South African Journal of Animal Science 2024, VOL 54

PCV2: The unexpected threat - How fish infection can transform industries and society: A pilot study

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(Submitted 10 July 2023; Accepted 23 February 2024; Published 26 March 2024)

Abstract

Integrated fish and livestock farming is an ancient practice and is used to increase production per unit area and reduce the environmental impact caused by untreated animal excreta. Although integrated aquaculture has been used in some countries, including Brazil, this practice has declined. However, many pig farmers maintain their ponds and/or have rivers near their pig farms, allowing raw manure to drain to them. The objective of this work was to verify the possibility of infection of fish by *porcine circovirus* 2 (PCV2) and, thus, serving as a reservoir species. Eleven specimens of Nile tilapia (*Oreochromis niloticus*), negative for PCV2, were inoculated with PCV2 intramuscularly and observed for 61 d post-inoculation (dpi), when they were euthanized and had their intestines, livers and gills removed to evaluate for presence of PCV2. The faeces were collected directly from the fish aquarium every four days up to 12 dpi and then once a week up to 61 dpi. The organs and faeces were tested by qualitative polymerase chain reaction (qPCR) for PCV2. This preliminary study showed that PCV2 inoculation was successful in infecting fish. In addition, PCV2 was eliminated in the faeces intermittently during the 61 d of experimentation.

Keywords: infection, fish farming, *Oreochromis niloticus*, virus
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Introduction

Integrated fish and livestock farming is an ancient practice. The goal is to increase production per unit area using animal manure instead of high-cost inputs, such as fish feed and industrialized fertilizers. This system/practice also aims to reduce the environmental impact caused by the excreta of untreated animals. In addition, fish tanks and ponds serve as an easy and inexpensive waste treatment system for processing manure. Thus, the recycling/decomposition of organic waste, using this system, addresses two main issues in aquaculture and livestock production: avoiding the disposal of untreated animal waste in the environment and providing economic benefits (Kumaresan *et al*., 2009; Bhatt *et al*., 2011).

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v54i1.11

Integrated aquaculture is an ancient practice in China and has been introduced in other southern and Southeast Asian countries such as Bangladesh, India, Indonesia, and Vietnam (Li *et al*., 2017). In Brazil, the practice of integrated aquaculture was widely used in the 1980s and 1990s (Guivant, 1997). Since then, this practice has declined. However, many pig farmers maintain their ponds and/or have rivers near their pig farms, allowing raw manure to drain to them.

Porcine *Circovirus* 2 (PCV2) is a small, unenveloped virus of the family Circoviridae, genus *Circovirus*, which is associated with several disease conditions, collectively known as porcine circovirus-associated diseases (PCVAD). PCV2 has a high mutation rate and eight PCV2 genotypes (PCV2a to PCV2h) have been described so far. PCV2 has demonstrated a high adaptability in different cell lines and is able to infect different species (Firth *et al*., 2009; Segalés *et al*., 2013a; Bao *et al*., 2018; Yao *et al*., 2019; Saporiti *et al*., 2021). In addition to pigs, viral DNA has been detected in rodents *(Mus musculus* and *Rattus rattus*), insects (*Musca* sp*., Blatta orientalis, Muscina stabulans, Stomoxys calcitrans,* and flies of the family *Syrphidae),* arachnids, canines, cattle, buffalo, shellfish, and in faecal samples from dogs and raccoons (*Nyctereutes procyonoides*) (Blunt *et al*., 2011; Pinheiro *et al*., 2013a; Halami *et al*., 2014; Krog *et al*., 2014; Herbst & Willems, 2017; Zhai *et al*., 2017). It is known that PCV2a, PCV2b and PCV2d are the most prevalent genotypes worldwide and result in moderate to severe clinical signs. The PCV2c, PCV2e and PCV2f genotypes have lower prevalence and are considered non-pathogenic; there is still little information available on prevalence and pathogenesis of PCV2g and PCV2h genotypes (Segalés *et al*., 2013b; Collins *et al*., 2017; Bao *et al*., 2018; Ge *et al*., 2021; Saporiti *et al*., 2021). In this context, due to the proximity of pig and fish production in Brazil, the present study was conducted to determine the ability of PCV2 to infect and be eliminated by fish, possibly acting as a reservoir species, keeping PCV2 in the farm environment. This research stands out as a fundamental pilot study, since, after a thorough review of the literature, no similar study was found. Therefore, their outcome can open up new perspectives and provide valuable insights for understanding and managing health in the interactions between pigs and fish and the potential impact on farm biosecurity.

