Research in Veterinary Science 103 (2015) 70–72

Contents lists available at ScienceDirect

Research in Veterinary Science

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

Short Communication

Preliminary evidence of age-dependent clinical signs associated with porcine circovirus 2b in experimentally infected CH3/Rockefeller mice

Alessandra M.M.G. de Castro [a,b,*], Taís F. Cruz [d,e], Katarina B. Yamada [a], Priscilla F. Gerber [c], Michelle P. Gabardo [c], João P. Araújo Jr. [d,e], Roberto M.C. Guedes [c], Cinthia K. Mori [a], Camila P. Oliveira [a], Sueli S. Santos [a], Leonardo J. Richtzenhain [a]

[a] Departamento de Medicina Veterinária Preventiva e Saúde Animal, da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, Cidade Universitária, 05508 270, São Paulo, SP Brazil
[b] Complexo Educacional Faculdades Metropolitana Unidas, Veterinária, Rua Ministro Nelson Hungria, 541, Real Parque, Morumbi, São Paulo, SP, Brazil
[c] Departamento de Clínica e Cirurgia Veterinária, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627 — Pampulha, 31270-901, Belo Horizonte, MG, Brazil
[d] Departamento de Imunologia e Microbiologia, Instituto de Biociência, Universidade Estadual Paulista "Julio de Mesquita Filho", Campus de Botucatu, Botucatu, SP, 18618-970, Brazil
[e] Biotechnology Institute – IBTEC – Sao Paulo State University – UNESP – Campus de Botucatu, SP

ARTICLE INFO

Article history:
Received 8 April 2015
Received in revised form 12 August 2015
Accepted 9 September 2015

Keywords:
Rodents
Swine virus
Viral load
PCV2

ABSTRACT

Mice and rats are susceptible to porcine circovirus 2b (PCV2) infection under field and experimental conditions. However, whether PCV2 induces disease in rodents remains a matter of debate. The objectives of the present study were to determine whether PCV2-induced disease in mice is age-dependent and whether intranasally inoculated animals are able to infect animals they come into contact with. Twenty-five CH3/Rockefeller mice were divided into six groups and intranasally inoculated with 25 μL of either PCV2b or PBS on days 0, 3 and 6. One group remained untreated. Two age groups were tested: 3-week-old mice and 6-week-old mice. The administration of three PCV2 intranasal inoculations at intervals of three days was able to induce infection and support virus transmission in susceptible mice, regardless of the age at inoculation. The clinical signs associated with PCV2 infection were more severe in younger mice, and PCV2-DNA load was higher in their faeces. In conclusion, PCV2 induced disease in mice.

