VIVIANE SISDELLI ASSAO

DETECTION AND EVALUATION OF GENETIC DIVERSITY OF *Porcine circovirus 3* IN BRAZILIAN SWINE FARMS, EXPRESSION AND PURIFICATION OF THE CAPSID PROTEIN

Thesis submitted to the Veterinary Medicine Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

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Co-advisers: Ricardo Seiti Yamatogi
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Assent:

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To my lovely family.

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To God for everything you have done for me

To my parents, Mario and Mariusa, thank you both for your love, support, and for always being there for me. You always encourage and believe in me.

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“I am among those who think that science has great beauty” (Marie Curie).

ABSTRACT

ASSAO, Viviane Sisdelli, D.Sc., Universidade Federal de Viçosa, February 2021. **Detection and evaluation of genetic diversity of *Porcine circovirus 3* in Brazilian swine farms, expression and purification of the capsid protein**. Advisor: Abelardo Silva Júnior. Co-advisers: Maria Aparecida Scatamburlo Moreira and Ricardo Seiti Yamatogi.

Porcine circovirus 3 (PCV3) is a recently discovered virus. PCV3 has been associated with several signs, but its pathogenicity is still being debated. PCV3 has been detected and circulating in the swine population worldwide. Due to the fact that there are a few studies of PCV3 in Brazilian swine herds, this work aimed to investigat PCV3 in Brazil. We evaluated two molecular diagnostic tests (real-time PCR and Nested PCR) for PCV3 detection, and the real-time PCR assay had better results than the Nested PCR assay used in this study. We investigated the genetic diversity of PCV3 in swine farms from Minas Gerais, the fourth largest pork producing state in Brazil. Our results revealed the presence of at least two PCV3 main lineages circulating in Brazil. Furthermore, for the first time, we reported the detection of two different PCV3 strains in the same pig. We also surveyed PCV3 in swine farms affected by several health problems in Paraná, the second-largest pork producing state in Brazil. Our results showed that 36.88% of the swine were PCV3 positive. PCV3 DNA was detected in swine with different health problems, including animals with vesicular lesions that had not been previously documented. Finally, we expressed the truncated PCV3 capsid protein using a prokaryotic expression system and partially purified it using a two-steps purification with the goal for future development of vaccines and diagnostic tests.

Keywords: Porcine circovirus 3. Genetic diversity. Real-time PCR. Nested PCR. Capsid protein.

RESUMO

ASSAO, Viviane Sisdelli, D.Sc., Universidade Federal de Viçosa, fevereiro de 2021. **Detecção e avaliação da diversidade genética de *Porcine circovírus 3* em fazendas de suínos brasileiras, expressão e purificação da proteína do capsídeo**. Orientador: Abelardo Silva Júnior. Coorientadores: Maria Aparecida Scatamburlo Moreira e Ricardo Seiti Yamatogi.

Porcine circovirus 3 (PCV3) é um vírus que foi descoberto recentemente. PCV3 tem sido associado a diversos sintomas, mas a sua patogenicidade ainda está sendo discutida. PCV3 tem sido detectado circulando na população suína em todo o mundo. Devido ao fato de que existem poucos estudos sobre PCV3 no rebanho suíno brasileiro, esse trabalho teve como objetivo estudar a presença de PCV3 no Brasil. Neste trabalho nós investigamos a diversidade genética de PCV3 em fazendas de suínos localizadas em Minas Gerais. Os resultados mostraram que pelo menos duas linhagens de cepas de PCV3 estão circulando no Brasil. Pela primeira vez, um estudo relata a detecção de duas cepas diferentes de PCV3 no mesmo suíno. Nós avaliamos duas técnicas de diagnóstico molecular (real-time PCR e Nested PCR) para a detecção de PCV3, e a técnica de real-time PCR obteve resultados superiores em comparação com a técnica de Nested PCR usada neste estudo. Nós investigamos a presença de PCV3 em fazendas de suínos que relatavam problemas sanitários no Paraná, o segundo maior estado produtor de carne suína. Nossos resultados mostraram que 36,88% dos suínos eram positivos para PCV3. O DNA de PCV3 foi detectado em suínos com diversos problemas de saúde, incluindo suínos com lesões vesiculares o que não foi descrito em trabalhos anteriores. Além disso, nós expressamos a proteína truncada do capsídeo viral de PCV3 usando um sistema de expressão procarioto e purificamos parcialmente a proteína usando um protocolo de purificação em duas etapas para ser utilizada no futuro para o desenvolvimento de vacinas e testes de diagnóstico.

Palavras-chave: *Porcine circovirus 3.* Diversidade genética. Real-time PCR. Nested PCR. Proteína do capsídeo.

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LIST OF ABBREVIATIONS

ORFs Open reading frames.

REP Replicase.

CAP Capsid.

PCV1 *Porcine circovirus 1*.

PCV2 *Porcine circovirus 2*.

PCV3 *Porcine circovirus 3*.

PCV4 *Porcine circovirus 4*.

ICTV International Committee on the Taxonomy of Viruses.

PDNS Porcine dermatitis and nephropathy syndrome.

VLPs Virus-like particles.

PCR Polymerase chain reaction.

NLS Nuclear localization signal

AA Amino acid

NT Nucleotides

SUMMARY

1. INTRODUCTION

The swine industry is an organized segment of Brazilian agribusiness. Brazil ranks fourth in pork production and exportation in the world. Despite the pandemic impacts, in 2020, we exported 1.021 million tons of pork (natural and processed products), which is 36.1% higher than registered in 2019.

Swine producers are constantly facing several problems as diseases, pathogens control, changes in the virulence of the pathogens, antimicrobial resistance, and vaccine problems.

The advent of new technologies has enabled the discovery of novel infectious agents. In 2016, a new virus was detected in swine with different health problems in the United States of America, and it was named *Porcine circovirus 3* (PCV3). Since then, PCV3 detection in swine herds has been increasing around the world. This emergent pathogen raised the concern of the swine industry since it is unknown if PCV3 represents a threat to the health of the swine.

As a new member of the family *Circoviridae* and genus *Circovirus,* PCV3 has a common genomic organization, although PCV3 is distantly related to other known circoviruses*.* PCV3 is a non-enveloped virion with icosahedral symmetry, has an ambisense genome organization, with 1999-2001 nucleotides, encodes three open reading frames (ORFs). ORF1 encodes the replicase (rep) protein, ORF2 encodes the capsid (cap) protein, ORF3 encodes a protein with unknown function. Similar to other circoviruses, PCV3 has a stem-loop structure between the intergenic region between ORF1 and ORF2.

As a newly discovered virus, PCV3 pathogenicity is not fully understood yet. Until now, PCV3 has been associated with porcine dermatitis and nephropathy syndrome like clinical signs, reproductive failure in sow (Palinski et al., 2016), multisystemic inflammation, the occurrence of stillbirths, myocarditis, digestive disease, respiratory disease (Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Savic et al., 2020, neurological disorders (Phan et al., 2016; Hayashi et al., 2018b; Zheng et al., 2020). At the same time, PCV3 has also been detected in asymptomatic animals. Considering that *Porcine circovirus 2* has caused

important economic losses to the swine industry, PCV3 should be carefully investigated.

This work aimed to diagnostic and study PCV3 in Brazil. We compared two molecular tests (real-time PCR and Nested PCR) for PCV3 detection. We investigated the genetic diversity of PCV3 in swine farms from Minas Gerais, the fourth largest pork producing state in Brazil. Also, we investigated the presence of PCV3 in swine farms reported several health problems in Paraná, the second-largest pork-producing state in Brazil. In addition, we expressed and partially purified the recombinant PCV3 capsid protein.

2. LITERATURE REVIEW

Introduction

Numerous members of the family *Circoviridae* have been characterized in many species (fish, insects, avian, and mammals) and up to date, four species of circovirus are known to infect swine: *Porcine circovirus 1* (PCV1), *Porcine circovirus 2* (PCV2), *Porcine circovirus 3* (PCV3), and *Porcine circovirus 4* (PCV4) (Palinski et al., 2016; Phan et al., 2016; Klaumann et al., 2018a; Zhang et al., 2019). According to the International Committee on the Taxonomy of Viruses (ICTV), they are part of the genus *Circovirus*. *Porcine circovirus* is characterized as circular, single-stranded DNA viruses, enclosed in an icosahedral virion with a diameter of 17 nm (Ouyang et al., 2019).

PCV1 was discovered in 1974 by a German research group as a cell culture contamination by electron microscopy, it is found in wild and farmed pigs, and has not been associated with clinical disease (Tischer et al., 1974; Opriessnig et al., 2020).

PCV2 was discovered in the 1990s by multiple laboratories. PCV2 has been identified as an economically significant pathogen associated with several clinical diseases in swine such as porcine circovirus-associated diseases, postweaning multisystemic wasting syndrome, respiratory and enteric disease, porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure (Palinski et al., 2016; Ouyang et al., 2019; Opriessnig et al., 2020).

PCV3 was discovered in 2016, in the United States of America, using metagenomic sequencing, and has been found in swine with several clinical syndromes as PDNS, reproductive failure, myocarditis, and multisystemic inflammation (Palinski et al., 2016; Phan et al., 2016).

PCV4 emerged in 2019, in China, and was isolated in swine with several clinical diseases, including respiratory signs, enteric signs, and PDNS (Zhang et al., 2019).

Two researchers' groups reported the discovery of PCV3 as a new virus infecting swine in the United States of America (Palinski et al., 2016; Phan et al., 2016). The novel virus had the genetic and structural similarity to the genus *Circovirus* and less than 70% capsid amino acid sequence identity compared with other species, based on this information they proposed a new species named *Porcine circovirus 3* (Palinski et al., 2016; Phan et al., 2016).

Genetic organization

Circoviruses belong to the family *Circoviridae*, genus *Circovirus*, characterized as single-stranded circular DNA viruses, and are the smallest known autonomously replicating viruses (Palinski et al., 2016). Circoviruses are characterized by a non-enveloped virion with icosahedral symmetry, and a diameter ranging from 13 to 25 nm (Palinski et al., 2016; Wang et al., 2017; Klaumann et al., 2018a).

About the PCV3 genetic organization, PCV3 has an ambisense genome organization, with 1999-2001 nucleotides, and encodes three open reading frames (ORFs).

ORF1 is the largest ORF, encodes a 296-297 amino acid replicase (rep), which is 54-55% identical to the rep of bat circovirus and 48% identical to the rep of PCV2 (Palinski et al., 2016; Phan et al., 2016). ORF2 encodes a 214 amino acid capsid (cap), oriented in the opposite direction that rep, and it is 36-37% identical to the Cap of PCV2 and duck circovirus, and 35% identical to the Cap of bat circovirus (Palinski et al., 2016; Phan et al., 2016). ORF3 encodes a 231 amino acid protein, oriented on the same strand as ORF1, 94% identical to an ORF identified in PorkNW2/USA/2019 and 39% identical to murid herpesvirus M169, the function of the protein is unknown, and the initiation codon it is unclear (Palinski et al., 2016; Phan et al., 2016).

Besides that, and similar to other members of the genus *Circovirus*, the PCV3 genome has a stem-loop structure located in the intergenic region between ORF1 and ORF2 (Palinski et al., 2016; Phan et al., 2016).

It was observed that the *cap* gene is highly conserved among PCV3 strains from different countries, 96.3-100% amino acid homologies (Ouyang et al., 2019). The low identity (36%) between the Cap protein of PCV2 and PCV3 makes cross-protection seems unlikely (Palinski et al., 2016).

Some researchers have reported that PCV3 strains have high genetic stability and a low mutation rate (Arruda et al., 2019; Qi et al., 2019; Ha et al., 2020a). Considering that Cap protein is the major structural protein in circovirus, it is important to consider the mutations in this region. The occurrence of

mutations at Cap protein could affect the PCV3 immunogenicity, therefore, further studies are needed to verify the impact of mutations in PCV3 strains.

As a newly found circovirus species, it took some time to standardize the nomenclature for PCV3 genotype definition. Previously, PCV3 was being divided into three major clades PCV3a, PCV3b, and PCV3c (Fu et al., 2017); or in two main groups PCV3a and PCV3b, and subdivided into subgroups a1, a2, b1, and b2 (Fux et al., 2018).

Different research groups in consensus suggested the new PCV3 classification criteria and schemes (Franzo et al., 2020). Based on some criteria (bootstrap support>0.9 and maximum genetic distance of 3% at the complete genome and 6% at the ORF2 levels, the classification suggested two clusters (Clade 1 and Clade 2). Clade 1 included the vast majority of sequences and strains discovered from several countries. Clade 2 included only two distantly related sequences collected in 2006 from two different Chinese farms. These strains could represent either recently emerged variants or the last descendant of previously circulating genotypes (Franzo et al., 2020).

The consensus in PCV3 classification should be helpful in the understanding of PCV3 epidemiology and potential control measures (Franzo et al., 2020).

Genetic Evolution

Retrospective studies detected PCV3 DNA in swine samples from 1967, indicating that PCV3 was circulating and has been present in swine populations worldwide for a long time (Rodrigues et al., 2020), and just recently was detected, probably because of the advances in technology.

For a better understanding of PCV3 evolutionary origin, Saraiva et al. (2018) analyzed a data set containing PCV3 strains and different species of circoviruses and suggested that PCV3 did not emerge through recombination events among currently known circoviruses. PCV3 has a distinct origin from other porcine circoviruses, and it shares an ancient common ancestor with the bat circoviruses (Saraiva et al., 2018).

The origin of PCV3 is still unknown. Li et al. (2018) suggested a potential bat circovirus origin since PCV3 was closely related to bat circovirus and generally, bats are considered to be important reservoirs of novel emerging infectious diseases. Fu et al. (2017) suggested that PCV3 may have originated as a result of the bat-associated circovirus cross-species transition from bats into swine, approximately 215 years ago. This could have occurred through intermediate contact with wild boards continued to circulate and evolve in wild boards and swineherd until PCV3 emerged (Fu et al., 2017). Saraiva et al. (2018) predicted an origin for the current PCV3 strain between 1946 and 1987 and suggested that PCV3 lineages diverged around 50 years ago, which agrees with the time proposed by Fu et al. (2017).

Epidemiology

Since 2016, cases of PCV3 detection have been reported in several countries. Until now, PCV3 was identified in the United States of America (Palinski et al., 2016; Phan et al., 2016); China (Shen et al., 2017); Korea (Kwon et al., 2017); Poland (Stadejek et al., 2017); Italy (Faccini et al., 2017); Brazil (Tochetto et al. 2017); United Kingdom (Collins et al., 2017); Denmark, Spain (Franzo et al., 2018a); Germany (Fux et al., 2018); Japan (Hayashi et al., 2018a); Thailand (Kedkovid et al., 2018b); Sweden (Ye et al., 2018); Russia (Yuzhakov et al., 2018); Colombia (Vargas-Bermudez et al., 2019a); Hungary (Deim et al., 2019); Serbia (Savic et al., 2020); Malaysia (Tan et al., 2020); Poland (Woźniak et al., 2020); Slovenia (Plut et al., 2020); Argentina (Serena et al., 2020), and Taiwan (Chang et al., 2020).

The high rate of countries reporting the presence of PCV3 and the haplotype network analysis performed by Saraiva et al. (2018) indicates that PCV3 is highly spread globally.

PCV3 transmission routes are not yet well characterized. Some researchers considered direct contact (Kedkovid 2018), fecal-oral (Zhai et al., 2017), and oronasal route (Jiang et al., 2019; Wang et al., 2019b) as possible transmissions route. The transplacental route was suggested considering the detection of the virus in sows with reproductive failures, mummified fetus, and stillbirth (Kedkovid et al., 2018a; Deim et al., 2019). The detection of PCV3 in

mummified/stillborn fetuses and 1-day suckling piglets support the PCV3's ability to cause intrauterine infection (Sukmak et al., 2018a; Saporiti et al., 2020). Besides that, PCV3 was also detected in semen, which could suggest a risk of horizontal transmission (Ku et al., 2017).

Hosts

PCV3 was first identified in swine (Palinski et al., 2016; Phan et al., 2016). There are reports of PCV3 detection in other hosts as the dog (Zhang et al., 2017; Sun et al., 2019); wild boar (Klaumann et al., 2018b; Franzo et al., 2019; Prinz et al., 2019; Giudici et al., 2020); laboratory mice (Jiang et al., 2019); cattle (Wang et al., 2019); ticks, chamois (Franzo et al., 2019); red/roe/fallow deer (Franzo et al., 2019; Czyżewska-Dors et al., 2020); mouflon (Franzo et al., 2019; Czyżewska-Dors et al., 2020); baboons with xenotransplantation of swine hearts (Krüger et al., 2019); and mosquitoes (Ha et al., 2020b).

