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Vaccination strategies to curb environmental spread of Porcine Circovirus 2

D.F. Silva[1,2], V.H. Silva[2], L.M. Barros[2], T.S. Oliveira[2], C.I.S. Macedo[2], R.G. Mesquita[2], A.R. Silvestrini[3], I.R. Silvestrini[3], S. Ferraz[2], A.M.M.G. Castro[4#]

[1]School of Nursing at the University of São Paulo-EEUSP, São Paulo, Brazil
[2,4] Universidade Paulista -UNIP, Stricto Sensu Postgraduate Program in Environmental and Experimental Pathology, São Paulo, Brazil
[3] United Metropolitan Colleges University Center of São Paulo, Brazil

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Abstract

Integrated farming is an established agricultural practice aimed at increasing productivity per unit area by utilizing animal manure. However, there has been a growing concern regarding swine manure's potential as a disseminator of pathogens in the environment. Among these pathogens is Porcine Circovirus 2 (PCV2), which can persist in the environment for extended periods. The objective of this study is to demonstrate the impact of introducing two PCV2 vaccination protocols on PCV2 load in faecal samples and the surrounding environment. To achieve this, fifty-seven pigs aged 21 d were divided into three groups (A, B, and Control group) and received intramuscular injections of two different PCV2 vaccines as per the manufacturer's instructions. Faecal and environmental samples were collected using polyester swabs and tested for PCV2 using SYBR green quantitative real-time PCR (qPCR). The introduction of vaccination in a PCV2-positive herd led to a reduction in viral load in both faecal samples and the surrounding environment.

Keywords: environment, Porcine Circovirus 2, pig farming, swine, viral elimination
[#]Corresponding author: alessandra.castro8@docente.unip.br

Introduction

Integrated farming of fish and livestock has been a longstanding practice; the goal is to enhance production per unit area by utilizing animal manure instead of costly inputs such as fish feed and industrial fertilizers. This approach also seeks to mitigate the environmental impact caused by untreated livestock waste. Consequently, by recycling and decomposing organic waste through this system, two primary issues are addressed: preventing the disposal of untreated animal waste into the environment and yielding economic benefits (Kumaresan *et al*., 2009; Bhatt *et al*., 2011).

The pollutant potential of swine manure has been associated with a high charge of phosphorus, nitrogen, organic matter, and heavy metals. However, in recent years, there has been a growing concern that swine manure is a disseminator of pathogens in the environment. Pathogens that remain stable in faeces after dispersal into the environment, such as Swine Adenovirus (PAdV), Norovirus, Sapovirus, Rotavirus, Swine Parvovirus, and Hepatitis A and E Virus, have received special attention (Costantini *et al*., 2007; Viancelli *et al*., 2011; Bθtner & Belsham, 2012; Fongaro *et al*., 2015; Gentry-Shields *et al*., 2015).

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Porcine circovirus 2 (PCV2) belongs to the family *Circoviridae*, genus *Circovirus*, and is a small, non-enveloped virus. It is associated with various disease conditions collectively known as porcine circovirus-associated diseases (PCVAD). The two major genotypes, PCV2a and PCV2b, have a global distribution, highlighting their important role in transmission and persistence in the environment (Opriessnig *et al*., 2007). The virus is eliminated through various pathways, including faeces, where animals with clinical signs may reach a viral load of up to 10^9 copies of viral DNA per gram of faeces. Furthermore, PCV2 is excreted in faeces by animals at different production stages. The detection of PCV2 in groundwater emphasises the importance of faecal matter in environmental dissemination (McIntosh *et al*., 2008; Segalés, 2012).

The utilization of PCV2 vaccines has been crucial in mitigating the effects of PCV2 infection in pig herds by reducing clinical signs and improving performance. Moreover, both experimental and field studies have demonstrated that vaccination substantially lowers the viral load in the faeces of infected animals (Horlen *et al*., 2008; Fraile *et al*., 2012; Jeong *et al*., 2015; Czyżewska-Dors *et al*., 2018). Nevertheless, there is a lack of studies demonstrating a reduction in PCV2 load in faeces and the environment following the introduction of PCV2 vaccines. This study aims to elucidate the impact of various PCV2 vaccination protocols on PCV2 load in faecal matter and the surrounding environment.

Materials and methods

This study was conducted in a PCV2-positive pig herd that had never used a PCV2 vaccination protocol. All procedures followed the ethical standards of the institution or practice where the studies took place. The Institutional Committee for Animal Care and Use at the Veterinary School of the Centro Universitário das Faculdades Metropolitanas Unidas in Brazil approved the studies under approval number CE 093/17. A randomized controlled experiment was conducted to assess the effect of the intervention, specifically the PCV2 vaccine (Horlen *et al*., 2008). A summary of the experimental design is shown in Figure 1.

