susceptibility of PCV3 in wild boars suggested a relevant variability and the absence of closely related strains originating from domestic pigs (Franzo et al., 2018). In contrast, we suggest that wild boar strains are closely correlated with the domestic pig strains. The disagreement of the results may have occurred because our analyzes were based on the amino acid sequences rather than the nucleotide sequences. Therefore, we suggest that the wild boars may play an important epidemiological role as a reservoir of PCV3 for commercial swine; however this hypothesis will require further investigations.

In this study, we analyzed porcine samples from the main producing regions of Brazil collected between 2006 and 2007. PCV3 could be identified in all states from the Midwest, Southeast and South regions of Brazil. Interestingly, no clinical signs were noticed in singular PCV3 infection. Moreover, the positive PCV3 rate in healthy pigs was higher than that found in sick pigs, suggesting that most pigs could live with PCV3 infection without any clinical infection sign. PCV3 sequences collected in 2006 and 2007 shared high identity with the Brazilian strains obtained in 2016, indicating a genetic stability of PCV3 Brazilian strains over the past 10 years. Multiple events of PCV3 introduction in Brazil may have occurred. In the phylogenetic tree, PCV3 strains were clustered in two main groups and two of the Brazilian strains clustered with the strains obtained from wild boars, suggesting that it may play an important epidemiological role as a reservoir of PCV3 for commercial swine. The results of this study contributed to find new strains and to understand the genetic diversity and molecular evolution of PCV3 in Brazil.

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Conflict of interest

All authors have declared no conflict of interest.

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Table 1. Detailed information of 67 tissue samples collected from pigs between 2006 and 2007 from nine states of Brazil

Strain	Geographical origin	Year of collection	clinical signs	PCV2 infection	PCV3 infection
78	Minas Gerais	2006	-	-	-
1029	Minas Gerais	2007	-	-	+
1034	Goias	2007	+	+	-
1042	Minas Gerais	2007	-	-	-
1050	Minas Gerais	2007	-	-	+
1061	Minas Gerais	2007	-	-	-
1066	Minas Gerais	2006	-	-	-
1107	Minas Gerais	2006	-	-	-
1125	Minas Gerais	2007	+	+	+
1144	Santa Catarina	2007	+	+	-
1155	Minas Gerais	2007	-	-	-
1162	Minas Gerais	2007	+	+	-
1163	Minas Gerais	2007	+	+	-
1164	Minas Gerais	2007	-	-	+
1167	Minas Gerais	2006	-	+	-
1169	Minas Gerais	2007	-	-	-
1171	Minas Gerais	2007	+	+	-
1218	Minas Gerais	2007	+	+	-
1222	Minas Gerais	2007	+	+	-
1231	Minas Gerais	2007	+	+	-
1291	Minas Gerais	2007	-	-	-
1322	Minas Gerais	2006	+	+	-
1329	Rio Grande do Sul	2007	-	-	+
1343*	Minas Gerais	2006	+	+	+
1350	Minas Gerais	2007	-	-	-
137	Minas Gerais	2007	-	-	+
1373*	São Paulo	2007	-	-	+
1396	Rio Grande do Sul	2007	-	-	+
1405	Minas Gerais	2006	+	+	-
1410	Minas Gerais	2006	+	+	-
1429	Santa Catarina	2007	+	+	+
1445	nd	2006	-	-	-
1456	nd	2007	-	-	-
147	São Paulo	2006	-	-	-
1473	Minas Gerais	2006	+	+	-
200	Minas Gerais	2006	-	+	+
295*	Espírito Santo	2007	+	+	+
297	Minas Gerais	2007	-	-	+
348	Minas Gerais	2007	-	-	-
408	Minas Gerais	2007	+	+	-
474	Minas Gerais	2006	-	-	-
488	São Paulo	2007	+	+	+

489	Minas Gerais	2007	+	+	-
526	Espírito Santo	2007	+	+	+
532	Minas Gerais	2007	-	-	+
600	Mato Grosso	2007	+	+	+
622*	Mato Grosso	2007	-	-	+
633	Minas Gerais	2007	-	-	-
648	Minas Gerais	2007	-	-	+
666	Rio Grande do Sul	2007	+	+	+
679	Minas Gerais	2007	-	-	-
68	nd	2007	-	-	+
70	Minas Gerais	2007	-	-	+
727	Minas Gerais	2007	+	+	+
837	Mato Grosso	2007	+	+	+
839	Paraná	2007	-	-	+
872*	Goias	2007	-	-	+
876	Minas Gerais	2007	-	-	+
893	Minas Gerais	2007	-	-	-
916*	Paraná	2007	-	-	+
955	Minas Gerais	2006	-	-	-
958	Mato Grosso	2007	-	-	-
966	Mato Grosso	2007	-	-	+
986*	Mato Grosso do Sul	2007	+	+	+
987	Minas Gerais	2006	-	-	-
988	Minas Gerais	2007	-	-	+
993	Minas Gerais	2007	-	-	+

nd= no data available. *****Sequenced samples

6. GENERAL CONCLUSIONS

- The most common recent ancestor (tMRCA) of current PCV3 strains may have emerged 50 years ago.

- PCV3 is not genetically closely related with PCV1 or PCV2 and no sign of recombination between PCV3 and known circoviruses could be detected.

- PorkNW2 and SFBeef are actually defective PCV3 strains or replicative intermediates and PCV3 may be evolving undetected for some time in swine and bovine population.

- PCV3 is globally dispersed, considering that groups of genetically related isolates of PCV3 originated from different countries were found.

- PCV3 was identified in all analyzed states of the Midwest, Southeast and South regions of Brazil, with a detection rate of 47.8%.

- The positive PCV3 rate in healthy pigs was higher than that found in sick pigs, suggesting that most pigs could live with PCV3 infection without any clinical infection sign.

- PCV3 sequences collected in 2006 and 2007 shared high identity with the Brazilian strains obtained in 2016, indicating a genetic stability of PCV3 Brazilian strains over the past 10 years.

- PCV3 has not shown a differentiated independent molecular evolution in Brazil and multiple events of introduction may have occurred.

- Brazilian strains were grouped into two distinct phylogenetic groups in the phylogenetic tree, PCV3a and PCV3b.

- Two Brazilian strains were grouped with strains obtained from wild boars, suggesting that it may play an important epidemiological role as a reservoir of PCV3 for commercial swine in Brazil.