PCV3 detection in different species suggests that PCV3 possesses cross-species transmission abilities and has an unexpectedly broad range and circulation in the wild (Ouyang et al., 2019). Wild animals might be potential reservoirs for PCV3 (Franzo et al., 2019). However, it is too soon to confirm such reservoir status for PCV3, and further studies are necessary (Klaumann et al., 2018b).

Arthropods have been investigated if they could act as PCV3 reservoirs in nature. Franzo et al. (2019) identified two ticks (*Ixodes Ricinus*) PCV3 positive and they were collected from PCV3-negative roe deer. However, Grasi et al. (2020) studied ticks and suggested that ticks seemed not to be involved in PCV3 spread in an investigation with wild rodent species. We cannot exclude the possibility that other wild species could be involved in PCV3 epidemiology (Grassi et al., 2020). Further studies should be conducted to investigate if ticks are vectors to PCV3.

Some researchers have reported the presence of a circovirus-like in pork (PorkNW2) and beef (SFBeef) (Li et al., 2010; Li et al., 2011; Zhang et al., 2014). Saraiva et al. (2018) demonstrated that PorkNW2 and SFBeef are defective PCV3 strains or replicative intermediates. Also, a analysis of host-specific

adaptation, using codon adaptation index analysis, compared potential hosts and revealed that *Homo sapiens* had a similar value to *Sus scrofa,* and *Canis familiaris* (Li et al., 2018b), that were already identified as PCV3 DNA in this hosts. Based on the analysis, the authors hypothesized that *Homo sapiens* may be a potential host of PCV3 and that PCV3 might pose a potential risk to public health (Li et al., 2018b).

Pathogenicity

The PCV3 pathogenicity is not well understood yet, and now with the knowledge of PCV3 isolation (Mora-Díaz et al., 2020; Oh and Chae, 2020), it could help future studies to elucidate the PCV3 pathogenesis.

Until now, PCV3 has been described worldwide in animals affected by different clinical conditions as PDNS-like clinical signs and reproductive failure in sow (Palinski et al., 2016), multisystemic inflammation (Phan et al., 2016), the occurrence of stillbirths (Tochetto et al., 2018), myocarditis (Phan et al., 2016; Arruda et al., 2019), digestive and respiratory disease (Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Savic et al., 2020), neurological disorders (Phan et al., 2016; Hayashi et al., 2018b; Zheng et al., 2020), and even in cases of apparently healthy animals (Wen et al., 2017; Klaumann et al., 2019).

Reproductive failure and multisystemic inflammation seem to be the most consistently reported clinical signs (Mora-Díaz et al., 2020). A study suggested that PCV3 might be an important factor in reproductive failure and a significant threat to swine production. It was identified a higher PCV3 positivity and mean viral load associated with reproductive failure than with other clinical signs (diarrhea, respiratory disease, PMWS, PNDS, others) (Guo et al., 2020). Some studies have demonstrated that the positive rate of PCV3 in stillbirth is higher than in other life stages, confirming that PCV3 plays an important role in reproductive failure (Fu et al., 2017; Assao et al., 2021 unpublished work). The fact that PCV3 is involved in reproductive failure in sow is a big concern, as it is known that sow health is fundamental for the success of the gestation.

Phan et al. (2016) reported the presence of PCV3 in two cases sharing characteristics of cardiac and multisystemic inflammation, further supporting a

pathogenic role of PCV3. Using histopathology and in situ hybridization, Arruda et al. (2019) demonstrated that PCV3 could be causing multisystemic inflammation. Recently, swine experimental inoculation studies reported the presence of lesions consistent with multisystemic inflammation in different tissues (Jiang et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2020). These studies demonstrated that PCV3 has the potential to cause multisystemic inflammation.

The hypothesis that PCV3 could be causing myocarditis cases (Phan et al., 2016) is supported by the in situ hybridization witch provided evidence of PCV3 replication in the heart (Arruda et al., 2019); the fact that PVC3 was isolated from perinatal pigs with myocarditis (Mora-Díaz et al., 2020); and a study demonstrating that infectious clone of PCV3 can infect the myocardium of Kunming mice (Jiang et al., 2020). PCV3 appears to have a proclivity for the cardiovascular system like PCV2 (Arruda et al., 2019). The PCV3 mechanism of inducing myocarditis in swine needs to be elucidated.

Jiang et al. (2018) reported the successful reproduction of PDNS-like clinical disease using an infectious PCV3 DNA clone in piglets. However, in other studies that performed a swine experimental inoculation study using PCV3, the swine did not develop the clinical disease (Mora-Díaz et al., 2020; Temeeyasen et al., 2020).

Several studies have suggested that the PCV3 infection is associated with digestive disease (Phan et al., 2016; Zhai et al., 2017; Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Guo et al., 2020; Savic et al., 2020; Zhang et al., 2020). Saporiti et al. (2019) did not identify the association of PCV3 with the occurrence of diarrhea. However, Zhang et al. (2020) induced diarrhea in piglets performing an oral inoculation with PCV3 sole-positive intestinal contents. Also, PCV3 infection can cause changes in the gut microbiota community resulting in a severe inflammatory response and weight loss (Hou et al., 2020). Considering all that, PCV3 infection might cause digestive disease, but more studies should be performed to elucidate this PCV3's pathogenic mechanisms.

PCV3 DNA was detected in swine with respiratory signs (Phan et al., 2016; Kim et al., 2017; Zhai et al., 2017; Fux et al., 2018; Xu et al., 2018; Qi et al., 2019;

Saporiti et al., 2019; Chung et al., 2020; Savic et al., 2020). Qi et al. (2019) suggested that PCV3 may have a tissue tropism for the respiratory tract. However, Saporiti et al. (2019) identified PCV3 in serum samples from diseases and healthy swine, suggesting that PCV3 does not seem to be causally associated with respiratory disorders. Studies that inoculated PCV3 in swine had controversial results regarding the observation of respiratory signs (Jiang et al., 2019; Mora-Díaz et al., 2020; Temeeyasen et al., 2020). Although the PCV3 pathogenesis is unclear, the hypothesis that PCV3 infection could have a potential role in developing respiratory signs need to be investigated.

PCV3 DNA was also identified in swine with neurological signs (Phan et al., 2016; Hayashi et al., 2018; Zheng et al., 2020). Co-infection with PCV3 and other pathogens may induce other signs (Hayashi et al., 2018). It is necessary to further investigate the pathogenicity of both PCV3 singular infection and co-infection cases (Hayashi et al., 2018).

There are several reports of PCV3 detection in animals without clinical signs (Zheng et al., 2017; Wen et al., 2017; Franzo et al., 2018b; Xu et al., 2018; Ye et al., 2018; Zou et al., 2018; Kedkovid et al., 2018b; Guo et al., 2019; Xia et al., 2019; Yuqi Liu et al., 2019a; Klaumann et al., 2019; Qi et al., 2019; Zhang et al., 2019; Saporiti et al., 2019; Oh and Chae, 2020; Savic et al., 2020). This suggests that co-factors may be required for the expression of PCV3 full virulence (Franzo et al., 2018b) and/or that PCV3 could cause subclinical infection once some samples presented a low viral load (Klaumann et al., 2018b, 2019). Temeeyasen et al. (2020) inoculated piglets with PCV3, and the results demonstrated a prolonged viremia, viral replication in tissues, multisystemic inflammation but no evidence of clinical signs. These results support that PCV3 can induce subclinical infection (Temeeyasen et al., 2020).

PCV3 was detected in different samples, such as lung, heart, lymph nodes, thymus, intestine, kidney, spleen, nasal swab, oral swab, semen, serum, liver, cerebrum, tonsil, feces, colostrum, and umbilical cord (Ku et al., 2017; Zheng et al., 2017; Fu et al., 2017; Li et al., 2018b; Yuzhakov et al., 2018; Zou et al., 2018; Franzo et al., 2018; Ha et al., 2018; Kedkovid et al., 2018b; Klaumann et al.,

2018b; Sukmak et al., 2018a; Arruda et al., 2019; Deim et al., 2019; Yingyi Liu et al., 2019; Guo et al., 2019; Han et al., 2019).

PCV3 was detected in all life stages, but the analyses of the PCV3 prevalence based on life stage are unclear. Kim et al. (2018a) observed the highest prevalence of PCV3 in sows and gilts. However, Stadejek et al. (2017) showed that PCV3 was most common among weaned and finishers swine. Some researchers have reported a low positive rate of PCV3 in suckling piglets (Stadejek et al., 2017; Kim et al., 2018a) suggesting a protective role of maternal immunity, which agrees with Kedkovid et al. (2018a) of the possibility of the presence of protective antibodies against PCV3 in the colostrum.

It is known that the immune system interacts with epitopes of the pathogen, and thinking about that it is important to evaluate PCV3 epitopes for developing a vaccine. Bioinformatics tools have been used to predict PCV3 epitopes (Li et al., 2018a; Zhang et al., 2019; Chen et al., 2019; Liu et al., 2020; Jiang et al., 2020). Some studies predicted potential B and T cell epitopes (Li et al., 2018a; Zhang et al., 2019; Chen et al., 2019; Liu et al., 2020; Jiang et al., 2020). Two epitopes were located in the nuclear localization signal (NLS) sequence of the PCV3 cap, implying that the NLS-truncated cap and full-length cap may react differently with PCV3 antibodies in pig serum (Jiang et al., 2020). Conservative analyses showed that most of the epitopes are conserved among diverse PCV3 strains (Jiang et al., 2020). However, some studies observed that amino acid site 24, 56, and 77 of *cap* was predicted as a potential epitope and to be under positive selection during evolution, which may be pivotal to the escape of PCV3 from the host immune system (Li et al., 2018a; Chen et al., 2019; Jiang et al., 2020). Jiang et al. (2020) observed that one epitope (140-146 amino acid) was exposed on the surface of cap protein and buried in VLP.

The confirmation of the ability of PCV3 to self-assemble into virus-like particles (VLPs) provided a new tool for studing PCV3 and potential treatments and diagnostics (Wang et al., 2020). The cryo-EM structure of PCV3-VLP revealed some structural details of PCV3 capsid protein, as PCV3-VLP displays a typical icosahedral symmetry, and there are significant structural variations among the surface-exposed loops of PCV3 and PCV2-VLPs (Bi et al., 2020).

Therefore, it is important to study the structural details of PCV3 VLP and epitopes to a better understanding of the virus to develop a vaccine against PCV3.

Detection

As PCV3 infection has been reported in many countries and coinfection of PCV3 with other pathogens is also prevalent in swine herds; it is necessary to develop rapid, specific, and sensitive methods for virus detection or differentiation (Ouyang et al., 2019).

Several diagnostics methods were developed for PCV3 detection, such as polymerase chain reaction (PCR) (Palinski et al., 2016; Ku et al., 2017; Giovanni Franzo et al., 2018; Ye et al., 2018), real-time PCR (Palinski et al., 2016; Kim et al., 2017; Wang, 2017; Chen et al., 2018), Nested PCR (Zhang et al., 2017), droplet digital PCR (Zhang et al., 2019a; Yuqi Liu et al., 2019b), loop-mediated isothermal amplification (Park et al., 2018; Zheng et al., 2018; Wang et al., 2019), polymerase spiral reaction (Ji et al., 2019), enzyme-linked immunosorbent assay (Deng et al., 2018; Geng et al., 2019; Zhang et al., 2019; Wang et al., 2020), real-time recombinase-aided amplification assay (Li et al., 2020), and colorimetric isothermal multiple-self-matching-initiated amplification (Gou et al., 2020).

PCV3 was isolated in PK-15 cell line and the virus replication was demonstrated using real-time PCR, indirect immunofluorescence assay, and RNAscope (Mora-Díaz et al., 2020).

Co-infection

Many studies have been reporting co-infection in swine and wild boards of PCV3 and other pathogens, such as PCV1, PCV2, *Porcine reproductive and respiratory syndrome virus*, porcine epidemic diarrhea virus, torque teno sus virus, porcine bocavirus, pseudorabies virus, classical swine fever virus, porcine parvovirus, *Leptospira* spp., *Streptococcus spp.* (Zhai et al., 2017; Zheng et al., 2017, 2020; Wang et al., 2017; Wen et al., 2017; Ye et al., 2018; Ha et al., 2018; Li et al., 2018b; Chen et al., 2019; Yuqi Liu et al., 2019b; Zhao et al., 2019; Prinz et al., 2019; Wang et al., 2019a; Chung et al., 2020; Dal Santo et al., 2020; Dei Giudici et al., 2020).

One possible explanation of the frequent reports of coinfection between PCV3 and other pathogens was suggested by Jiang et al. (2019) that PCV3 infection might increase susceptibility to infections by primary and secondary pathogens. And, considering that PCV2 has immunosuppressive activity, PCV3 infection might compromise the swine immune system to a certain extent, and consequently facilitating concomitant infection with other viruses (Dei Giudici et al., 2020).

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3. COMPARISON OF REAL-TIME PCR AND NESTED PCR FOR PCV3 DETECTION IN PIG SERUM SAMPLES

Short Communication

Summary

Porcine circovirus 3 (PCV3) is spread worldwide and it is being associated with different clinical signs, which could be a threat to the global swine industry. In this study, we compared two molecular methods (real-time PCR and Nested PCR) for PCV3 detection using the conserved capsid protein as a target. The methods were tested in 386 swine serum samples collected between 2018-2019 in Paraná, Brazil. The results showed that the two methods had a fair agreement, but the frequency of PCV3 detection using this real-time PCR protocol was higher than the Nested PCR protocol.

Introduction

Swine production is an agricultural sector of great importance for the world economy, and Brazil is the fourth-largest producer and fourth-largest exporter of pork (ABPA, 2019). Prevention of diseases is a fundamental tool for swine production since prevention avoids the economic losses caused by diseases. In the last 30 years, the number of viral infectious diseases in swine has significantly increased (Klaumann et al., 2018a).

In 2016, two research groups reported the discovery through a metagenomics approach of this new porcine circovirus. It had a genetic and structural similarity to the genus *Circovirus* and less than 70% capsid amino acid sequence identity compared with other species, so they proposed a new species: *Porcine circovirus 3* (PCV3) (Palinski et al., 2016; Phan et al., 2016). The importance of this new member of the Circovirus genus is still under discussion, once PCV3 was detected in both healthy and diseased animals (Klaumann et al., 2018a). However, there are more reports of PCV3 detection in diseased animals.

Retrospective studies detected PCV3 DNA in swine samples from 1967 in Brazil (Rodrigues et al., 2020), in 1993 in Sweden (Ye et al., 2018), and in 1996 in Spain and China (Klaumann et al., 2018c; Sun et al., 2018). These findings indicated that PCV3 has been present and circulating in swine populations worldwide for a long time (Rodrigues et al., 2020). The fact that PCV3 is infecting clinically healthy animals may have facilitated the spread of the virus around the world through the animal trade and circulation.

Until now, PCV3 was detected in more than 20 countries. It is known that pathogen surveillance is essential to control the dissemination, specially PCV3 that was detected in different species, in all ages of swine, and it is circulating in several countries. Currently, PCV3 detection is based on molecular techniques (conventional PCR and real-time PCR) and its characterization by Sanger sequencing or next-generation sequencing. In this context, this study aimed to compare two PCV3 detection methods using swine serums samples from 10 farms with health problems located in Paraná, Brazil.

Material and methods

A total of 386 swine serums were collected in 2018 and 2019 from 10 weaner farms from Paraná, Brazil. The farms reported health problems as encephalitis, arthritis, weight loss, and diarrhea.

Total DNA was extracted from samples using the Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions.

The DNA extracted was tested by PCR using endogenous gene 18S rRNA of swine previously described (Assao et al., 2019). After, Nested PCR was performed using a combination of primers described by Ku et al. (2017) (table 1) that amplified the PCV3 ORF2 sequence. The first reaction of the Nested PCR was performed following the conditions: 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 s, 61ºC for 45 s, 72ºC for 45 s, and 72ºC for 5 min. Followed by the second reaction of the Nested PCR that was performed following the conditions: 95ºC for 5 min, followed by 35 cycles of 95ºC for 30 s, 56.5ºC for 45 s, 72ºC for 45 s, and 72ºC for 5 min.