1. Introduction

Porcine circovirus 2 (PCV2) is a small, non-enveloped virus of the family *Circoviridae*, genus *Circovirus*, which is associated with various disease conditions, collectively known as porcine circovirus-associated disease (PCVAD). Two major genotypes, PCV2a and PCV2b, are present worldwide (Opriessnig et al., 2007). PCV2 has been detected in other animals in addition to domestic pigs (Lorincz et al., 2010, Pinheiro et al., 2013 and Rose et al., 2012), and several studies have been conducted to investigate the susceptibility of mice to PCV2 infection. However, whether PCV2 induces disease in rodents remains a matter of debate. PCV2 replicates in BALB/c mice and can be detected in infected mice through in situ hybridisation and polymerase chain reaction (PCR) (Kiupel et al., 2001). Microscopic lesions consistent with PCVAD have not been observed in BALB/c, C57BL/6, C3H/HeN (Opriessnig et al., 2009) or ICR-CD1 mice following infection with PCV2 (Quintana et al., 2002), although PCV2 DNA can be recovered from the tissues of inoculated mice, regardless of their genetic lineage (Opriessnig et al., 2009). Recently, macroscopic and microscopic lesions similar to those caused by PCVAD in pigs have been reported in Kunming mice (Deng et al., 2011 and Li et al., 2010). A previous study provided evidence of PCV2 transmission between mice (Cságola et al., 2008) and demonstrated the potential of using Kunming mice as animal models of PCVAD (Deng et al., 2011). The current study was conducted to determine whether PCV2-induced disease in mice is age-dependent and whether intranasally inoculated animals are able to infect animals that they come into contact with. The experimental protocol was approved through the Institutional Animal Care and Use Committee from the Veterinary School of the University of São Paulo. Twenty-five CH3/Rockefeller mice, aged either 3 or 6 weeks old, were obtained from the bioterium, inspected, identified and randomly assigned to six groups (G1–G6). Their cages (41 x 34 x 16 cm) were separated to avoid cross-contamination, and each contained a self-feeder and an automatic watering device. A summary of the experimental design is presented in Table 1. The inoculum, which was the PCV2b virus strain (GenBank number KC924956), was obtained through serial passage in ST cell cultures (Cruz and Araujo Jr, 2014) that were propagated in 490 cm^2 roller bottles. The inoculum was negative for other swine agents (data not shown). The animals were intranasally inoculated with 25 μL of the PCV2b inoculum ($10^{6.91}$ 50% tissue culture infective dose ($TCID_{50}$) per mL) and monitored daily; any clinical signs of

* Corresponding author.
E-mail address: alessandramarnie@gmail.com (A.M.M.G. de Castro).

http://dx.doi.org/10.1016/j.rvsc.2015.09.008
0034-5288/

Table 1
Experimental design and frequencies of animals with clinical signs, dead mice, and gross (non-collapsed) and microscopic lung lesions (moderate to severe). The inoculated mice were PCV2b-challenged three times via intranasal inoculation.

Group	Treatment	Age (Weeks)	n	Clinical signs	Dead	Gross lesions[a]	Microscopic lesions[b]
G1	Inoculated[c]	3	5	2/5	2/5[d]	2/3	3/3
G2		6	4	2/4	0/4	1/4	3/4
G3	Contactants	3	5	2/5	0/5	2/5	5/5
G4		6	5	0/5	0/5	0/4[e]	2/4[f]
G5	Sham inoculated[f]	3	3	0/3	0/3	0/3	0/3
G6		6	3	0/3	0/3	0/3	0/3

[a] Description of non-collapsed lungs.
[b] Refers to the number of mice showing mild to severe lung lesions.
[c] Inoculated intranasally with PCV2b (GenBank accession number KC924956) on days 0, 3 and 6.
[d] Mice C4 and C5 died on days 22 and 29 after inoculation, respectively.
[e] One lung sample was missed.
[f] Inoculated intranasally with 25 μL of phosphate-buffered saline buffer on days 0, 3 and 6.