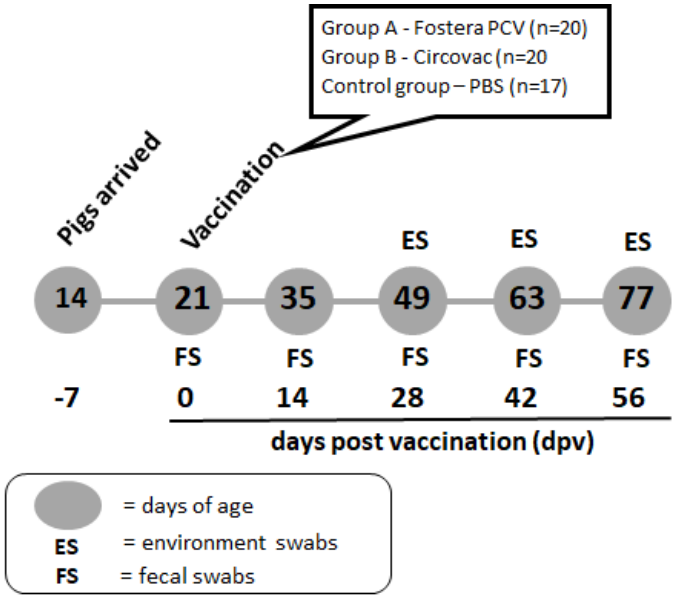

Figure 1 Summary of experimental design to elucidate the impact of PCV2 vaccination protocols on PCV2 load in faecal matter and the surrounding environment

Fifty-seven piglets from 20 sows were distributed into two vaccinated groups and one control group (17 ± 3 pigs/group). To minimize sow variation, approximately four 14-day-old piglets were randomly selected from each sow and allocated to each group using the random number generation function in Excel (Microsoft Corporation, Redmond, WA, USA).

Vaccination Protocols

- Group A: Received 2.0 mL of the Fostera PCV vaccine (Zoetis) intramuscularly at 21 d old, with a second dose at 35 d old

- Group B: Received 2.0 mL of the Circovac vaccine (Merial) intramuscularly at 21 d old, with no booster dose
- Control Group: Received 2.0 mL of phosphate-buffered saline (PBS) intramuscularly at 21 d of age

Piglets from the vaccinated groups (A and B) were randomly distributed into three pens (approximately 15/pen), whereas piglets from the control group remained in a pen with other animals from the herd. The general and feeding management of the pig herds was maintained constant throughout the experimental period up to 56 d post-vaccination (dpv).

Faecal Samples were collected every 14 d, from 0 dpv to 56 dpv, from each pig using polyester swabs. Sixty environmental samples were collected to investigate the presence of viral nucleic acids in the environment from day 28 to 56 dpv. Swabs were run horizontally, vertically, and diagonally over 10 cm^2 swab sites in random places (five per pen, totalling four pens per time point). The faecal and environmental swabs were stored in 2 mL microtubes with 1 mL of sterile saline solution and kept at -20°C until DNA extraction. DNA was extracted using the QIAamp DNA Mini Kit, following the manufacturer's instructions.

Genomic DNA copy numbers of PCV2 were quantified using SYBR Green quantitative real-time polymerase chain reaction (qPCR). The primer pair, SybPCV2F (ATA ACC CAG CCC TTC TCC TAC C) and SybPCV2R (GGC CTA CGT GGT CTA CAT TTC C) was used to amplify a 145-base pair (bp) segment of the open reading frame 2 (ORF2) of PCV 2 (Yang *et al*., 2007). The qPCR was set up with 12 µL of Maxima® SYBR® Green/ ROX qPCR MasterMix (Fermentas®, Canada), 4 µL of extracted DNA, 0.2 µM of each primer (SybPCV2F and SybPCV2R), and sterile water to 25 µL. The qPCR conditions were 10 min at 95 °C, 1 min at 60 °C, and 40 cycles of 15 s at 95 °C and 1 min at 60 °C, followed by melting curve analysis on a QuantiStudio3 (Applied Biosystems, USA). PCV2 cycle-threshold (C_T) values were converted into copy number per swab using standard curve data. A positive control (PCV2-positive sample) and a negative control (water) were included in every group of samples tested.

The analytical sensitivity of qPCR was established at 12 copies of DNA per microliter for environmental samples and 10 copies of DNA per microliter for faecal samples. Any samples that did not produce a signal by reaching a C_T value of 38 were classified as negative, indicating that the number of DNA copies was below the analytical sensitivity threshold (number of DNA copies < analytical sensitivity).