A duplex real-time PCR was performed using primers and probe previously described by Palinski et al. (2016) that amplified PCV3 ORF2 sequence, and primers and probes previously described by Duvigneau et al. (2005) that amplified β-actin (table 1.1). The amplification parameters were set as 95ºC for 5 minutes, followed by 45 cycles of 95ºC for 15 seconds, and 60ºC for 60 seconds. A standard curve was obtained using one PCV3-positive sample, confirmed by Sanger sequencing. The amplicon of this positive sample was ligated into a cloning vector, Clone JET PCR Cloning kit (Thermo Fisher), and transformed into *Escherichia coli* DH5α competent cells. The plasmid DNA was recovered using Fast-n-Easy Plasmid Mini-prep (Cellco) and quantified using QuantiFluor ONE dsDNA System (Promega) and a Qubit (Thermo Fisher). The standard curve was generated using ten-fold dilutions of the plasmid DNA, with a detection range of $1.4x10^0$ to $1.4x10^9$ molecules. Samples with threshold cycles (Ct) values ≤ 38 and with a typical amplification curve were considered positive. The values of the quantification cycle (Cq) were 7.56 to 38.17 cycles with a linear relationship (R) of 0.98 and reaction efficiency (E) of 90%.

To measure the agreement between Nested PCR and real-time PCR, Cohen's Kappa indices was calculated using the Data Analysis Supplement for Excel™ (Microsoft Corp). And the estimation of several diagnostic parameters (sensitivity, specificity, PCV3 prevalence, positive predictive value, negative predictive value, and accuracy) was calculated using the web-based software MedCalc's diagnostic test evaluation calculator (https://www.medcalc.org/calc/diagnostic_test.php).

Table 1.1 - Method and sequences of the primers used to detect PCV3 (Nested PCR and real-time PCR), and the endogenous control (PCR).

Method	Target	Primer Forward (5'-3')	Primer Reverse (5'-3')	Product
Nested PCR	PCV3 ORF2 (1st)	TTGCACTTGTGTACAATTATTGCG	ATCTTCAGGACACTCGTAGCACCAC	1075 bp
	PCV3 ORF2 (2nd)	TTACTTAGAGAACGGACTTGTAACG	AAATGAGACACAGAGCTATATTCAG	649 bp
Real-time PCR	PCV3 ORF2	AGTGCTCCCCATTGAACG	ACACAGCCGTTACTTCAC	112 bp
		Probe: 6-carboxyfluorescein/ACCCCATGG/Zen/CTCAACACATATGACC/IowaBlack		
	β-actin	CTCGATCATGAAGTGCGACGT	GTGATCTCCTTCTGCATCCTGTC	114 bp
		Probe: HEX/ATCAGGAAG/Zen/GACCTCTACGCCAACACGG/3IABkFGHex		
PCR	18S rRNA	GCCTCGAA AGAGTCCTGTATTG	CTGAGAAACGGCTACCACATC	107 bp

Results

In this study, real-time PCR was able to measure $1.4x10^1$ to $1.4x10^9$ copies/µL of PCV3. All the samples were positive for both the endogenous controls. PCV3 was present in all farms investigated (table 1.2). The Nested PCR assay detected that 10.62% (41/386) of the serum samples were positive for PCV3. The real-time PCR assay detected that 40.93% (158/386) of the serum samples were positive for PCV3. Real-time PCR detected more PCV3 DNA in the clinical serum samples than Nested PCR.

Table 1.2 - Results of the detection of PCV3 in 10 farms using the Nested PCR and real-time PCR assay.

Farm	Nested PCR	Real-time PCR
A	18.92% (7/37)	35.14% (13/37)
B	4.65% (2/43)	25.58% (11/43)
C	2.70% (1/37)	40.54% (15/37)
D	0% (0/44)	40.91% (18/44)
E	20% (7/35)	42.86% (15/35)
F	40.63% (13/32)	15.63% (5/32)
G	0% (0/32)	65.63% (21/32)
H	0% (0/40)	42.5% (17/40)
I	8.33% (4/48)	66.67% (32/48)
J	18.42% (7/38)	28.95% (11/38)
TOTAL	10.62% (41/386)	40.93% (158/386)

A contingency table was constructed to compare the tests with each other, and to estimate the diagnostic parameters (table 1.3).

Table 1.3 - Comparison between results obtained from the Nested PCR and real-time PCR on samples from the farms.

	Real-time PCR positive	**Real-time PCR negative**	**Total**
Nested PCR positive	33	8	41
Nested PCR negative	117	228	345
Total	150	236	386
Sensitivity	22% (95% CI 15.65% to 29.49%)		
Specificity	96.61% (95% CI 93.43% to 98.53%)		
Positive Predictive Value	80.49% (95% CI 66.20% to 89.68%)		
Negative Predictive Value	66.09% (95% CI 64.08% to 68.04%)		
Accurary	67.62% (95% CI 62.70% to 72.26%)		

The tests had 68% of agreement and Cohen's k: 0.215. The diagnostic sensitivity of 22% (95% CI 15.65% to 29.49%) and diagnostic specificity of 96.61% (95% CI 93.43% to 98.53%). The positive predictive value, which is probability that the virus is present when the test is positive, was 80.49% (95% CI 66.20% to 89.68%), whereas the negative predictive value, which indicates the probability that the virus is absent when the test is negative, was 66.09% (95% CI 64.08% to 68.04%). The overall accuracy of the tests was determined to 67.62% (95% CI 62.70% to 72.26%).

Discussion

PCV3 is a novel virus and is a potential threat to the swine industry worldwide. The hypothesis that PCV3 is a potential pathogen is due to the PCV3 detection in swine with clinical signs similar to those produced by PCV2 infections (Zhang et al., 2019b). Several studies have focused on the genetic characterization of individual isolates, clinical and epidemiological investigations (Li et al., 2018a)

Molecular methods as Nested PCR and real-time PCR are widely used for pathogens detection. Up to now, no data are comparing Nested PCR and real-time PCR for PCV3 detection using the same target. The ORF2 sequence was selected as a molecular target to be used in both methods because it is highly conserved among PCV3 strains from different countries (Ouyang et al., 2019) and has the highest diversity between other porcine circovirus (Wang, 2017).

Nested PCR is a modification of PCR, and was developed to improve the sensitivity and specificity of DNA amplification, however, the potential for carryover contamination of the reaction is typically increased due to additional manipulation of amplicon products (Carr et al., 2010). Compared to the Nested PCR, real-time PCR provided a faster detection once it does not require agarose gel electrophoresis. The advantages of real-time PCR included quantification of target DNA sequence, has high sensitivity and specificity, has the potential for automation for high-throughput analysis, and no manipulation is required after the amplification (Hyong et al., 2008; Kralik and Ricchi, 2017). The disadvantage of real-time PCR is the cost, the complexity, and it requires trained staff.

In this study, we compared two molecular methods for PCV3 detection. We successfully develop a simultaneous detection of PCV3 ORF2 sequence and β-actin gene using real-time PCR. For the targets ORF2 sequence and β-actin, we choose different fluorophores, FAM and HEX, respectively, making the results easier to interpret. We successfully performed a Nested PCR using a combination of primers previously described that amplified the PCV3 ORF2 sequence without contamination problems.

To investigate the degree of agreement between real-time PCR and Nested PCR, a total of 386 swine serum samples were selected, and the Kappa index was measured for all farms as a whole. The results imply that there is a fair agreement between these two tests.

Based on the results obtained from this study, it is possible to observe that real-time PCR detected more positive samples (40.93%) than Nested PCR (10.62%). Our study indicates that the real-time PCR assay is more sensitive than the Nested PCR assay performed in this study.

The results difference might indicate a low viral concentration in the serum samples that were not enough to be detected using the Nested PCR. The real-time PCR assay was able to detect PCV3 even low viral titer ($1.4x10^1$ copies/µL) according to the standard curve. Some researchers have suggested that the low viral loads of PCV3 could cause subclinical infection (Klaumann et al., 2018b; Feng et al., 2019). In addition, this could be a survival mechanism that PCV3 has to develop to avoid the host's immune system (Savic et al., 2020). However, in this study, the clinical samples were collected from swine with and without clinical signs. Considering all that, we suggest that other pathogens might be involved and developing the clinical signs. Further studies are needed to elucidate the pathogenic potential of PCV3 and the role of other pathogens in PCV3 infections.

The real-time PCR assay were faster, amplified two targets (ORF2 sequence and β-actin) at the same time, and quantify the viral load of the samples. The Nested PCR required an extra PCR assay for detection of the endogenous control, it is not possible to quantify the viral load and required an additional electrophoresis step to observed the results.

Conclusion

In conclusion, this study developed and evaluated two molecular diagnostic tests that targeted the PCV3 ORF2 sequence. In this particular assay, the real-time PCR protocol was more suitable than Nested PCR for PCV3 detection. Real-time PCR is a fast and reliable method for PCV3 detection in serum samples and could be used to monitor the presence of PCV3 in the field, which is useful for monitoring the health status of the farms.

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4. GENETIC DIVERSITY OF *PORCINE CIRCOVIRUS 3* (PCV3) STRAINS IN BRAZIL AND THE FIRST DETECTION OF TWO DIFFERENT PCV3 STRAINS COINFECTING THE SAME HOST

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Abstract

Porcine circovirus 3 (PCV3) is a recently emerged circovirus discovered in 2016, which has drawn the attention of the swine industry worldwide. In this study, we evaluated the genetic diversity of PCV3 strains in pig farms. A total of 261 samples from sows, weaning pigs, growing pigs, and stillborn/mummified fetuses were analyzed by quantitative real-time PCR. The results revealed that PCV3 strains have at least two main lineages circulating in Brazil. For the first time, it was possible to detect two different PCV3 strains in the same host.

Short communication

Porcine circovirus 3 (PCV3) was discovered in 2016 through a survey using a metagenomics approach in swine with clinical signs of porcine dermatitis and nephropathy syndrome, reproductive failure, and cardiac and multisystemic inflammation [1, 2]. Until now, four species of circovirus were known to infect swine. PCV1 has not been associated with clinical disease. PCV2 is an economically significant pathogen associated with a diverse range of clinical diseases [3]. PCV3 and PCV4 emerged recently and had been detected in swine with several clinical diseases [1, 2, 4].

As a newly discovered member of the *Circovirus* genus, PCV3 has conserved elements in the genomic organization in common with other species; however, PCV3 is only distantly related to the other known circoviruses [5]. The classification proposed divided PCV3 into two clades [13]. Clade 1 (PCV3a) included PCV3 sequences from different countries. Clade 2 included only two Chinese sequences could represent either recently emerged variants or the last descendant of previously circulating genotypes [13]. Researchers from many countries worldwide have reported the detection of PCV3 in swine, showing different clinical signs, and even in asymptomatic animals. The detection of PCV3 in apparently healthy swine could indicate subclinical infections [6], which has led to whether PCV3 has clinical relevance in the field [7]. Considering the economic importance of PCV2 to the swine industry, PCV3 as an emergent pathogen and a new member of the same family should not be neglected.

This work aimed to detect PCV3 in several Brazilian[farms using quantitative real-time PCR (qPCR) and sequencing to elucidate some questions

about PCV3: (I) Is there genetic diversity among Brazilian PCV3 strains? (II) Is there a main PCV3 strain circulating among different Brazilian swineherds? (III) Is PCV3 found more frequently in fetuses from reproductive failure cases compared to other age groups?

We analyzed 261 swine samples (serum, vaginal swab, umbilical cord, intestine, spleen, liver, heart, lung, cerebrum, and lymph nodes). The samples were divided according to age group: sows (92), weaning pigs (17), growing pigs (65), and stillborn/mummified fetuses (87). These samples were collected from healthy sows, weaning and slow-growing pigs, and fetuses in 2019 from 19 commercial farms located in Minas Gerais State, which is a crucial swine-producing state in Brazil.

Total DNA was extracted from samples using the Wizard SV Genomic DNA purification system (Promega). To detect and quantify the PCV3 viral load, we used qPCR primers and probes, as previously described [1]. As an endogenous control, primers that amplified a region of 107 base pairs of the 18S ribosomal gene of swine were used [8]. One PCV3-positive sample, confirmed by Sanger sequencing was used to obtain a standard curve. The amplicon of this positive sample was ligated into a cloning vector (Clone JET PCR Cloning kit, ThermoFisher). Samples with threshold cycle (Ct) values of ≤38 and a typical amplification curve were considered positive. Data were analyzed statistically. A chi-squared test was used to evaluate the association between positive samples in different age groups, and ANOVA and Tukey's multiple comparison tests were used to compared viral load among different samples, using a p-value of <0.05.

We sequenced 17 strains in this work. ORF2 sequences were obtained from positive samples [Supplementary Material, (SI)] that were amplified by nested PCR, using a combination of primers, as previously described [9]. The Sanger sequencing data were trimmed and assembled into contigs using CLC Genomics Workbench version 8.5.4 (Qiagen) [Supplementary Material, (SI)]. Sequences were further clustered using the cd-hit-est tool of CD-HIT version 4.7 [10] to remove redundancy [Supplementary Material, (SII)].

A dataset containing 17 ORF2 sequences of Brazilian PCV3 strains and 83 ORF2 sequences of reference PCV3 strains [11] from different countries was

downloaded from GenBank [Supplementary Material, (SIII)]. The ORF2 sequences were aligned by MAFFT version 7.307 and the polymorphisms identified were screened using MEGA version 10.1.6 [Supplementary Material, (SIV)].

To expedite the construction of the phylogenetic tree, the model HKY+G was chosen as the best-fit model of nucleotide substitution from the full alignment using jModelTest version 2.1.10 [12]. The phylogenetic tree was calculated using the Bayesian Markov Chain Monte Carlo method using MrBayes 3.2.7a [13], in two runs with 5,000,000 generations. In the end, the average standard deviation of the split frequencies was 0.008550. The chains reached a stationary distribution after 500,000 generations, and 10% of the trees generated were burned to produce the consensus tree, which was annotated using Iroki [14].

At the farm level, 78.94% (15/19) of the farms were PCV3-positive. At the animal level, 39.85% (104/261) of the swine tested were PCV3-positive. When we analyzed the age groups, sows had 28.26% (26/92), weaning pigs 35.29% (6/17), stillborn/mummified fetuses 51.72% (45/87), and growing pigs 41.54% (27/65) PCV3 positivity (Fig. 1.1A). Among the tested samples, we detected PCV3 DNA in all the 10 different types of samples, including vaginal swabs (3/5).

We observed that different age groups had a different frequency of PCV3 positivity (Fig. 1.1A). A statistically significant difference was observed between sows and stillborn/mummified fetuses. Therefore, we investigated the viral load of the samples from these groups. However, no significant differences were observed (Fig. 1.1B). No significant difference was found among different farms (data not shown).

Among the PCV3-positive samples, 17 had the ORF2 of their viral strains sequenced. The partial sequences of ORF2 were clustered into four non-redundant sequences, named UFV01/BR/MG/2019, UFV02/BR/MG/2019, UFV03/BR/MG/2019, and UFV04/BR/MG/2019 [Supplementary Material, (SII)] and deposited in the GenBank database under accession numbers MT497513, MT497514, MT497515, and MT497516, respectively.

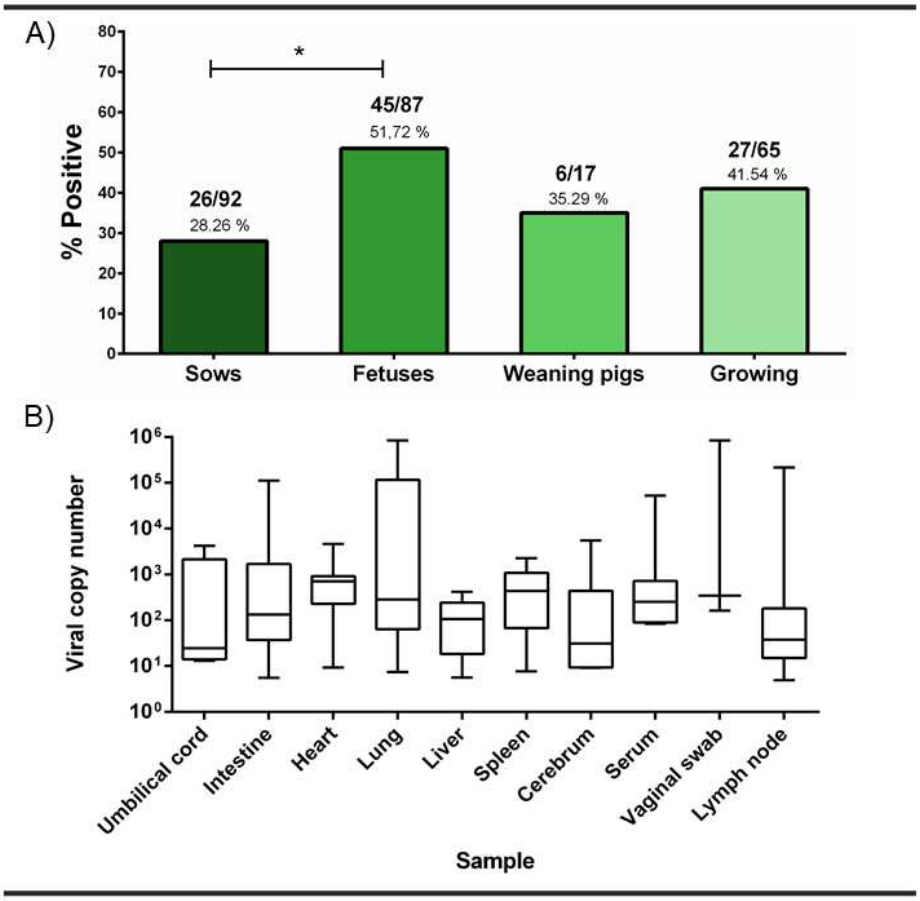

Figure 1.1 - Detection and quantification of PCV3 using qPCR. A) Percentage of PCV3 positivity in different age groups (sows, stillborn/mummified fetuses, weaning pigs, and growing pigs). A chi-squared test was performed (*p<0.05). B) PCV3 viral copy numbers.