respiratory disease or lethargy were recorded. At 42 dpi, all of the mice were euthanized via intraperitoneal overdose of ketamine/xylazine, followed by cervical dislocation. Faecal samples were collected at 1, 4, 8, 12, 16, 20, 24, 28, 32, 36 and 40 dpi. At 42 dpi, serum and sections of lung, spleen, liver, kidney, heart, intestine and brain were collected for assessment in a double-antibody sandwich ELISA (DAS-ELISA) and fixation in 10% neutral-buffered formalin. Formalin-fixed and paraffin-embedded tissue sections were evaluated by a blinded veterinary pathologist and were submitted to immunohistochemistry (IHC) for detection of PCV2-specific antigens using rabbit polyclonal anti-PCV2 serum as previously described (Pinheiro et al., 2013). DNA was extracted from the tissues and faeces using the phenol-chloroform-proteinase K method (Sambrook et al., 1989) and a DNA extraction QIamp® DNA Stool Mini Kit (Qiagen, USA), respectively. The extracts were used to quantify a 145-nt-long PCV2 DNA fragment via SYBR Green qPCR as previously described (Yang et al., 2007). The reactions were performed using a StepOne™ Real-Time PCR System (Applied Biosystems-Life Technologies Inc., USA). The number of viral DNA copies was determined by comparison with a standard curve, and the viral concentration was expressed as copies of PCV2 DNA per 20 mg of tissue sample and as copies of PCV2 DNA per 100 ng of total DNA in the faeces samples. β-Actin served as a control for the PCR, as previously described (Hui et al., 2004). The 145-nt-long fragments were bidirectionally sequenced using BigDye® Terminator v3.1 (Applied Biosystems-Life Technologies Inc., USA) and were aligned against the PCV2b inoculum sequence. The qPCR data are reported as the means ± standard deviation unless otherwise indicated. The Shapiro–Wilk test was used to normalize the data distribution of the examined variables. Nonparametric Kruskal–Wallis ANOVA analysis was employed to evaluate the PCV2 load between the contact and inoculated animals and between age groups. Differences among the interacting groups that were indicated by the repeated measures ANOVA were assessed using the Mann–Whitney test with a significance level of $p < 0.05$. All of the control group mice were negative for PCV2 regardless of age. The intranasal PCV2 inoculations were successful in infecting and transmitting the virus to the contact animals. Previous results regarding PCV2 infection in mice have been contradictory, and the lineages of the animals that were used in these studies could explain the differences in the results, as genetic variations in the host modulate the pathways and mechanisms involved in specific disease tolerance traits. MHC I molecules play a pivotal role in immune recognition of intracellular pathogens. The mutational and polymorphic patterns that have been linked to MHC-I haplotypes in BALB/c (H2 d) and CH3 (H2 k) mice may explain why both of these strains are more susceptible to PCV2 infection than C57BL/6 mice (Opriessnig et al. 2009). Despite the absence of information on MHC-I haplotypes in Kunming mice (Cságola et al., 2008, Li et al., 2010 and Deng et al., 2011) and CH3/Rockefeller mice, which were used in the current study, genetic differences should be considered when seeking understanding of the varying levels of disease pathogenesis that have been observed in different studies.

Two mice from G2 and two mice from G3 were lethargic from 14 to 20 dpi and 17 to 30 dpi, respectively. Two mice from G1 showed lethargy and respiratory distress at 14 dpi; one died at 22 dpi, and the other died at 29 dpi (Table 1). The clinical respiratory signs associated with PCV2 infection were shown to be age-dependent in mice. The route and number of inoculations, as well as the intervals between them, might have influenced the death rate and rate of lesion occurrence in the present study. In previous studies, both intramuscular and intraperitoneal routes, sometimes combined with oral or intranasal challenge, have been used. However, longer inoculation intervals of 7 or 14 days were used in previous studies versus the 3-day interval used herein. The use of a shorter interval might impede the development of a specific adaptive response, resulting in more severe clinical signs and the occurrence of microscopic lesions, primarily in the lungs of younger mice (Kiupel et al., 2001, Opriessnig et al., 2009, Deng et al., 2011 and Li et al., 2010). Younger animals presented higher PCV2 loads in their tissues in both the infected (mean ± standard deviation 4.72 ± 2.28 vs. 2.96 ± 3.05, $p < 0.01$) and contact groups (mean ± standard deviation 4.92 ± 2.46 vs. 3.63 ± 3.75, $p < 0.01$) compared with 6-week-old mice. The same pattern was observed when assessing PCV2 load in the faeces (Fig. 1). There was no difference in PCV2 load in any of the evaluated organs between the 3- and 6-week-old animals (data not shown). PCV2 DNA shedding in the faeces was detected earlier in the younger contact mice than in the older contact mice (4 vs. 8 dpi, respectively) (Fig. 1). The 145-nt-long sequence corresponding to PCV2-ORF2 that was obtained from tissue (n = 4) and faecal (n = 4) samples, which were selected for evaluation based on PCV2 DNA load, showed 100% identity with the inoculum used (data not shown). All of the serum samples were negative for anti-PCV2 antibodies. An absence of