Each DNA sample were evaluated using an internal control with qualitative PCR, confirming the lack of inhibitors that might interfere with PCR or any problems during DNA extraction. The primer pairs AC1 (TGA GAC CTT CAC GCC) and AC2 (ATC TGC AAG GTG GAC) were used to amplify an 850-pb fragment from the *β-actin* gene for this assessment (Hui *et al*., 2004). The reactions were carried out in a final volume of 25 µL, comprising 2.5 µL of extracted DNA, 12.5 µL of DreamTaq™ Green PCR Master Mix (2X) (Fermentas, USA), 0.2 µM of each primer (AC1 and AC2) and DNAse-free water to final volume. Sterile ultrapure water was used as the negative control for the reaction and extraction processes. The PCR conditions using a Peltier thermal cycler (MJ Research) were: initial denaturation at 94 °C for 3 min, 45 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 10 s, extension at 72 °C for 10 s, and a final extension step at 72 °C for 10 min.

Statistical analysis was performed using IBM SPSS v.23 software. Analysis of variance (ANOVA) was used for cross-sectional assessment of PCV2 copy numbers per swab. Real-time PCR results (copies per swab) were $\log_{10}$ transformed prior to statistical analysis. The significance level was $P < 0.05$, followed by pair-wise testing using Tukey–Kramer adjustments to identify the groups that were different.

Results and Discussion

The results of this study clearly demonstrate that in a herd without a history of using a PCV2 vaccination protocol, vaccinating against PCV2 substantially reduced the viral load both in faeces and in the surrounding environment. PCV2 is widely distributed in the global pig population, with a high proportion of infected animals exhibiting clinical signs, particularly on farms without vaccination protocols (Opriessnig *et al*., 2007; Segalés, 2012). Although the farm where the study was conducted had animals showing clinical signs of PCVAD, none of the animals in the control group exhibited such conditions during the study period.

The commercial PCV2 vaccine is widely recognized for its efficacy in controlling PCV2 infection in pigs by reducing PCV2 viremia and lymphoid lesions in field conditions. Subsequent studies have also demonstrated that these vaccines improve productivity by lowering mortality rates in pig herds (Jeong *et al*., 2015; Silva *et al*., 2016; Czyżewska-Dors *et al*., 2018; Karuppannan & Opriessnig, 2017). In the current study, although no clinical signs or productivity parameters were specifically analysed, there was an observed enhancement in animal performance among vaccinated animals. This improvement was evidenced by a higher weight gain between 28 and 56 days post-vaccination (dpv), although detailed data is not presented here (data not shown).

The internal amplification control (IAC) C is a non-target DNA sequence that exists within the same sample tube and is co-amplified with the target sequence. In PCR procedures without an IAC, a negative result might indicate the absence of the target sequence in the reaction. However, it could also signify inhibition of the reaction due to the presence of inhibitory substances in the sample matrix or errors in nucleic acid extraction. PCR targeting constitutive cell genes such as *β-actin*, *β-globin*, and *albumin* has been used. A few components like bile salts and complex polysaccharides in faeces have been recognized as PCR inhibitors. This underscores the importance of employing an IAC to enhance test sensitivity and accuracy, thus decreasing the probability of false negatives. In our study, all samples yielded positive results for the *β-actin* gene, confirming the absence of inhibitors and extraction errors (Horlen *et al*., 2008; Kompalic-Cristo *et al*., 2007; Hoorfar *et al*., 2004).

The PCR assay has been used to detect PCV2 in different clinical samples and quantitative PCR assays have facilitated the linking of PCVAD to the quantity of PCV2 load in tissues and blood (Opriessnig *et al*., 2007). In the present study, the SYBR® Green-based detection system was used to quantify the PCV2 DNA in faecal samples and in the environment. However, this system requires dissociation curve analysis (Yang *et al*., 2007). In the present study, the qPCR analytical sensitivity was 12 and 10 copies of DNA/µL for environment and faecal swabs, respectively, with a *Ct* cutoff of 38.

In the present study, the viral load in faeces was substantially lower in vaccinated groups than in the control group, regardless of the vaccine used. The prevalence and mean Log_{10} value of viral load in faeces in the study are shown in Table 1. The mean Log_{10} value of the viral load in faeces of the pigs from control and vaccinated groups (A and B) was 2.56 and 1.6, respectively. Within the vaccinated groups (A and B), the mean Log_{10} value was 1.64 and 1.62 for groups A and B, respectively. A reduction in viral load between the vaccinated and control groups was observed (P <0,05; data not shown) from 28 to 56 dpv. Moreover, the vaccine led to a decrease in the number of animals with detectable viral DNA in their faeces among vaccinated groups, i.e., 08/20 and 09/20 at 56 dpv compared to 16/17 in the control group.

Previous experimental and field studies showed that the faeces represent a crucial route of viral elimination, with viral DNA loads reaching up to 10^9 copies of viral DNA/g of faeces in animals displaying clinical signs. Additionally, PCV2 is shed in faeces by animals across various production stages, and this amount correlates with the systemic or tissue PCV2 viral load. The PCV2 shed in faeces contributes to the dissemination of the virus in the herds, since the pig infections are dependent on exposure or an infectious dose or both (Segalés *et al*., 2005; McIntosh *et al*., 2008).