It is interesting to observe that four different strains, i.e., MT497513, MT497514, MT497515, and MT497516 were present on the same farm, suggesting that different PCV3 strains can circulate in the same herd. Two strains, MT497513 and MT497514, were obtained from different tissue samples (lymph node and intestine, respectively) from the same animal, indicating co-infection.

Analysis of polymorphisms confirmed high conservation among the ORF2 sequences of PCV3 strains [Supplementary Material, (SIV)]. The MT497513 sequence is identical to that of the PCK3-1701 strain (MF611876.1), which was identified in South Korea (2016), and of PCV3-CN-JL22-2018 (MK178309.1), originating from China (2018). MT497514 differs by one synonymous substitution from two PCV3 strains from Brazil (MK645718.1 and MK645719.1), three strains

from China (MK645718.1, MK645719.1, and MK178321.1), one strain from Italy (MF162298.1), and one strain from South Korea (MK503331.1). MT497515 differs by two synonymous and one non-synonymous substitution from MT497514. MT497516 differs by one synonymous substitution from MT497513.

Taking into consideration only the sequences of Brazilian PCV3 strains (Fig. 1.2), most of the substitutions are located at third-codon positions of ORF2. The overall mean number of synonymous substitutions (dS) is equal to 2.96, and the number of non-synonymous (dN) is equal to 1.35, with a dN/dS rate of 0.46 among the Brazilian strains. Nine amino acid residues were shown to be polymorphic among sequences of the Cap protein, and three of them (V24A, K27R, and S77T|G) were polymorphic in at least four strains. In the phylogenetic tree (Fig. 1.3), all Brazilian strains were classified in the monophyletic clade of the PCV3a genotype, according to the most recent genotyping proposal for PCV3 [11].

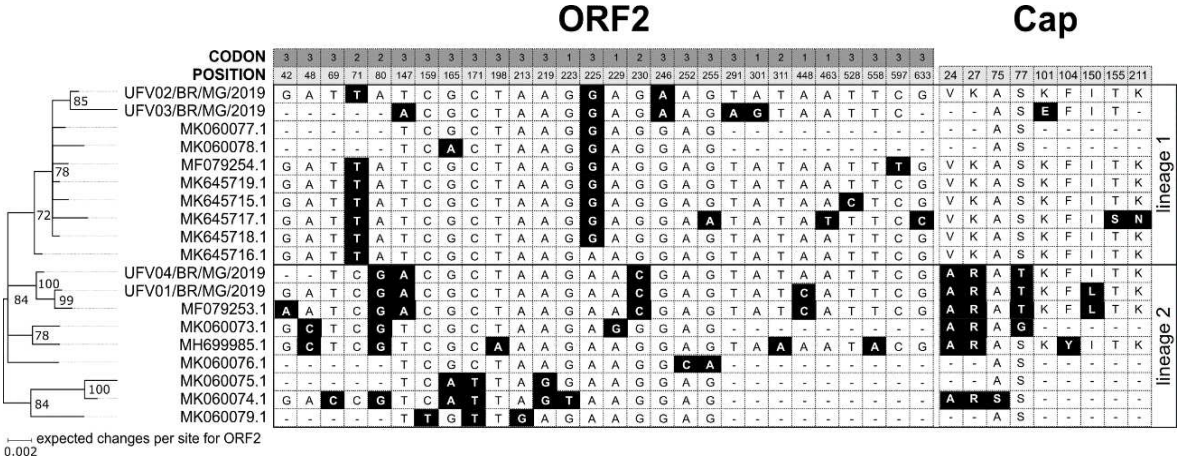

Figure 1.2 - Polymorphisms identified in nucleotide sequences of ORF2 and amino acid sequences of the Cap protein of Brazilian PCV3 strains. Each column corresponds to the positions of ORF2 and Cap that are variable.

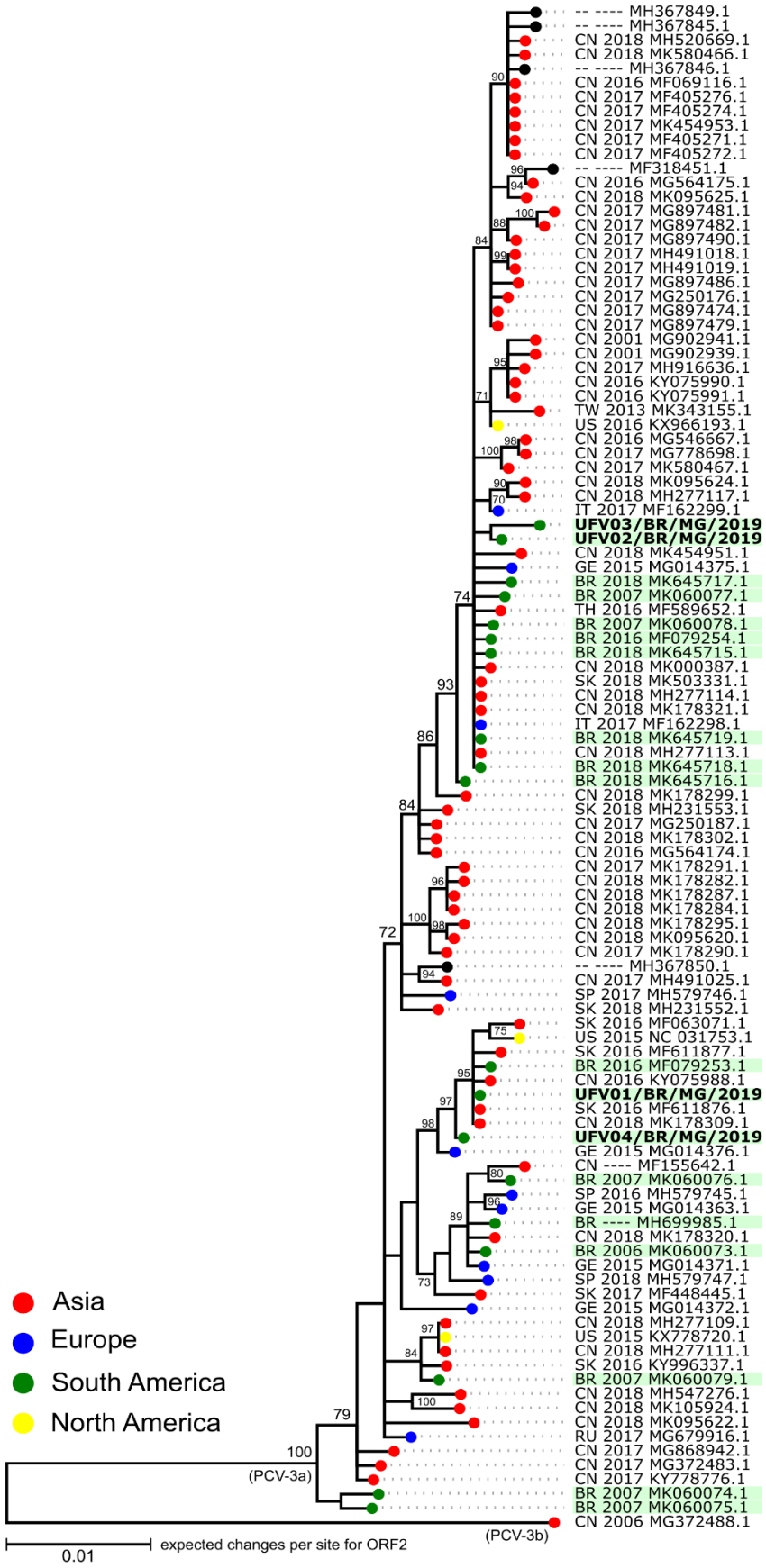

Figure 1.3 - Phylogenetic tree of ORF2 sequences of PCV3 strains. Analysis of 19 Brazilian strains of PCV3 and 83 reference PCV3 strains. The posterior probability (PP) values are shown beside each node only for those with high support (PP>70). The sequences obtained in this study are highlighted in bold.

PCV3 infection is related to several health problems. However, reproductive failure and multisystemic inflammation seem to be the most consistently reported clinical signs [7]. The association of PCV3 with several clinical presentations suggests that PCV3 could be a potential threat to the swine industry. This study aimed to contribute to the knowledge on PCV3 strains.

In this study, different swine samples of different age groups from 19 farms were collected and subjected to qPCR to detect PCV3. We detected PCV3 DNA in all different samples and PCV3 was detected in all different age groups. The detection of PCV3 DNA in 78.94% of the farms corroborates the results of other researchers that PCV3 is disseminated throughout Brazil [15, 16].

The PCV3 positivity rate was homogeneous in samples from weaning and growing pigs, ranging from 35.29 to 41.54%. These results corroborate with other researchers who suggested that PCV3 has a homogeneous frequency of positivity in different age groups [6].

Our results demonstrate that stillborn/mummified fetuses had a higher PCV3 positivity rate (51.72%). PCV3 DNA was detected in internal organs (intestine, spleen, liver, heart, lung, and cerebrum) and umbilical cord samples from fetuses, showing that PCV3 is present in a diverse range of tissues. However, no differences were observed in viral loads among different samples. PCV3 was detected in six sows with reproductive failure, and their respective stillborn/mummified fetuses were also PCV3-positive. Also, we identified the strain MT497513 in samples from one sow with a reproductive problem and three of her stillborn fetuses. These results support the hypothesis that PCV3 can be transmitted vertically [17-19] and reinforce this as a possible route of PCV3 transmission.

Horizontal transmission of PCV3 from sows to weaning pigs could be possible, according to Kedkovid et al. [25]. Our results show that PCV3 was detected in 70% (21/30) of the sera from clinically healthy sows. The average viral load in serum samples from clinically healthy sows was 4.12×10^3 copies/µL. The low viral load of PCV3 could be caused by a subclinical infection, which explains the swine being asymptomatic [6, 20]. It is crucial to evaluate the

impact of subclinical PCV3 infection because animal health is important, especially that of sows, as it is vital for reproductive success.

In this study, we also analyzed vaginal swabs from healthy sows that had stillborn piglets; the swabs were collected immediately after parturition. We identified that 3/5 of the vaginal swabs were PCV3-positive. The PCV3 DNA detection in vaginal swabs could indicate a risk of horizontal transmission, especially in farms that carry out natural insemination.

Two strains were obtained from different tissue samples from the same animal, which presented clinical signs of wasting. This result confirms that more than one PCV3 strain can infect the same animal. This is the first time that different PCV3 strains have been detected in different tissue samples from the same pig. PCV3 co-infection with different PCV3 strains could increase the chances of viral recombination within a single host.

We were able to obtain 17 sequences of ORF2 of PCV3, which were clustered into four non-redundant sequences. Analysis of the genome sequences showed a high identity of nucleotides (98.98-100%) and amino acids (97.66-100%) among different PCV3 strains from other countries available on GenBank.

Considering the fact that Cap is the major structural protein and main antigen of PCV3 [21], it is vital to analyze amino acid mutations in the Cap protein. The amino acid mutations observed in the PCV3 Cap protein suggest that different PCV3 strains circulate in Brazilian swine farms. We performed a phylogenetic analysis based on the PCV3 Cap sequence using the 17 PCV3 Brazilian strains were identified in this study and 15 Brazilian strains were previously deposited into GenBank. Our results demonstrate that the Brazilian PCV3 strains can be arranged into four different clusters by considering 10 amino acids of the Cap protein. This result reinforces the evidence of genetic diversity among PCV3 strains with at least two main lineages circulating in Brazilian herds.

The phylogenetic tree showed that the strains sequenced in this study were grouped with reference strains of genotype PCV3a. The four Brazilian strains sequenced in this study were clustered into different subclades together with strains from Asia, Europe, and North America.

This study identified four PCV3 strains in samples collected from Brazilian pig farms in Minas Gerais State. The phylogenetic and polymorphism analyses indicate two main lineages of PCV3 strains circulating in Brazilian herds. This is the first description of two PCV3 strains coinfecting the same animal. We identified the DNA of PCV3 in samples collected from pigs of all age groups and fetuses from reproductive failure cases, displaying a higher frequency of PCV3 detection.

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Supplementary Material

Table SI. Clustering of ORF2 sequences. Seventeen samples [(Sample identification (ID)] had the ORF2 of their viral strains sequenced, and these sequences were clustered (Cluster ID) into non-redundant sequences.

Sample	ORF2 sequence	Cluster ID
OR.S1	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTG GCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAG AATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGC AATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCT CCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCT GGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTT ATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAAC TACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCA ACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAA AAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCC	UFV01/ BR/MG/ 2019
OR.S2	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTG GCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAG AATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGC AATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCT CCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCT GGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTT ATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAAC TACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCA ACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAA AAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCCGT TCTC	UFV01/ BR/MG/ 2019
PI.S1	GGAACCCCTCAGAATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGA ATGGGAAACTGCAATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAG CCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATC TAGACGGCGCCTGGACCACA	UFV01/ BR/MG/ 2019
PI.S2	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTG GCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAG AATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGC AATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCT CCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCT GGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTT ATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAAC TACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCA ACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAA AAAACTGGAATGACA	UFV01/ BR/MG/ 2019
PI.S3	CGACGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAG CTGGCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCT CAGAATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAAC TGCAATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATT TCTCCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCG	UFV01/ BR/MG/ 2019

	CCTGGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAA GTTATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGG AACTACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGC TCAACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCG GAAAAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTC C	
PI.S4	GCTGGCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCC TCAGAATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAA CTGCAATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAAT TTCTCCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCG CCTGGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAA GTTATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGG AACTACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGC TCAACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCG GAAAAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTC CGTTCTC	UFV01/ BR/MG/ 2019
PI.S5	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTG GCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAG AATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGC AATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCT CCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCT GGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTT ATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAAC TACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCA ACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAA AAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCC	UFV01/ BR/MG/ 2019
PI.S7	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTG GCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAG AATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGC AATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCT CCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCT GGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTT ATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAAC TACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCA ACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAA AAAACTGGAATGACAGAC	UFV01/ BR/MG/ 2019
PM.S1	ATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACAGAAGGCGCTATGCCA GAAGAAGACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAAGAAATACTCC ACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCACGCCAACCA CTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTATAAGATACT AAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTATGTT CGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGGCTCCAAGAC GACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGCCGT TACTTCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAAGCC TCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATGG GGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTACGG CACCAAAGAAGTTTGGATTCGTTACAAGTCCGTT	UFV01/ BR/MG/ 2019
PM.S2	TTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACAGAAGGCGCTATGCCAGA AGAAGACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAAGAAATACTCCAC CATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCACGCCAACCACT	UFV01/ BR/MG/ 2019

	TCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTATAAGATACTAA AGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTATGTTCG GGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGGCTCCAAGACGA CCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGCCGTTA CTTCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAAGCCTC TTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATGGGG AGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTACGGCA CCAAAGAAGTTTGGATTCGTTACAAG	
PM.S3	ATGAGACACAGAGCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACA GAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTGGCACATACTA CACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGC CCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTT GAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAG CAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAA CACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTA AAAAAAAACACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCT CACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGA CCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAA TGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCCGTTCTC	UFV01/ BR/MG/ 2019
PM.S4	CACAGAGCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACAGAAGGC GCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAAG AAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCA CGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTA TAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAA AACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGG CTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAA CACAGCCGTTACTTCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCTCACCCAG GACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACC GTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGA CTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCCGTT	UFV01/ BR/MG/ 2019
RC.S1	GCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACAGAAGGCGCTATG CCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAAGAAATAC TCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCACGCCAA CCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTATAAGAT ACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTAT GTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGGCTCCAA GACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGC CGTTACTTCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAA GCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAAT GGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTAC GGCACCAAAGAAGTTTGGATTCGTTACAAGTCCGTT	UFV01/ BR/MG/ 2019
RC.S4	AGACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAAGAAATACTCCACCAT GAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCACGCCAACCACTTCA TTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTATAAGATACTAAAGA TGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTATGTTCGGG CACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGGCTCCAAGACGACC CTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGCCGTTACT TCACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAAGCCTCTT CTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATGGGGAG CACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGAC	UFV01/ BR/MG/ 2019

RC.S2	ATGAGACACAGAGCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACA GAAGGCGCTATGTCAGAAGAAAACTATTCATTAGGAGGCCCACAGCTGGCACATACTAC ACAAAGAAATACTCCACCATGAACGTCATTTCCGTTGGAACCCCTCAGAATAACAAGCC CTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCGATTAGCTTTG AATATTATAAAATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGC AAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAAC ACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAA AAAAAACACAGCCGTTACTTCACCCCCAAACCAATTCTGGCGGGAACTACCAGCGCTCA CCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACC CCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATG ACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCC	UFV02/ BR/MG/ 2019
RC.S3	TACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAAC AAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCGATTAG CTTTGAATATTATAAAATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCA CAGCAAACAGAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCA CAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACT TCTAAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCAATTCTGGCGGGAACTACCAG CGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACAT ATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACT GGAATGACAGACTTCTAC	UFV03/ BR/MG/ 2019
RC.S5	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTG GCACATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAG AATAACAAGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGC AATTACCTTTGAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCT CCGGCTCAGCAAACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCT GGACCACAAACACTTGGCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTT ATGACTTCTAAAAAAAAACACAGCCGTTACTTCACCCCCAAACCAATTCTGGCGGGAAC TACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCA ACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTTGGAGCATTTATGTCCCGGAA AAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGGATTCGTTACAAGTCCGT TCTC	UFV04/ BR/MG/ 2019

Table SII. Representative sequences of ORF2 (nt) and the Cap protein (aa) from the PCV3 strains analyzed in this study.