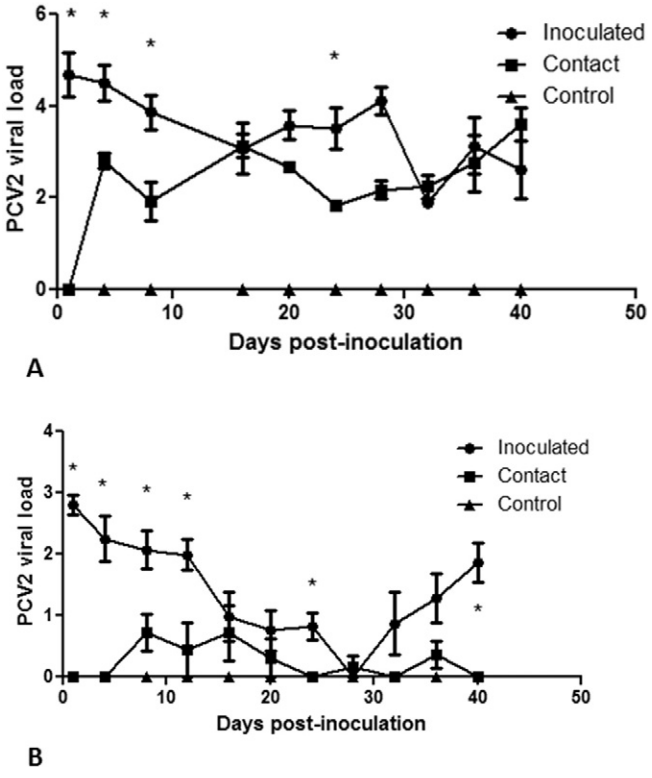

Fig. 1. The mean loads of log10 PCV2 DNA in faecal samples obtained from 3- (A) and 6-week-old (B) mice at 0, 10, 20, 30 and 40 days post intranasal inoculation. The asterisks indicate significant ($p < 0.05$) differences between the group mean loads of log10 PCV2 DNA at selected dpi. The error bars represent standard errors.

detectable anti-PCV2 IgG in infected mice was previously described by Opriessnig et al. (2009), which was in disagreement with other studies that reported seroconversion in mice after 7 dpi (Kiupel et al., 2001 and Li et al., 2010). This intra-study discrepancy might reflect the different assays that were used to detect anti-PCV2 antibodies. Additionally, it has been demonstrated that PCV2 can remain associated with macrophages and dendritic cells without showing any signs of replication (Darwich and Mateu, 2012). Considering the migratory capacity of dendritic cells, such virus-host interactions might represent an effective vehicle for virus spread within the body, which may explain the detection of PCV2 in tissues and faeces via qPCR in the present study, despite the seroconversion and/or lack of viraemia (Vincent et al., 2003). The detection of viral DNA in faeces on the first day post inoculation might have resulted from the ingestion of inoculum, as has been observed in swine (Patterson and Opriessnig, 2010). With the exception of the 6-week-old contact group mice, none of the remainder of the animals from the inoculated and contact groups presented collapsed lungs. Microscopic lesions were observed in the lungs, regardless of age (Table 1). In the 3-week-old mice, 6 of 11 mice showed mild to severe neutrophilic and lymphocytic bronchointerstitial pneumonia, whilst 2 of 11 of the 6-week-old mice showed mild to moderate neutrophilic bronchointerstitial pneumonia. Furthermore, numerous megakaryocytes were observed in the red pulp of the spleen in 4 of 12 of the 6-week-old mice. Hepatitis and pyelitis were observed in 4 of 12 of the 6-week-old mice, whilst only one 3-week-old animal from G1 had hepatitis, and no 3-week-old animals showed pyelitis. Mild multifocal areas of mineralisation were described in the hearts of two of the mice from G2. No lesions were observed in the other organs tested. Severe staining was observed in the spleens of animals from G1 (1/3), G2 (1/4) and G3 (4/5), whilst moderate staining was observed in the animals from G1 (1/3), G2 (2/4) and G4 (2/5). The discrepancies between the PCR and IHC results might be associated with the intrinsic sensitivities of the assays. It has previously been determined that an estimated viral load of a minimum of 10^8 PCV2 genomes per 500 ng DNA is required to yield visible IHC staining in swine samples. Moreover, in the absence of virus replication, PCV2 DNA persists in DCs (Vincent et al., 2003) without presenting antigens to the cell surface, preventing IHC detection. Thus, although gross microscopic lesions and clinical signs were observed in the present study, these characteristics were primarily observed in the lungs because of the lesions that were present in this tissue. Therefore, it is likely that viral replication in the mice occurred below the analytical sensitivity threshold for IHC assay. Finally, we demonstrated that administering three intranasal inoculations of PCV2 using an interval of three days between each led to viral infection and supported viral transmission to uninfected animals, regardless of the age of the animal at the time of inoculation; however, the clinical signs caused by viral infection were more severe in younger mice.