Materials and methods

The experimental protocol was approved by the Institutional Committee for the Care and Use of Animals of the Veterinary School of the University Center of the United Metropolitan Colleges (protocol number 009/15 B). Eleven *Oreochromis niloticus aged* between 10 and 12 cm in length from the Fishermen's Refuge, located in the municipality of Santana de Parnaíba, State of São Paulo, negative for PCV2 infection, were clinically inspected and randomly distributed into three groups. The fish of group 1 (G1) were injected with 0.1 mL of inoculum containing PCV2, the fish of group 2 (G2) received 0.2 mL of inoculum, and the fish of the control group (CG) were not inoculated. The groups were allocated to different aquariums, which were kept separate from each other to avoid cross-contamination.

The CG aquarium was placed in a different room to increase safety. The fish had their water changed daily and were fed a commercial cichlid feed. A summary of the experimental design is presented in Figure 1. The PCV2 inoculum was provided by the Biological Institute of São Paulo. The inoculum presented a viral load of 108 copies of DNA/µL and was negative for other common swine viruses detected in Brazil, i.e., *porcine circovirus* 1 (PCV1), *Torque teno sus virus* 1 (TTVSu1), *Torque Teno Sus Virus Species* k2 (TTSuVk2), *Porcine parvovirus* 1 (VPP1), and VPP4 (data not shown). Fish faeces were collected directly from the fish aquarium every 4 d up to 12 d post-inoculation (dpi) and then once a week up to 61 dpi. After 61 dpi, all fish were euthanized and had their intestines, liver, and gills removed. All organ samples were kept in microtubes containing sterile saline solution at 0.9% at -20 °C.

DNA was extracted from tissues and faeces using the EasyPure Genomic DNA Extraction Kit (TransGen, Sinapse Biotecnologia Ltd) and the DNA extraction QIamp® DNA Stool Mini Kit (Qiagen, USA), respectively (Yang *et al*., 2007).The purified DNA was sequenced in both directions (forward and reverse primers) in an ABI 3500 sequencing genetic analyser with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA). The sequences were aligned with the sequence inoculated with the inoculated PCV2 and a sequence identity matrix was performed using the BioEdit software version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Statistical analysis was performed using IBM SPSS v.23 software. Analysis of variance (ANOVA) was used for cross-sectional evaluation of the number of copies of PCV2 in tissue and faecal samples. Real-time PCR results (tissue and faecal copies) were $\log_{10}$ transformed before statistical analysis. The level of significance adopted

was P <0.05, followed by a paired test with a Tukey–Kramer adjustment to identify the groups that differed.

This study did not use a formal sample size calculation. Instead, it worked with a small sample of eleven, which can be considered a reduced size, particularly in fish-related research. This methodological choice was driven by the exploratory and preliminary nature of the study, which serves as a pilot study intended to generate initial data and insights that can inform more extensive and detailed future research.

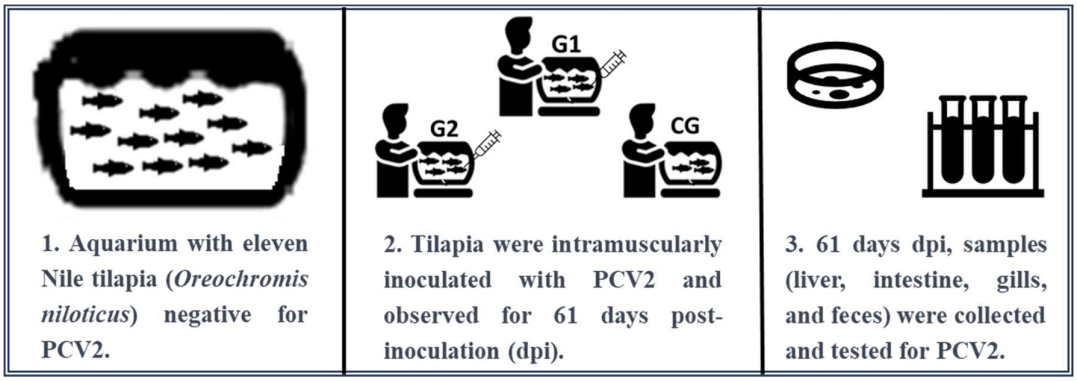

Figure 1. Summary of the experimental design of PCV2-infection in tilapia. Step-by-step depiction of the research findings, indicating the presence of PCV2 in different tissues, including the liver, intestine, gills, and faeces, after a 61-d observation period

Results and Discussion

PCV2 is ubiquitous in pig herds and recently, some of its new genotypes (PCV2a, PCV2b, PCV2c and PCV2e), have been described in Brazil (Rose *et al*., 2012a; Segalés *et al*., 2013c; Franzo & Segalés, 2018). *Escherichia coli* was found in fish raised in an integrated system in Vietnam (Dang & Dalsgaard, 2012). *Salmonella* spp. were found in fish mucus (20.0%), fish gut (40.0%) and pig faeces (11.1%) from integrated ponds, and fish mucus (40.0%) and fish gut (40.0%) from non-integrated ponds in China (Li *et al*., 2017).