Cluster ID	GenBank accession number	ORF2 sequence	Cap protein sequence
UFV01/ BR/MG/ 2019	MT497513	ATGAGACACAGAGCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACAGAA GGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAA GAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCACG CCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTTGAATATTATAAGA TACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTATGT TCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGGCTCCAAGACGA CCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGCCGTTACTT CACCCCCAAACCACTTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTT TCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATGGGGAGCACTGCTT TGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTG GATTCGTTACAAGTCCGTTCTC	MRHRAIFRRRPRPRRRRRHRRRYARRRLFIRRPTAGTYYTKKYST MNVISVGTPQNNKPWHANHFITRLNEWETAITFEYYKILKMKVTLSP VISPAQQTKTMFGHTAIDLDGAWTTNTWLQDDPYAESSTRKVMTS KKKHSRYFTPKPLLAGTTSAHPGQSLFFFSRPTPWLNTYDPTVQW GALLWSIYVPEKTGMTDFYGTKEVWIRYKSVL
UFV02/ BR/MG/ 2019	MT497514	ATGAGACACAGAGCTATATTCAGAAGAAGACCCCGCCCAAGGAGACGACGACGCCACAGAA GGCGCTATGTCAGAAGAAAACTATTCATTAGGAGGCCCACAGCTGGCACATACTACACAAAG AAATACTCCACCATGAACGTCATTTCCGTTGGAACCCCTCAGAATAACAAGCCCTGGCACGC CAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCGATTAGCTTTGAATATTATAAAAT ACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAAACAAAAACTATGTT CGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGGCTCCAAGACGAC CCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACACAGCCGTTACTTC ACCCCCAAACCAATTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAAGCCTCTTCTTTTT CTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATGGGGAGCACTGCTTT GGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTACGGCACCAAAGAAGTTTGG ATTCGTTACAAGTCC	MRHRAIFRRRPRPRRRRRHRRRYVRRKLFIRRPTAGTYYTKKYST MNVISVGTPQNNKPWHANHFITRLNEWETAISFEYYKILKMKVTLSP VISPAQQTKTMFGHTAIDLDGAWTTNTWLQDDPYAESSTRKVMTS KKKHSRYFTPKPILAGTTSAHPGQSLFFFSRPTPWLNTYDPTVQW GALLWSIYVPEKTGMTDFYGTKEVWIRYKS
UFV03/ BR/MG/ 2019	MT497515	TACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACAAG CCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCGATTAGCTTTGA ATATTATAAAATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCACAGCAAAC AGAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTGG CTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACAC AGCCGTTACTTCACCCCCAAACCAATTCTGGCGGGAACTACCAGCGCTCACCCAGGACAAA GCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATGG GGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTAC	YYTKKYSTMNVISVGTPQNNKPWHANHFITRLNEWETAISFEYYKIL KMKVTLSPVISPAQQTETMFGHTAIDLDGAWTTNTWLQDDPYAES STRKVMTSKKKHSRYFTPKPILAGTTSAHPGQSLFFFSRPTPWLNT YDPTVQWGALLWSIYVPEKTGMTDFY
UFV04/ BR/MG/ 2019	MT497516	CGACGCCACAGAAGGCGCTATGCCAGAAGAAGACTATTCATTAGGAGGCCCACAGCTGGCA CATACTACACAAAGAAATACTCCACCATGAACGTCATATCCGTTGGAACCCCTCAGAATAACA AGCCCTGGCACGCCAACCACTTCATTACCCGCCTAAACGAATGGGAAACTGCAATTACCTTT GAATATTATAAGATACTAAAGATGAAAGTTACACTCAGCCCTGTAATTTCTCCGGCTCAGCAA ACAAAAACTATGTTCGGGCACACAGCCATAGATCTAGACGGCGCCTGGACCACAAACACTTG GCTCCAAGACGACCCTTATGCGGAAAGTTCCACTCGTAAAGTTATGACTTCTAAAAAAAAACA CAGCCGTTACTTCACCCCCAAACCAATTCTGGCGGGAACTACCAGCGCTCACCCAGGACAA AGCCTCTTCTTTTTCTCCAGACCCACCCCATGGCTCAACACATATGACCCCACCGTTCAATG GGGAGCACTGCTTTGGAGCATTTATGTCCCGGAAAAAACTGGAATGACAGACTTCTACGGCA CCAAAGAAGTTTGGATTCGTTACAAGTCCGTTCTC	RRHRRRYARRRLFIRRPTAGTYYTKKYSTMNVISVGTPQNNKPWH ANHFITRLNEWETAITFEYYKILKMKVTLSPVISPAQQTKTMFGHTAI DLDGAWTTNTWLQDDPYAESSTRKVMTSKKKHSRYFTPKPILAGT TSAHPGQSLFFFSRPTPWLNTYDPTVQWGALLWSIYVPEKTGMTD FYGTKEVWIRYKSVL

Table SIII. Sequence dataset of ORF2 sequences analyzed in this study.

GenBank accession	Country	Abbreviation	Isolation Year
KX778720.1	United States of America	US	2015
KX966193.1	United States of America	US	2016
KY075988.1	China	CN	2016
KY075990.1	China	CN	2016
KY075991.1	China	CN	2016
KY778776.1	China	CN	2017
KY996337.1	South Korea	SK	2016
MF063071.1	South Korea	SK	2016
MF069116.1	China	CN	2016
MF079253.1	Brazil	BR	2016
MF079254.1	Brazil	BR	2016
MF155642.1	China	CN	----
MF162298.1	Italy	IT	2017
MF162299.1	Italy	IT	2017
MF318451.1	not available	--	----
MF405271.1	China	CN	2017
MF405272.1	China	CN	2017
MF405274.1	China	CN	2017
MF405276.1	China	CN	2017
MF448445.1	South Korea	SK	2017
MF589652.1	Thailand	TH	2016
MF611876.1	South Korea	SK	2016
MF611877.1	South Korea	SK	2016
MG014363.1	Germany	GE	2015
MG014371.1	Germany	GE	2015
MG014372.1	Germany	GE	2015
MG014375.1	Germany	GE	2015
MG014376.1	Germany	GE	2015
MG250176.1	China	CN	2017
MG250187.1	China	CN	2017
MG372483.1	China	CN	2017
MG372488.1	China	CN	2006
MG546667.1	China	CN	2016
MG564174.1	China	CN	2016
MG564175.1	China	CN	2016
MG679916.1	Russia	RU	2017
MG778698.1	China	CN	2017
MG868942.1	China	CN	2017
MG897474.1	China	CN	2017
MG897479.1	China	CN	2017
MG897481.1	China	CN	2017
MG897482.1	China	CN	2017
MG897486.1	China	CN	2017
MG897490.1	China	CN	2017
MG902939.1	China	CN	2001
MG902941.1	China	CN	2001
MH231552.1	South Korea	SK	2018
MH231553.1	South Korea	SK	2018
MH277109.1	China	CN	2018
MH277111.1	China	CN	2018
MH277113.1	China	CN	2018
MH277114.1	China	CN	2018
MH277117.1	China	CN	2018
MH367845.1	not available	--	----

MH367846.1	not available	--	----
MH367849.1	not available	--	----
MH367850.1	not available	--	----
MH491018.1	China	CN	2017
MH491019.1	China	CN	2017
MH491025.1	China	CN	2017
MH520669.1	China	CN	2018
MH547276.1	China	CN	2018
MH579745.1	Spain	SP	2016
MH579746.1	Spain	SP	2017
MH579747.1	Spain	SP	2018
MH699985.1	Brazil	BR	----
MH916636.1	China	CN	2017
MK000387.1	China	CN	2018
MK060073.1	Brazil	BR	2006
MK060074.1	Brazil	BR	2007
MK060075.1	Brazil	BR	2007
MK060076.1	Brazil	BR	2007
MK060077.1	Brazil	BR	2007
MK060078.1	Brazil	BR	2007
MK060079.1	Brazil	BR	2007
MK095620.1	China	CN	2018
MK095622.1	China	CN	2018
MK095624.1	China	CN	2018
MK095625.1	China	CN	2018
MK105924.1	China	CN	2018
MK178282.1	China	CN	2018
MK178284.1	China	CN	2018
MK178287.1	China	CN	2018
MK178290.1	China	CN	2017
MK178291.1	China	CN	2017
MK178295.1	China	CN	2018
MK178299.1	China	CN	2018
MK178302.1	China	CN	2018
MK178309.1	China	CN	2018
MK178320.1	China	CN	2018
MK178321.1	China	CN	2018
MK343155.1	Taiwan	TW	2013
MK454951.1	China	CN	2018
MK454953.1	China	CN	2017
MK503331.1	South Korea	SK	2018
MK580466.1	China	CN	2018
MK580467.1	China	CN	2017
MK645715.1	Brazil	BR	2018
MK645716.1	Brazil	BR	2018
MK645717.1	Brazil	BR	2018
MK645718.1	Brazil	BR	2018
MK645719.1	Brazil	BR	2018
NC_031753.1	United States of America	US	2015

Table SIV. Quantification of polymorphisms in the ORF2 (nt) and Cap (aa) sequences among PCV3 strains.

	UFV01/BR/MG/2019	UFV02/BR/MG/2019	UFV03/BR/MG/2019	UFV04/BR/MG/2019	UFV01/BR/MG/2019	UFV02/BR/MG/2019	UFV03/BR/MG/2019	UFV04/BR/MG/2019	UFV01/BR/MG/2019	UFV02/BR/MG/2019	UFV03/BR/MG/2019	UFV04/BR/MG/2019	UFV01/BR/MG/2019	UFV02/BR/MG/2019	UFV03/BR/MG/2019	UFV04/BR/MG/2019
	ORF2 (nt) Substitutions				**ORF2 (nt) Synonymous**				**ORF2 (nt) Non-Synonymous**				**Cap (aa) Substitutions**			
UFV01/BR/MG/2019	-	7	6	**1**	-	3	3	**0**	-	4	3	**1**	-	4	3	**1**
UFV02/BR/MG/2019	7	-	**3**	6	3	-	**2**	3	4	-	**1**	3	4	-	**1**	3
UFV03/BR/MG/2019	6	3	-	5	3	2	-	3	3	1	-	2	3	1	-	2
UFV04/BR/MG/2019	1	6	5	-	0	3	3	-	1	3	2	-	1	3	2	-
KX778720.1	9	10	10	8	7	8	9	7	2	2	1	1	2	2	1	1
KX966193.1	7	2	4	6	3	2	3	3	4	0	1	3	4	0	1	3
KY075988.1	1	8	7	2	1	4	4	1	0	4	3	1	0	4	3	1
KY075990.1	8	3	5	7	4	3	4	4	4	0	1	3	4	0	1	3
KY075991.1	8	3	5	7	4	3	4	4	4	0	1	3	4	0	1	3
KY778776.1	4	9	9	5	4	5	6	4	0	4	3	1	0	4	3	1
KY996337.1	10	9	9	9	7	8	8	7	3	1	1	2	3	1	1	2
MF063071.1	3	10	8	3	1	4	4	1	2	6	4	2	2	6	4	2
MF069116.1	7	3	5	5	3	3	4	2	4	0	1	3	4	0	1	3
MF079253.1	1	8	6	**1**	1	4	3	**0**	0	4	3	**1**	0	4	3	**1**
MF079254.1	7	2	5	6	3	2	4	3	4	0	1	3	4	0	1	3
MF155642.1	9	10	8	6	5	6	6	4	4	4	2	2	4	4	2	2
MF162298.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
MF162299.1	7	2	4	5	3	2	3	2	4	0	1	3	4	0	1	3
MF318451.1	6	5	8	7	2	3	5	2	4	2	3	5	4	2	3	5
MF405271.1	7	3	5	5	3	3	4	2	4	0	1	3	4	0	1	3
MF405272.1	7	3	5	5	3	3	4	2	4	0	1	3	4	0	1	3
MF405274.1	7	3	5	5	3	3	4	2	4	0	1	3	4	0	1	3
MF405276.1	7	3	5	5	3	3	4	2	4	0	1	3	4	0	1	3
MF448445.1	8	9	9	6	4	5	7	4	4	4	2	2	4	4	2	2
MF589652.1	8	3	5	7	4	3	4	4	4	0	1	3	4	0	1	3
MF611876.1	**0**	7	6	**1**	**0**	3	3	**0**	**0**	4	3	**1**	**0**	4	3	**1**
MF611877.1	2	9	8	3	1	4	4	1	1	5	4	2	1	5	4	2
MG014363.1	7	8	8	5	2	3	4	1	5	5	4	4	5	5	4	4
MG014371.1	6	7	6	4	3	4	4	2	3	3	2	2	3	3	2	2
MG014372.1	10	9	9	9	8	7	8	8	2	2	1	1	2	2	1	1
MG014375.1	9	4	5	8	3	2	3	3	6	2	2	5	6	2	2	5
MG014376.1	3	6	7	2	2	3	5	2	1	3	2	0	1	3	2	0
MG250176.1	7	3	6	6	3	3	5	3	4	0	1	3	4	0	1	3
MG250187.1	8	5	7	7	3	4	6	3	5	1	1	4	5	1	1	4
MG372483.1	3	6	6	4	2	3	4	2	1	3	2	2	1	3	2	2
MG372488.1	79	79	59	71	54	52	38	49	25	28	21	22	18	20	15	16