Table 1 Number of animals positive for PCV2 DNA per group and viral load (Log_{10}) in faeces (mean value ± standard deviation)

Group	Vaccine	Animals	0 dpv	14 dpv	28 dpv	42 dpv	56 dpv
A	Fostera PCV	20	18/20 (2.66 ± 0.73)[a]	20/20 (2.3 ± 0.74)[a]	16/20 (1.23 ± 1.15)[a,b]	12/20 (1.11 ± 1.66)[a]	08/20 (0.9 ± 1.37)[a]
B	Circovac	20	20/20 (2.00 ± 0.48)[a]	17/20 (2.52 ± 0.83)[a]	15/20 (1.29 ± 1.27)[a,b]	10/20 (1.21 ± 1.22)[a]	09/20 (1.07 ± 1.55)[a]
Control	Saline	17	15/17 (2.46 ± 0.99)[a]	13/17 (2.23 ± 0.99)[a]	16/17 (2.43 ± 0.98)[b]	16/17 (2.66 ± 0.99)[b]	16/17 (3.2± 0.92)[b]

The mean Log_{10} value of the viral load was 1.1 and 1.8 in pens containing vaccinated and control animals, respectively (Figure 2). A decrease in viral load in the environment was observed in pens housing both vaccinated and non-vaccinated animals (P <0.05; data not shown) at 28, 42, and 56 days post-vaccination (dpv). The global spread of PCV2 in swine herds highlights the virus's horizontal transmission, coupled with its resilience in the environment, which sustains its viability. As noted by various authors (Viancelli *et al*., 2011; Fongaro *et al*., 2015), the detection of PCV2 in groundwater shows its potential for environmental contamination. Furthermore, recent observations have found PCV2 in animal species beyond domestic swine, including rats, dogs, cattle, foxes, amphibians, and fish (Tarján *et al*., 2014; Herbst & Willens, 2017). This is attributed to its environmental resistance and high mutation rate, confirming PCV2's ability to break the interspecies barrier. The positive impact of different PCV2 vaccination protocols on pig health and pig herd productions is well known (Karuppannan & Opriessnig, 2017). Hence, emphasizing the vaccine's effect on virus elimination in faeces is crucial, as reducing viral load also diminishes contamination risks (Chae *et al*., 2012).

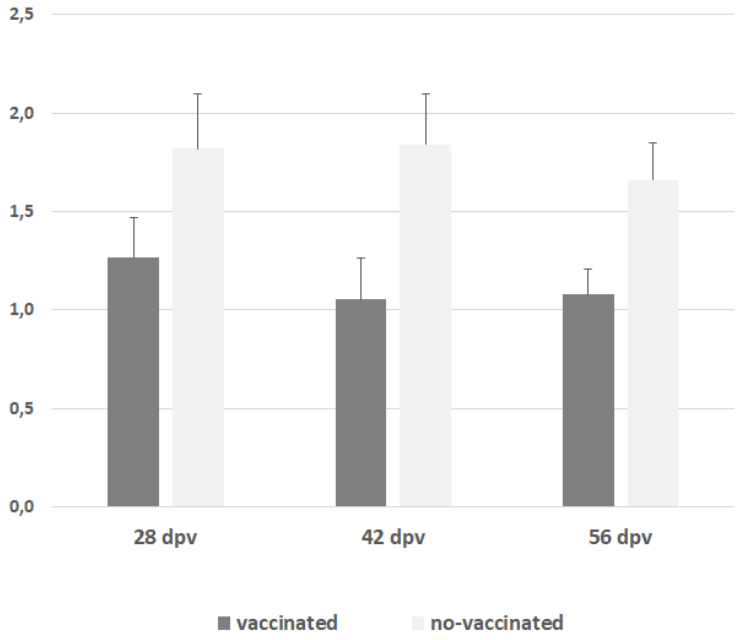

Figure 2 Number of positive animals for PCV2 DNA per group and viral load (Log_{10})

Conclusion

There is a beneficial effect of PCV2 vaccination on the environmental viral load, emphasizing the importance of the PCV2 vaccination for environmental health. Introducing PCV2 vaccination in a PCV2-positive herd that previously lacked a PCV2 vaccination protocol resulted in reduced viral load in faeces and the surrounding environment.

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Authors' Contributions

DFS, VHS, RGM, AMMGC: Planned the experimental project, data collection, writing, statistical analysis, and interpretation of results. LMB, TSO, CIS: Assisted in data collection and writing. ARS, IRS, SF: Writing, revising, and formatting the manuscript.

Conflict of interest statement and funding

All authors declare no conflict of interests. No funding was received.

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