The present study demonstrated that fish can also be infected with PCV2 and eliminate the virus through their faeces, as observed for species of bacteria present in pig herds. The results are presented in Table 1. All fish in the control group (CG) were negative for PCV2. The gill samples collected from fish of groups G1 and G2 were negative for PCV2. PCV2 was recovered from fish intestines (3/4 individuals) and livers (2/4 individuals) in G1 and G2. In the intestine, the viral load was slightly higher, with no difference (P >0.05), between G2 (1.1×10^4 to 4.5×10^4 copies/mg of tissues) and G1 (1.3×10^3 to 2.4×10^3 copies/mg of tissues). The same was observed in the liver, in which the viral load in G2 fish (4.3×10^2 to 1.1×10^3 copies/mg of tissues) was not statistically higher (P >0.05) in relation to G1 (1.1×10^2 to 3.2×10^2 copies/mg of tissues). The detection of PCV2 in fish liver may indicate that, as in pigs, PCV2 infection has a predilection for lymphoid tissues. In addition, PCV2 was detected in the intestine and faeces of fish, as previously observed in pigs. Therefore, these organs may be serving as a replication site and pathway for the spread of PCV2 in fish. However, it is important to note that the viral load in tissues and faeces was lower than that observed for pigs, which may indicate an adaptation of the virus to the new host (Opriessnig *et al*., 2007; Rose *et al*., 2012b; Segalés *et al*., 2013d; Tarján *et al*., 2014; Fehér *et al*., 2022).

PCV2 was detected in faeces on the 4th, 26th, 33rd, and 61st dpi (Figure 2), with viral load ranging from 1.2×10^2 to 8.3×10^2 copies of PCV2-DNA/mg of faeces (Table 1), showing intermittent elimination by this route, as observed in pigs. However, the dpi intervals were much longer than in pigs. This can be attributed to the management of the animals during the period of the experiment, in which water was changed daily. Previous studies have shown the presence of hepatitis E virus (HEV) in raw sewage water. The HEV strains were closely related to those found in human and animal populations in both developed and developing countries (Li *et al*., 2017). In addition, intact particles of porcine adenovirus (PAdV) and porcine circovirus 2 (PCV2) were detected in wastewater from manure treatment systems consisting of an equalization tank, a settling tank, an anaerobic reactor, an aerobic

reactor, and a secondary settling tank (Franzo *et al*., 2015; Pinheiro *et al*., 2013b; de Castro *et al*., 2015). In the present preliminary experimental study, fish infections by PCV2 were investigated. Considering future research, the undertaking of additional studies is proposed to elucidate the interactions related to natural PCV2 infection under field/environmental conditions.

It is relevant to highlight that this study did not use a formal calculation to determine the sample size and, therefore, the sample analysed is considerably reduced. However, it is important to emphasize that this study is the pioneer in addressing the interaction of *porcine circovirus virus type 2* (PCV2) with fish. In this context, it is crucial to note that pilot studies often share this characteristic of small samples, as they aim at an initial exploration of emerging topics (Chongviriyaphan, 1999; Hamilton-Reeves *et al*., 2016; Ljubobratovic *et al*., 2017). This research, therefore, establishes an initial milestone for the investigation of the ecology of PCV2 in fish, encouraging future studies to deepen this line of research.

The detection of PCV2 virus in fish faeces, regardless of the amount of inoculum, has significant implications for One Health. One Health understands the interconnectedness between human, animal, and environmental health, recognizing that public health challenges often transcend boundaries between these domains. The persistence of PCV2 in the environment of fish farms and the possible role of fish as reservoirs of the virus may raise concerns regarding the transmission of PCV2 to other species, including humans, through the food chain or environmental contamination. This finding underlines the importance of integrated health approaches and highlights the need for ongoing surveillance to mitigate potential threats to public health and the health of aquatic ecosystems (Opriessnig *et al*., 2020).

Given the findings of this study and the implications of the presence of PCV2 in fish, the need for future research that deepens this area of study is evident. Subsequent studies can broaden sampling and consider different fish species, environments, and environmental variables for a more comprehensive understanding of the dynamics between PCV2 and fish. In addition, further investigations may explore prevention and control strategies aimed at public health and the conservation of aquatic ecosystems. Continued research in this field is critical to a more complete understanding of the ecology and epidemiology of PCV2 in fish and its impact on One Health.