MG546667.1	9	4	7	8	5	4	6	5	4	0	1	3	4	0	1	3
MG564174.1	8	5	8	7	4	5	7	4	4	0	1	3	4	0	1	3
MG564175.1	8	4	7	7	3	3	5	3	5	1	2	4	5	1	2	4
MG679916.1	7	8	9	6	4	5	7	4	3	3	2	2	3	3	2	2
MG778698.1	9	4	7	8	5	4	6	5	4	0	1	3	4	0	1	3
MG868942.1	6	10	10	7	5	6	7	5	1	4	3	2	1	4	3	2
MG897474.1	6	2	5	5	2	2	4	2	4	0	1	3	4	0	1	3
MG897479.1	6	2	5	5	2	2	4	2	4	0	1	3	4	0	1	3
MG897481.1	10	6	8	8	5	5	7	5	5	1	1	3	5	1	1	3
MG897482.1	9	5	7	7	4	4	6	4	5	1	1	3	5	1	1	3
MG897486.1	8	4	7	7	3	3	5	3	5	1	2	4	5	1	2	4
MG897490.1	7	3	5	5	2	2	4	2	5	1	1	3	5	1	1	3
MG902939.1	10	5	7	9	4	3	4	4	6	2	3	5	6	2	3	5
MG902941.1	10	5	7	9	5	4	5	5	5	1	2	4	5	1	2	4
MH231552.1	9	6	7	7	5	4	6	5	4	2	1	2	4	2	1	2
MH231553.1	9	6	9	8	5	6	8	5	4	0	1	3	4	0	1	3
MH277109.1	9	10	10	8	7	8	9	7	2	2	1	1	2	2	1	1
MH277111.1	9	10	10	8	7	8	9	7	2	2	1	1	2	2	1	1
MH277113.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
MH277114.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
MH277117.1	9	4	5	6	5	4	4	3	4	0	1	3	4	0	1	3
MH367845.1	9	5	7	7	4	4	5	3	5	1	2	4	5	1	2	4
MH367846.1	8	4	5	6	3	3	4	2	5	1	1	4	5	1	1	4
MH367849.1	9	5	7	7	4	4	5	3	5	1	2	4	5	1	2	4
MH367850.1	8	8	10	7	4	5	7	4	4	3	3	3	4	3	3	3
MH491018.1	7	3	6	6	3	3	5	3	4	0	1	3	4	0	1	3
MH491019.1	7	3	6	6	3	3	5	3	4	0	1	3	4	0	1	3
MH491025.1	9	8	10	8	6	7	9	6	3	1	1	2	3	1	1	2
MH520669.1	8	4	5	5	3	3	4	2	5	1	1	3	5	1	1	3
MH547276.1	11	11	12	10	7	7	9	7	4	4	3	3	4	4	3	3
MH579745.1	8	9	9	6	4	5	6	3	4	4	3	3	4	4	3	3
MH579746.1	10	8	10	9	6	6	8	6	4	2	2	3	4	2	2	3
MH579747.1	7	8	7	5	4	5	5	3	3	3	2	2	3	3	2	2
MH699985.1	7	8	8	5	4	5	6	3	3	3	2	2	3	3	2	2
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MK060074.1	10	13	13	11	7	8	9	7	3	5	4	4	3	5	4	4
MK060075.1	9	10	13	10	8	9	11	8	1	1	2	2	1	1	2	2
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MK060079.1	9	8	10	8	7	8	9	7	2	0	1	1	2	0	1	1
MK095620.1	9	8	8	7	6	7	7	5	3	1	1	2	3	1	1	2

MK095622.1	13	14	11	11	10	11	10	10	3	4	1	1	3	3	1	1
MK095624.1	9	4	5	6	5	4	4	3	4	0	1	3	4	0	1	3
MK095625.1	8	4	6	7	4	4	5	4	4	0	1	3	4	0	1	3
MK105924.1	11	12	11	9	7	8	8	6	4	4	3	3	4	4	3	3
MK178282.1	10	9	8	7	6	7	7	5	4	2	1	2	4	2	1	2
MK178284.1	9	8	8	7	6	7	7	5	3	1	1	2	3	1	1	2
MK178287.1	9	8	8	7	6	7	7	5	3	1	1	2	3	1	1	2
MK178290.1	9	8	9	7	6	7	8	5	3	1	1	2	3	1	1	2
MK178291.1	10	9	9	8	7	8	8	6	3	1	1	2	3	1	1	2
MK178295.1	10	9	8	7	6	7	7	5	4	2	1	2	4	2	1	2
MK178299.1	8	5	6	6	4	5	5	3	4	0	1	3	4	0	1	3
MK178302.1	8	5	8	7	4	5	7	4	4	0	1	3	4	0	1	3
MK178309.1	**0**	7	6	**1**	**0**	3	3	**0**	**0**	4	3	**1**	**0**	4	3	**1**
MK178320.1	7	8	7	5	4	5	5	3	3	3	2	2	3	3	2	2
MK178321.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
MK343155.1	11	6	8	10	3	2	3	3	8	4	5	7	8	4	5	7
MK454951.1	9	5	5	8	5	4	3	5	4	1	2	3	4	1	2	3
MK454953.1	7	3	5	5	3	3	4	2	4	0	1	3	4	0	1	3
MK503331.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
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MK580467.1	8	3	6	7	4	3	5	4	4	0	1	3	4	0	1	3
MK645715.1	7	2	5	6	3	2	4	3	4	0	1	3	4	0	1	3
MK645716.1	5	2	5	4	1	2	4	1	4	0	1	3	4	0	1	3
MK645717.1	9	4	6	8	3	2	4	3	6	2	2	5	6	2	2	5
MK645718.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
MK645719.1	6	**1**	4	5	2	**1**	3	2	4	**0**	1	3	4	**0**	1	3
NC_031753.1	3	10	8	4	3	6	5	3	0	5	3	1	0	4	3	1

5. *Porcine circovirus 3* DETECTION IN SWINE WITH DIFFERENT CLINICAL SIGNS IN PARANÁ, BRAZIL

Summary

The presence of *Porcine circovirus 3* (PCV3) affecting swine with different clinical signs around the world has been reported and drawn attention to swine health. This study aimed to investigate the presence of PCV3 in swine farms reporting various health problems in Paraná, Brazil. We performed a duplex real-time PCR for simultaneous detection of PCV3 ORF2 and β-actin. The 873 serum samples collected from 24 swine farms with health problems were tested by this assay and 36.88% of the swine were PCV3 positive, being that 40.93% were weaner and 33.68% were finishing swine. PCV3 positive varied from 15.63 to 66.67% in weaner farms, and 5.56 to 89.74% in finishing farms. PCV3 DNA was detected in swine with different health problems, including diarrhea, pneumonia, arthritis, vesicular lesion, and other clinical signs. We suggest that PCV3 in association with other pathogens, might be causing different health problems in swine farms located at Paraná, Brazil.

Introduction

Circoviruses belong to the family *Circoviridae*, genus *Circovirus*, and are the smallest known autonomously replicating viruses (Palinski et al., 2016). Until now, four species of circovirus were known to infect swine. *Porcine circovirus 1* (PCV1) was discovered in 1974 as a cell culture contamination and has not been associated with clinical disease. *Porcine circovirus 2* (PCV2) was discovered in the 1990s. PCV2 has been identified as an economically significant pathogen associated with a diverse range of clinical diseases such as porcine circovirus-associated diseases, postweaning multisystemic wasting syndrome, respiratory and enteric disease, porcine dermatitis and nephropathy syndrome (PDNS), and reproductive failure (Gillespie et al., 2009; Opriessnig et al., 2020). *Porcine circovirus 3* (PCV3) was discovered in 2016 and has been found in swine samples with several clinical syndromes (Palinski et al., 2016; Phan et al., 2016). *Porcine circovirus 4* (PCV4) emerged in 2019 and was isolated in swine with several clinical diseases, including respiratory signs, enteric signs, and PDNS (Y. Zhang et al., 2019a).

In 2016, two researchers group in the United States of America reported the discovery of PCV3 in swine with different clinical signs through a

metagenomics approach (Palinski et al., 2016; Phan et al., 2016). This new member of the genus *Circovirus* has the potential to be pathogenic and deserves further investigation (Ouyang et al., 2019). PCV3 has been reported in all continents, except for Africa and Australia (Franzo et al., 2020). Although the first time we heard about PCV3 was in 2016, a retrospective study detected PCV3 DNA in swine samples dating from 1967 in Brazil, and this is the oldest PCV3 partial capsid sequence described until now (Rodrigues et al., 2020).

PCV3 had been detected in swine, showing different clinical signs, and even in asymptomatic animals worldwide. The detection of PCV3 in apparently healthy swine could indicate subclinical infections (Klaumann et al., 2018b), which has led some researchers to the question of whether PCV3 has clinical relevance in the field (Mora-Díaz et al., 2020). The possibility that PCV3 might be causing immunosuppressive activity like PCV2 (Dei Giudici et al., 2020), and considering the economic importance of PCV2 to the swine industry worldwide, PCV3 as an emergent pathogen and as a new member of the same family should not be neglected (Klaumann et al., 2018a).

In this context, this work investigated PCV3 in pig farms with health problems from Paraná, the second-largest swine producer, and the third-largest swine exporter among Brazilian states.

Material and methods

A group of 24 swine farms (10 weaners and 14 finishing farms) has been having several health problems for more than one year. The farms are located in the Western Paranaense mesoregion (figure 2.1), the biggest pig producer mesoregion in Paraná, and are part of the Toledo microregion of Paraná, which has the highest number of swine among all the microregions of Paraná (IBGE, 2019).

Figure 2.1 – Localization of Paraná state in Brazil. The yellow star indicates the location where the samples were collected. Digital image (http://www.aen.pr.gov.br/modules/galeria/fotos.php?evento=57766) was manipulated.

Some reports were that initially, the animals had fever, arthritis, lethargy, and evolved to encephalitis and death in a few days. Also, some farms reported animals with pneumonia, three farms reported problems with diarrhea, and one farm reported animals with vesicular lesions. The mortality rate had raised, weaner farms had 5.89% to 13.77%, and finishing farms had 3.71% to 20.88% of mortality (table 2.1).

Table 2.1 – Clinical signs and mortality rate of the 10 weaner farms.

Farm		Clinical signs	Mortality rate
I	Weaner	Arthritis and encephalitis	6.84%
II		Arthritis and encephalitis (positive for *Streptococcus suis* serotype 9)	5.89%
III		Slow growing piglets and encephalitis (positive for *Streptococcus suis* serotype 9)	7.07%
IV		Arthritis and encephalitis (positive for *Streptococcus suis* serotype 9)	8.44%
V		Diarrhea	13.77%
VI		Arthritis and encephalitis (positive for *Streptococcus suis* serotype 9)	9.98%
VII		Slow growing piglets and encephalitis (positive for *Streptococcus suis* serotype 9)	7.05%
VIII		Slow growing piglets, arthritis, and encephalitis (positive for *Streptococcus suis* serotype 9)	8.10%
IX		Slow growing piglets and encephalitis (positive for *Streptococcus suis* serotype 9)	11.33%
X		Slow growing piglets and encephalitis (positive for *Streptococcus suis* serotype 9)	9.91%
XI	Finishing	Slow growing pigs, encephalitis, pneumonia	12.42%
XII		Pleuropneumonia and ileitis	8.17%
XIII		Pneumonia	10.58%
XIV		Slow growing pigs and bloody diarrhea	11.35%
XV		Pneumonia	8.92%
XVI		Vesicular lesions	3.71%
XVII		Pneumonia	10.25%
XVIII		Diarrhea (positive for *Brachyspira spp.*)	12.25%
XIX		Pneumonia	11.26%
XX		Slow growing pigs, encephalitis, and pneumonia	9.18%
XXI		Slow growing pigs, encephalitis, and pneumonia	8.69%
XXII		Slow growing pigs, encephalitis, and pneumonia	20.88%
XXIII		Encephalitis and pneumonia	8.87%
XXIV		Arthritis and pneumonia	9.52%

A total of 873 swine serums were collected between September 2018 until April 2019 from swine with different clinical signs and healthy swine. Total DNA

was extracted from samples using the Wizard SV Genomic DNA Purification System (Promega) according to the manufacturer's instructions.

A duplex real-time PCR was developed to detect PCV3 ORF2 sequence and β-actin gene as an endogenous control. The targets were a 112 base pairs region of the PCV3 ORF2 sequence (Palinski et al., 2016), and a 114 base pair region of the β-actin gene (Duvigneau et al., 2005) (table 2.2). The real-time PCR mixture contained 10ul iTaq Universal Probes Supermix (BioRad), 2.25ul of each PCV3 primers (900nM), 1.25 ul of each β-actin primers (500nM), 2 ul of each probe (100nM), 2 ul of DNA template, and sterile water to bring the final volume to 25 ul. The amplification parameters were set as 95ºC for 5 minutes, followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds. The threshold was established as the default of the CFX96^{TM} real-time PCR detection system (Bio-Rad Laboratories, Inc).

Table 2.2 - Sequences of the primers and probes used for the detection of PCV3 and β-actin by real-time PCR in swine serum samples.

Target	Primer Forward (5'-3')	Primer Reverse (5'-3')	Product
PCV3 ORF2	AGTGCTCCCCATTGAACG	ACACAGCCGTTACTTCAC	112 bp
	6-carboxyfluorescein/ACCCCATGG/Zen/CTCAACACATATGACC/IowaBlack		
β-actin	CTCGATCATGAAGTGCGACGT	GTGATCTCCTTCTGCATCCTGTC	114 bp
	HEX/ATCAGGAAG/Zen/GACCTCTACGCCAACACGG/3IABkFGHex		

Results

The 873 swine serum samples were screened by the duplex real-time PCR assay. PCV3 was present in 36.88% (322/873) of the clinical samples collected (table 2.3). All samples were positive for β-actin. The results showed that 40.93% of weaners and 33.68% of finishing animals were positive for PCV3. The frequency of detection was varied in the farms (figure 2.2).

Table 2.3 – The detection result of clinical samples using real-time PCR at weaner and finishing farms.

Farm		Nº samples	PCV3 +	% positivity
I	Weaner	43	11	25.58
II		37	15	40.54
III		37	13	35.14
IV		44	18	40.91
V		35	15	42.86
VI		32	5	15.63
VII		32	21	65.63
VIII		40	17	42.5
IX		48	32	66.67
X		38	11	28.95
XI	Finishing	40	3	7.5
XII		36	22	61.1
XIII		42	26	61.9
XIV		39	35	89.74
XV		38	4	10.53
XVI		6	2	33.33
XVII		40	4	10
XVIII		36	2	5.56
XIX		40	9	22.5
XX		40	12	30
XXI		39	3	7.69
XXII		11	2	18.18
XXIII		40	32	80
XXIV		40	8	20
TOTAL		**873**	**322**	**36.88**

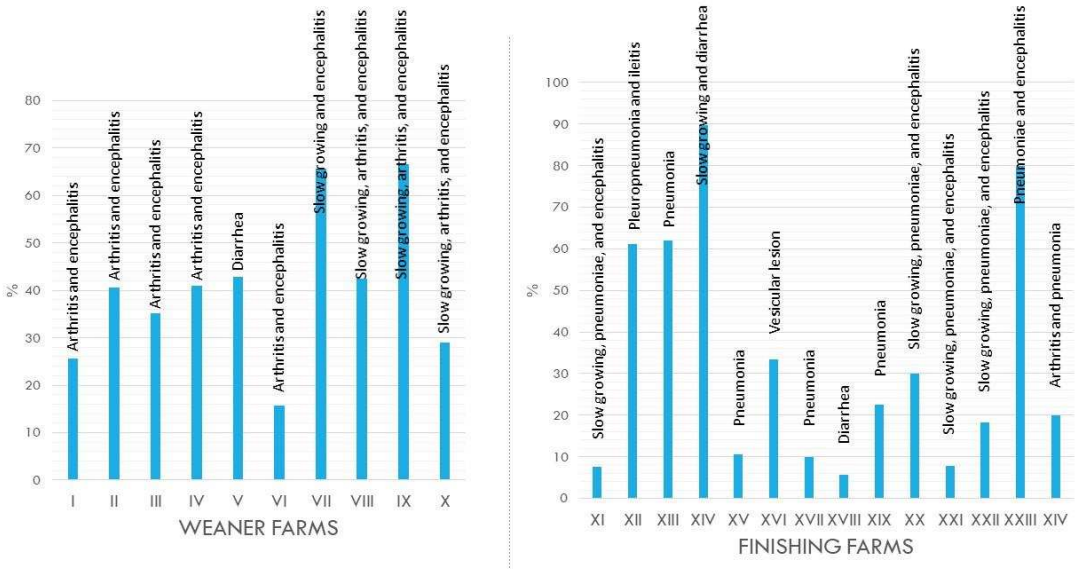

Figure 2.2 - PCV3 frequency of detection in the farms with different clinical signs.

Discussion

PCV3 infection is being related to several health problems as PDNS-like clinical signs and reproductive failure in sow (Palinski et al., 2016), cardiac and multi-systemic inflammation (Phan et al., 2016), the occurrence of stillbirths (Tochetto et al., 2018), digestive and respiratory disease (Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Savic et al., 2020). The association of PCV3 with several clinical presentations suggest that PCV3 could be a potential threat to the swine industry.

In this study, we examined samples from investigated weaner and finishing farms with several health problems from the biggest swine producer mesoregion of Paraná, Brazil. The farms were having problems with a mortality rate above the average [1.5% and 0.6% for weaners and finishing swine, respectively (Embrapa, 2006)]. We analyzed the samples using a duplex real-time PCR assay that amplified the PCV3 ORF2 sequence and β-actin gene simultaneously.

Our results showed that PCV3 DNA was detected in 36.88% of clinical samples collected between 2018 and 2019 from Paraná, Brazil. PCV3 was detected in all farms analyzed. The circulation of PCV3 in Brazilian swine farms was reported previously (Saraiva et al., 2019; Dal Santo et al., 2020; Rodrigues et al., 2020).

In the analyzed samples, weaner swine had a PCV3 prevalence of 40.93% and finishing swine had a PCV3 prevalence of 33.68%. Kwon et al. (2017) found similar results for weaner swine (49.3%) and higher for finishing swine (41.1%). Researchers from other countries reported a lower PCV3-positive rate of 9% to 15.8% for weaning and 3.5% to 10.88% for finisher swine (Hayashi et al., 2018b; Kim et al., 2018b; Klaumann et al., 2018c). The variation in the PCV3 detection could be due to the molecular diagnostic test used, the epidemic situation of the region, or the health status of the farms.

In Brazil, Assao et al. (2021 unpublished work) studied swine farms in Minas Gerais state and observed a PCV3 prevalence higher in finishing swine (41.54%) than in weaner (35.29%) swine. The higher PCV3 prevalence in weaner swine from Paraná farms compared to Minas Gerais farms could be associated with stress. In Paraná farms, each farm is responsible for growing pigs of a certain

age, so the piglets are transported from the farm specialized in piglet production to the weaner farm; and the farms analyzed in Minas Gerais were farrow-to-finishing operations, so they piglets remain on the same farm for the entire lifetime. It is known that stress may impair immune functions (Gimsa et al., 2018), which could be provoking the development of PCV3 infection in these animals.

The three farms with diarrhea problems were positive for PCV3. The association between PCV3 and diarrhea is still being discussed. Saporiti et al. (2019) results did not identify the association of PCV3 with the occurrence of diarrhea. However, several studies have suggested that the PCV3 infection is associated with digestive disease (Phan et al., 2016; Zhai et al., 2017; Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Guo et al., 2020; Savic et al., 2020; Zhang et al., 2020). Zhang et al. (2020) induced diarrhea in piglets performing oral inoculation with PCV3 sole-positive intestinal contents and described the pathogenicity induced by PCV3 associated with swine diarrhea. Also, PCV3 DNA had already been detected in swine feces (Zhai et al., 2017). Considering all that, PCV3 infection might cause diarrhea, but more studies should be conducted to elucidate this PCV3's pathogenic mechanisms causing diarrhea.

Eight weaner farms were positive for PCV3 and *Streptococcus suis* serotype 9 (previously investigated, data not shown). The presence of PCV3 and *Streptococcus suis* serotype 9 was previously reported (Hayashi et al., 2018b; Kedkovid et al., 2018b). This is the first time that PCV3 DNA was identified in swine with vesicular lesions in the legs.

PCV3 DNA was identified in 24 farms with different health problems, therefore it is not possible to claim that PCV3 was the only one responsible for causing the health problems in these farms. Considering that the detection of PCV3 and other pathogens are frequently reported, and the fact that PCV3 has been identified in asymptomatic animals, we hypothesize that PCV3 might be the primary pathogen or an opportunistic pathogen. This would agree with the suggestions that: (A) PCV3 could cause inflammatory lesions, lymphocytic dysplasia, and necrosis, leading to disruption of the immune system and consequently increased the susceptibility to other infections (H. Jiang et al., 2019); (B) and that PCV3 could be causing immunosuppressive activity like

PCV2, compromising the swine immune system and consequently facilitating concomitant infection with other pathogens (Dei Giudici et al., 2020). It is known that co-infection of viruses can complicate the infection status and makes it more difficult to control diseases (N. Chen et al., 2019), which would explain the farms were having health problems for quite some time. More studies are necessary to understand more about PCV3 infection and other pathogens to prevent and control future diseases.

Conclusion

In summary, this study identified PCV3 in swine farms presenting different clinical signs. We suggest that PCV3 in association with other pathogens, might play a role in the various clinical signs observed in these farms located in Paraná, Brazil.

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6. EXPRESSION AND PARTIAL PURIFICATION OF RECOMBINANT *PORCINE CIRCOVIRUS 3* CAPSID PROTEIN IN *ESCHERICHIA COLI*

Abstract

The discovery of a new circovirus infecting swine has raised concern once its pathogenesis is not fully understood yet. The capsid protein of *Porcine circovirus 3* (PCV3) has been widely used for diagnosis because it is highly conserved among PCV3 strains. In this study, we optimized the capsid gene of PCV3, inserted it into the pET28a expression vector, and used *Escherichia coli* Rosetta in auto-induction media to achieve a high level of expression. A two-steps purification was performed using anion-exchange and size-exclusion chromatography. The results showed an efficient expression using a prokaryotic expression system and partial purification of PCV3 capsid protein.

Introduction

In 2016, a new circovirus infecting swine was discovered and named *Porcine circovirus 3* (PCV3) (Palinski et al., 2016; Phan et al., 2016). PCV3 has an ambisense genome organization, with 1999-2001 nucleotides, and encodes three open reading frames (ORFs). ORF1 encodes an amino acid replicase, ORF2 encodes an amino acid capsid (Cap), and ORF3 encodes an amino acid protein with unknown function (Palinski et al., 2016; Phan et al., 2016).

Since the first report, PCV3 has been detected in many countries around the world, including some South American countries as Brazil (Tochetto et al., 2018; Saraiva et al., 2019; Rodrigues et al., 2020; Varela et al., 2020; Assao et al., 2021 unpublished work), Colombia (Vargas-Bermudez et al., 2019b), and Argentina (Serena et al., 2020).

PCV3 is being associated with different clinical conditions as PDNS-like clinical signs and reproductive failure in sow (Palinski et al., 2016), multi-systemic inflammation (Phan et al., 2016), the occurrence of stillbirths (Tochetto et al., 2018), myocarditis (Phan et al., 2016; Arruda et al., 2019), digestive and respiratory disease (Xu et al., 2018; Han et al., 2019; Qi et al., 2019; Savic et al., 2020), neurological disorders (Phan et al., 2016; Hayashi et al., 2018b; Zheng et al., 2020), and even in cases of apparently healthy animals (Wen et al., 2017; Klaumann et al., 2019). As PCV3 was discovered recently, its pathogenicity is not clear yet and further studies are needed to better understanding of PCV3.

The recent success of PCV3 isolation and the confirmation of the PCV3 ability to self-assemble into virus-like particles will help future studies to elucidate the pathogenicity, potential treatments, and diagnostics (Mora-Díaz et al., 2020; Oh and Chae, 2020; Wang et al., 2020). In biochemical and structural studies, the expression of recombinant proteins is fundamental (Muzika et al., 2018). This knowledge could help understand PCV3 infection. To improve recombinant protein expression yield in bacteria, auto-induction media are being used (Muzika et al., 2018). Auto induction media allows bacterial growth through the balance between medium components and different carbon sources (glucose, glycerol, and lactose); when glucose depletion happens, the lactose induces protein expression (Muzika et al., 2018). The method is based on the function of *lac* promoter and the target protein is produced automatically without the need to monitor cell density or inducer addition at the proper time (Fathi-Roudsari et al., 2018).

In this work, we synthesized, expressed, and partially the purified recombinant PCV3 Cap protein in *Escherichia coli* Rosetta expression system using auto-induction media for future biotechnological use to a better understanding of PCV3.

Material and methods

We optimized and synthesized the PCV3 *cap* gene without ten amino acids that are part of the nuclear localization signal (NLS) sequence located at the N-terminal end, in order to generate a 208 amino acids truncated, recombinant PCV3 Cap protein.

The *cap* synthesized was cloned into pET28a and pET28a-SUMO (figure 3.1). The recombinant plasmids were transformed into *E. coli* BL21 and *E. coli* Rosetta (DE3) (Novagen), then grown in Terrific Broth (TB) medium (12 g tryptone, 24 g yeast extract, 0.017M of KH2PO4, 0.072M of K2HPO4, 12 ml glycerol, per L) with kanamycin (50ug/ml) and chloramphenicol (25ug/ml) overnight at 37ºC with shaking.

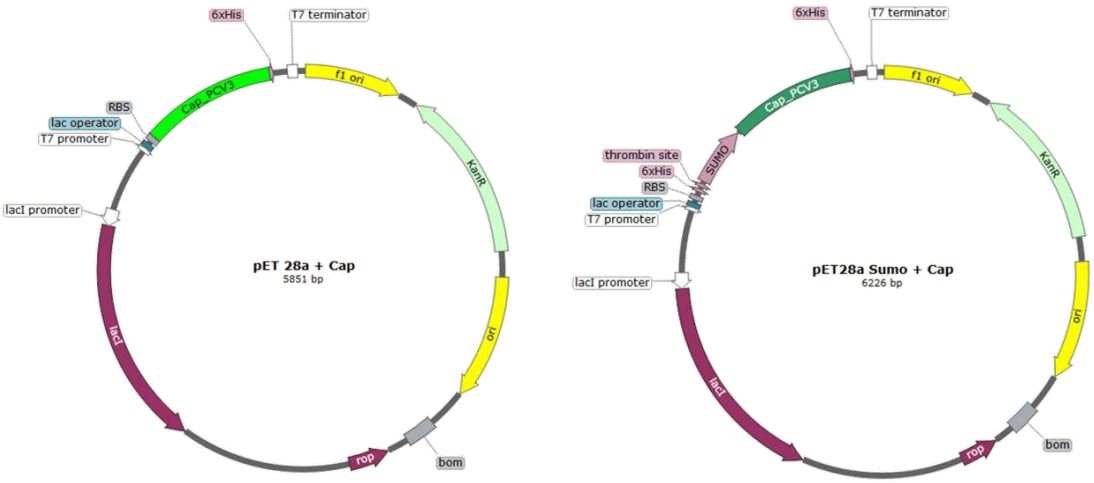

Figure 3.1- Schematic illustration of the construction of the plasmid pET28a and pET28a SUMO with recombinant PCV3 capsid protein.

Protein expression was induced using TB medium containing α-lactose, ZYM-5052 medium and TB medium containing IPTG. We tested different growth characteristics. The culture was diluted 1:10 in TB medium with kanamycin (50ug/ml) and chloramphenicol (25ug/ml). Different α-lactose concentrations (0.5, 1, 2, and 5g/L), IPTG concentrations (0.1 mM, 0.5 mM, and 1 mM), temperatures (15, 20, 25, 30, and 37ºC), and hours of induction (2, 4, 6, 8, 24, 48, 72 hours) were evaluated. Cultured samples were collected and centrifuged at 4000 rpm for 15 min at 4ºC. The supernatant was removed, and the cell pellets were resuspended in Lysis Buffer (60mM NaCl, 50mM $NaHCO_3$, pH:7.3) and sonicated or submitted to a high-pressure cell disruptor. After this, soluble and

insoluble cell fractions were separated by centrifugation at 4500 rpm for 20 min at 4ºC. The insoluble cell fraction was resuspended in Lysis buffer with 8M urea.

To evaluate the expression of the recombinant PCV3 capsid protein, SDS-PAGE and Western-blot analysis of total cell protein, soluble and insoluble protein fractions were performed. Samples were resuspended in an equal volume of sample buffer (1M Tris HCl, SDS, 0.1% Bromophenol blue, glycerol, and 14.3M β-mercaptoethanol) and heated at 100ºC for 10 min. Proteins were separated on 15% polyacrylamide gel, electrophoresis was conducted, and subsequently, SDS-PAGE gels were stained with Coomassie blue or transferred by electroblotting onto nitrocellulose membranes to performed Western-blot. The membranes were treated with a blocking solution (1x PBS-T with 5% BSA) overnight, followed by incubation with 6x-His Tag mouse monoclonal antibody (Invitrogen) (diluted 1:2000) for 1.5 hours. After this time, the membrane was washed with PBS and incubated with antibody anti-mouse (diluted 1:2000) for 1.5 hours. Membranes were washed and soaked in 1-Step Ultra TMB-Blotting Solution (Thermo Fisher Scientifc Inc.) for color development.

We tried to purify the recombinant PCV3 Cap protein using HisTrap FF crude, HiTrap DEAE FF, and Superdex 200 increase 10/300 GL. We used an automated FLPC system (AKTA, GE Healthcare Life Sciences, USA).

For HisTrap FF crude, we loaded the soluble fraction of the recombinant Cap protein expression into the column. After the column had been washed with binding buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20-40 mM Imidazole, pH 7.4). The recombinant Cap protein was eluted using a gradient of 10%, 30%, and 100% of elution buffer (20 mM NaH_2PO_4, 500 mM NaCl, 300-500 mM Imidazole, pH 7.4). We also tried adding 8M Urea in the buffers as recommended by the manual for inclusion bodies if the recombinant Cap protein was aggregating. The results were observed using SDS-PAGE gels stained with Coomassie blue.

The first step, of the two-steps purification, was performed using the HiTrap DEAE FF column, a weak anion column commonly used in ion exchange. We loaded the soluble fraction of the recombinant Cap protein expression into the column. After the column had been washed with start buffer (20mM Tris, pH 7.6-8.6), the recombinant protein was eluted using 10%, 30%, and 100% of

elution buffer (start buffer + 1M NaCl). The results were observed using SDS-PAGE gels stained with Coomassie blue. Then, the product of 30% of elution buffer was loaded into the column Superdex 200 increase 10/300 GL, which is a column for size exclusion chromatography, and it was used as the second step of purification. Using the buffer (50mM phosphate in 0.15M NaCl, pH 7.2), we collected samples of the peaks created. The results were observed using SDS-PAGE gels stained with Coomassie blue or transferred by electroblotting onto nitrocellulose membranes to performed Western-blot.

Results

The results of SDS-PAGE and Western-blot showed that the recombinant PCV3 Cap protein was successfully expressed using the *E. coli* Rosetta expression system and pET28a vector, and it was present in soluble and insoluble fractions (figure 3.2). However, the recombinant PCV3 Cap protein presented a better result in the soluble fraction. We did not have success using the *E. coli* BL21 expression system.

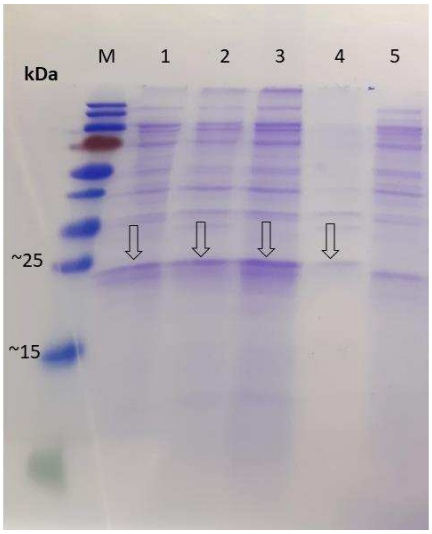

Figure 3.2 - SDS-PAGE of Cap PCV3 expression after 48 hours of induction with TB media with α-lactose. M – Prestained protein marker, 1 - Soluble fraction collected after 24h of induction, 2 - Insoluble fraction collected after 24h of induction, 3 - Soluble fraction collected after 48h of induction, 4 - Insoluble fraction collected after 48h of induction, 5 - negative control. The arrow indicates the recombinant PCV3 Cap protein (~24 kDa).

After the success of the expression, we tried to improve the expression of the recombinant PCV3 Cap protein using the plasmids pET28a and pET28a-SUMO, but the presence of SUMO did not increase the expression of the protein. Besides that, we tried different inducers (IPTG and lactose). We did not have success in the expression using this IPTG. We tried different auto-induction medium, and between TB media containing α-lactose and ZYM-5050 media, the results were similar.

Among the different lactose concentration (0.5, 1, 2, and 5g/L), temperatures (15, 20, 25, 30, and 37ºC), and time (2, 4, 6, 8, 24, 48, 72 hours) the best result was obtained using TB supplemented with 2g/L of α-lactose,

incubated at 20ºC, and 48 hours of induction with shaking, as indicated in line 2 of figure 3.3.

We did not have success purifying the recombinant protein using HisTrap FF crude. So we decided to try the two-steps purification. For the first step of the two-steps purification, we used the HiTrap DEAE FF column, the soluble fraction of the induction was loaded into the column and we eluted the protein with a gradient of elution buffer. We analyzed the fractions collected during the elution of the recombinant PCV3 Cap protein using SDS-phage. The results showed that the recombinant protein was being eluted with 30% of elution buffer (figure 3.4).