It is essential to recognize the limitations inherent in this study. First, this is an experimental study conducted in a single location and with a specific species of fish, which may limit the generalization of the results to other geographic areas and fish species. In addition, the sample used in this study was relatively small, which may influence the representativeness of the results on a broader scale. It is also important to note that the statistical test applied was specific to the conditions of this study and may not be directly applicable in other contexts. Notwithstanding these limitations, it is essential to highlight that this study constitutes the first successful effort to detect *porcine circovirus virus type 2* (PCV2) in fish faeces, shedding light on a hitherto unexplored area of research and establishing an essential starting point for subsequent investigations in this emerging area of study.

Table 1. Summary of polymerase chain reaction findings regarding *porcine circovirus* 2 (PCV2) inoculation in Nile tilapia

Group	Treatment	n	Intestine (Viral load copies/ mg of tissues)	Liver (Viral load copies/ mg of tissues)	Gill	Viral load copies/ mg of faeces (days post-infection)			
						4th	26th	33rd	61st
GC	Not inoculated	3	0/3 (-)	0/3 (-)	0/3	0	0	0	0
G1	0.1 mL of PCV2b inoculum	4	3/4 (1.3×10^3 to 2.4×10^{3a})	2/4 (1.1×10^2 to $3.2 \times 10^{2\ a}$)	0/4	1.2×10^2	4.1×10^2	3.8×10^2	6.0×10^2
G2	0.2 mL of PCV2b inoculum	4	3/4 (1.1×10^4 to $4.5 \times 10^{4\ a}$)	2/4 (4.3×10^2 to $1.1 \times 10^{3\ a}$)	0/4	3.7×10^2	5.7×10^2	5.0×10^2	8.3×10^2

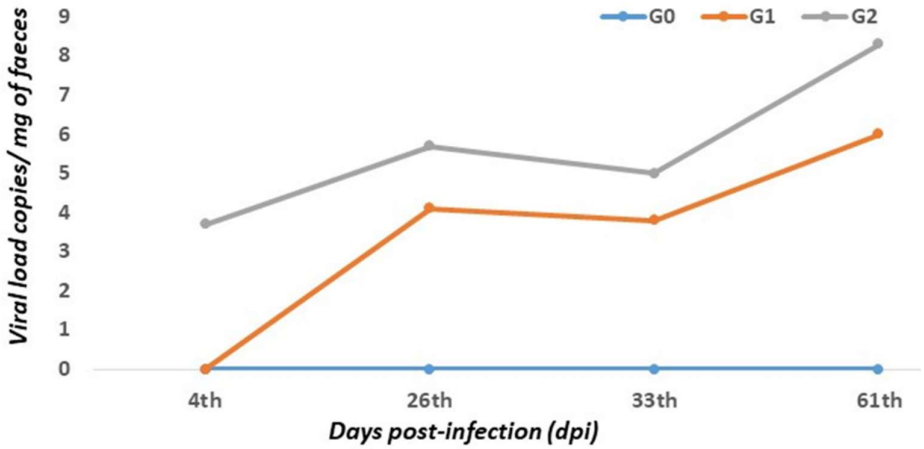

Figure 2. Temporal variation in viral load in Nile tilapia over time. Each point on the graph indicates the amount of viral load copies per milligram (mg) of faeces at different time intervals

Conclusion

This preliminary study provided promising evidence that Nile tilapia can be infected with PCV2, acting as a potential host and source of spread of the virus. The results presented here contribute to the scientific foundation of the theme and highlight the importance of future investigations to improve our understanding of this viral infection in fish and other living beings.

Acknowledgements

Scientific initiation scholarship for Rosecleer Rodrigues da Silva (Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP nº 2020/15758-0); Scientific initiation scholarship for Victor Hugo da Silva (FAPESP number 2017/21592-4).

Authors' Contributions

VHS (Orcid: 0000-0002-6466-5349) participated in animal experiments and laboratory analyses. FB (Orcid: 0000-0001-5405-300X) and JCSB (Orcid: 0000-0002-3947-4655) participated in the study elaboration, laboratory analysis, and writing of the manuscript. RRS (Orcid: 0000-0002-3223-3234), DFS (Orcid: 0000-0001-8275-7178), and LJB (Orcid: 0009-0000-7767-8694) participated in the preparation and review of the manuscript, final analysis and writing of the manuscript, and involvement in the preparation and review of the manuscript. AMMGC (Orcid: 0000-0003-4414-6661) outlined the study, animal experiment, and laboratory analysis; involvement in the writing and revision of the manuscript for important intellectual content; data analysis and interpretation; involvement in the preparation and revision of the manuscript.

Declaration of conflict of interest and funding

All authors declare no conflicts of interest. No funding was received.

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