For the second step of purification using the Superdex 200 increase 10/300 GL column, the fraction of 30% of elution buffer collected in the first step of the purification, was loaded into the column. We collected several fractions of this step of the purification and analyzed using SDS-phage. And the results showed that the recombinant PCV3 Cap protein was being eluted with other proteins (figure 3.5).

To confirm that the recombinant Cap protein was the protein with approximately 24kDa we performed a Western-blot analysis using the anti-6x His monoclonal antibody from mouse. And the results confirm that it was the recombinant PCV3 Cap protein (figure 3.6).

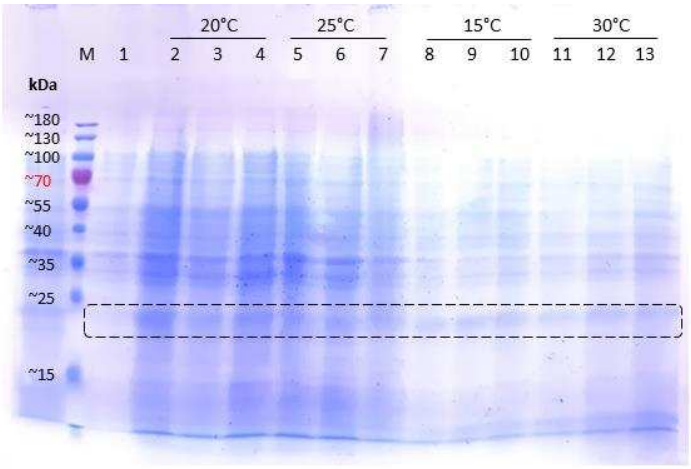

Figure 3.3 - SDS-PAGE of the recombinant PCV3 Cap protein expression with different temperatures and lactose concentration. M – Prestained protein marker, 1- negative control, 2- 2g lactose (20ºC), 3- 1g lactose (20ºC), 4- 0.5g lactose (20ºC); 5- 2g lactose (25ºC), 6- 1g lactose (25ºC), 7- 0.5g lactose (25ºC), 8- 2g lactose (15ºC), 9- 1g lactose (15ºC), 10- 0.5g lactose (15ºC), 11- 2g lactose (30ºC), 12- 1g lactose (30ºC), 13- 0.5g lactose (30ºC). The dashed line indicates the recombinant protein (~24 kDa).

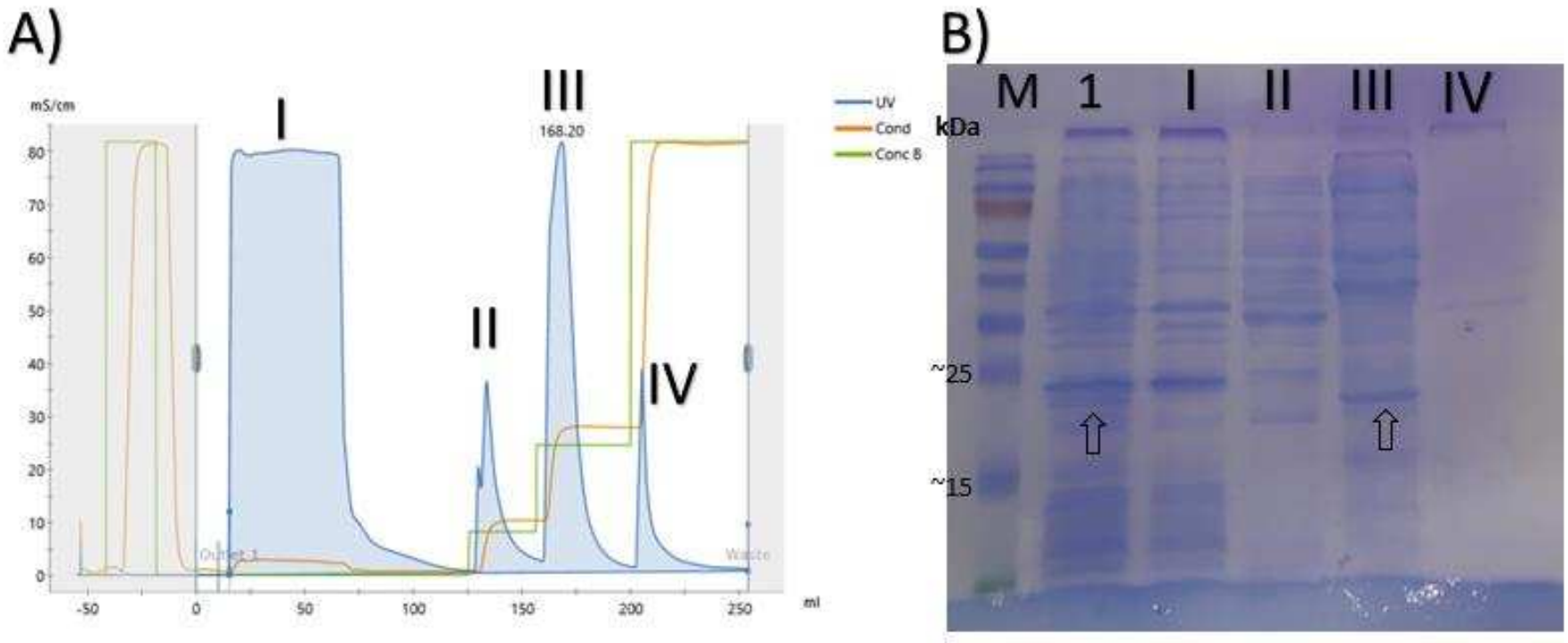

Figure 3.4 – A) First-step of the recombinant PCV3 Cap protein purification using HiTrap DEAE FF and the soluble fraction of 48 hours of induction of the protein in TB media with α-lactose at 20℃. I – unbound molecules eluted, II - elution with 10% of elution buffer, III - elution with 30% elution buffer, IV - elution with 100% of elution buffer. B) SDS-PAGE of the fractions collected during the purification: M – Prestained protein marker, 1 - total fraction, I - unbound molecules eluted, II - elution with 10% of elution buffer, III - elution with 30% of elution buffer, IV - elution with 100% of elution buffer. The arrow indicates the recombinant PCV3 Cap protein (~24 kDa).

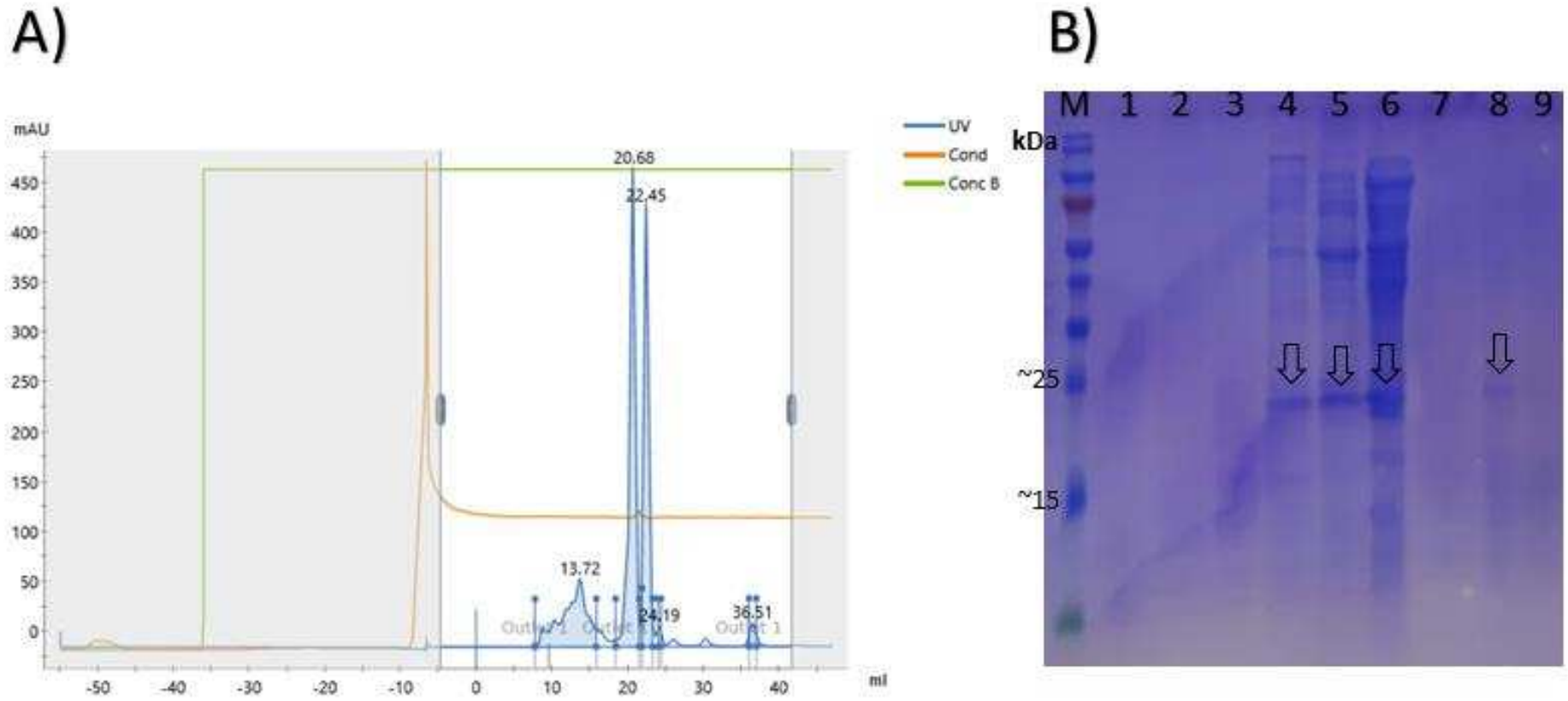

Figure 3.5 - A) Second-step of the recombinant PCV3 Cap protein purification using Superdex 200 increase 10/300 GL column. The column was loaded with 30% elution buffer of the first step of the purification with anion-exchange column. B) SDS-PAGE of the eluted fractions of the second step of the purification. M – Prestained protein marker, 1-9 fractions collected during the elution of the protein. The arrow indicates the recombinant protein (~24kDa).

Figure 3.6 - Western-blot of recombinant PCV3 Cap protein purified after two steps of purification. M – Prestained Protein marker, 1 - negative control, 2 – the product of the recombinant protein two-steps purification.

Discussion

Since the first reports in 2016, PCV3 was identified in many countries, including Brazil. The oldest PCV3 partial capsid sequence described was found in Brazil in 1967 (Rodrigues et al., 2020). It is important to investigate the current status of PCV3 infection in Brazil. However, PCV3 pathogenicity is not fully understood, and further studies are needed to better understand PCV3. Therefore, in this study, we expressed the recombinant PCV3 Cap protein using an auto-induction media to be used in future biotechnological use.

In this study, we used the *E. coli* expression system, which is a popular organism for recombinant protein production. Because it has a well-characterized genetic background, is relatively simple, has a low cost, and fast growth rate (Z. Li et al., 2011; Fathi-Roudsari et al., 2018; Wang et al., 2020). Cap PCV3 is frequently reported to be expressed using *E. coli* BL 21 (DE3) (Deng et al., 2018; X. Li et al., 2018a; Wang et al., 2020). However, we did not have success using *E. coli* BL 21. We used *E. coli Rosetta* (DE3) strains that are used to enhance the expression of proteins that contain codons rarely used in the *E. coli* expression system.

The decision of excluded some amino acids at the N-terminal sequence was based on the knowledge that the NLS domain is rich in arginine residues encoded by low-usage codons in *E. coli* that impede a foreign gene expression (S. Zhang et al., 2019; Wang et al., 2020). The N-terminal of the PCV3 capsid protein has 22 arginines among the top 40 amino acids (Liu et al., 2020). And removing the NLS domain has also been utilized to improve the expression efficiency and stability of the expressed protein (Wang et al., 2020). A recent study demonstrated that N-terminal 33 amino acids could be deleted without affecting the antigenicity of the capsid protein (Liu et al., 2020). Therefore, in this work a part of the NLS domain was removed, optimized, and we demonstrated that the deletion of the first ten amino acids was enough to the *E. coli Rosetta* expression system to express the recombinant PCV3 Cap protein with success.

Auto-induction medium contains a mixture of carbon and energy sources (glucose, lactose, and glycerol) that are used during different stages of cell growth and recombinant protein production (Blommel et al., 2007). Auto-induction media

have the advantages that make protein expression easier and more reproducible, as there is no need to monitor the culture OD_{600} or inducer addition at a proper time and it places the transition from the un-induced to an induced state under metabolic control of the expression host, which minimizes the required handling of cultures (Blommel et al., 2007; Muzika et al., 2018).

Considering that *E. coli* Rosetta supports the expression of proteins using lactose-inducible promoter-based systems (Muzika et al., 2018), autoinduction media was optimized for the recombinant Cap protein expression with success. Due to the complexity of producing ZYM-5050 media, we choose to use TB media containing α-lactose. The results of SDS-PAGE and Western-blot showed a band at the right molecular size (~24kDa).

We did not have success purifying the recombinant protein using HisTrap FF crude. As we were having problems with the purification, we tried pET28a-SUMO once SUMO can lead to increased expression levels and enhanced solubility of the recombinant protein. Our results demonstrated that the presence of Sumo did not increase the expression nor enhanced solubility of the recombinant protein.

After that, we decided to try the two-steps purification. PCV3 purification using a two-step chromatography technology was previously described (Wang et al., 2020). The first step was using an anion-exchange chromatography, and the second step was using size-exclusion chromatography. The first step of the purification showed that the recombinant PCV3 Cap protein was being eluted with 30% of elution buffer. The product of 30% of elution buffer was loaded into the size-exclusion column, and we observed some peaks. The results showed that the recombinant PCV3 Cap protein and some proteins of different molecular weights were being eluted together even after the two-step purification. The presence of impurities with the recombinant PCV3 Cap protein after the two-step purification was also observed by other researchers (Wang et al., 2020). Other researchers purified Cap using Ni2+NTA affinity chromatography (Deng et al., 2018; X. Li et al., 2018a; S. Zhang et al., 2019), however, we did not have success using this method.

Although we were able to express and partially purified the recombinant PCV3 Cap protein, more studies should be performed to improve the purification protocol of Cap protein. Once Cap protein purified can be used to develop diagnostic tests as ELISA, to produce antibodies, and can even be used as a vaccine candidate.

Conclusion

In conclusion, this work successfully optimized the auto-induction medium to express the recombinant PCV3 Cap protein. In addition, we partially purified the recombinant protein using a two-step purification.

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7. FINAL CONCLUSION

- There is a genetic diversity among PCV3 Brazilian strains with at least two main lineages circulating in Brazilian swine herds;
- PCV3 have a higher prevalence in fetuses from reproductive failure and horizontal transmission might be a transmission route;
- PCV3 might be a opportunistic or a primary pathogen;
- More studies are necessary to purify the cap protein for future use.

8. CEUA

CERTIFICADO

A Comissão de Ética no Uso de Animais - CEUA/UFV certifica que o processo nº 13/2018, intitulado **"Expressão heteróloga da proteína do capsídeo de *Porcine circovirus* 3 (PVC3): aplicação em ensaios de ELISA, candidatos vacinais e associação com falhas reprodutivas"**, coordenado pelo professor Abelardo Silva Júnior do Departamento de Veterinária, está de acordo com a Legislação vigente (Lei Nº 11.794, de 08 de outubro de 2008), as Resoluções Normativas editadas pelo CONCEA/MCTI, a DBCA (Diretriz Brasileira de Prática para o Cuidado e a Utilização de Animais para Fins Científicos e Didáticos) e as Diretrizes da Prática de Eutanásia preconizadas pelo CONCEA/MCTI, portanto sendo aprovado por esta Comissão em 14/05/2018, com validade de 12 meses.

CERTIFICATE

The Ethic Committee in Animal Use/UFV certify that the process number 13/2018, named **"Heterologous expression of porcine circovirus 3 (PVC3) capsid protein: application in ELISA assays, vaccine candidates and association with reproductive failures"**, is in agreement with the an actual Brazilian legislation (Lei Nº 11.794, 2008), Normative Resolutions edited by CONCEA/MCTI, the DBCA (Brazilian Practice Guideline for the Care and Use of Animals for Scientific Purposes and Teaching) and the Guidelines of Practice the Euthanasia recommended by CONCEA/MCTI therefore being approved by the Committee on May 14, 2018 valid for 12 months.

Profª. Átima Clemente Alves Zuanon
Presidente
Comissão de Ética no Uso de Animais – CEUA/UFV