Acknowledgments

The authors would like to thank the São Paulo Research Foundation (FAPESP), São Paulo State, Brazil (grant number 2007/57115-3), for financial support. The authors Guedes and Mori (grant number 2010-2899) would like to thank the Conselho Nacional de Desenvolvimento Científico (CNPq) for research fellowships. The authors Yamada (grant number 2011/00054-8) and Castro (grant number 2009/09175-2) would like to thank FAPESP for research fellowships.

References

Cruz, T.F., Araujo Jr., J.P., 2014. Cultivation of PCV2 in swine testicle cells using the shell vial technique and monitoring of viral replication by qPCR and RT-qPCR. J. Virol. Methods 196, 82–85.

Cságola, A., Kiss, I., Tuboly, T., 2008. Detection and analysis of porcine circovirus type 1 in Hungarian wild boars: short communication. Acta Vet. Hung. 56, 139–144.

Darwich, L., Mateu, E., 2012. Immunology of porcine circovirus type 2 (PCV2). Virus Res. 164, 61–67.

Deng, Z.B., Wang, N.D., Xu, D.J., Yuan, A.W., Ge, M., Luo, W., Xue, L.Q., Yu, X.L., 2011. Viral distribution and lesions in Kunming mice experimentally infected with porcine circovirus type 2b. Vet. Res. Commun. 35, 181–192.

Hui, R.K.H., Zeng, F., Chan, C.M.N., Yuen, K.Y., Peiris, J.S.M., Leung, F.C.C., 2004. Reverse transcriptase PCR diagnostic assay for the coronavirus associated with severe acute respiratory syndrome. J. Clin. Microbiol. 42, 1994–1999.

Kiupel, M., Stevenson, G.W., Choi, J., Latimer, K.S., Kanitz, C.L., Mittal, S.K., 2001. Viral replication and lesions in BALB/c mice experimentally inoculated with porcine circovirus isolated from a pig with postweaning multisystemic wasting disease. Vet. Pathol. 38, 74–82.

Li, J., Yuan, X., Zhang, C., Miao, L., Wu, J., Shi, J., Xu, S., Cui, S., Wang, J., Ai, H., 2010. A mouse model to study infection against porcine circovirus type 2: viral distribution and lesions in mouse. Virol. J. 7, 158.

Lorincz, M., Cságola, A., Biksi, I., Szeredi, L., Dán, A., Tuboly, T., 2010. Detection of porcine circovirus in rodents — short communication. Acta Vet. Hung. 58, 265–268.

Opriessnig, T., Meng, X.J., Halbur, P.G., 2007. Porcine circovirus type 2 associated disease: update on current terminology, clinical manifestations, pathogenesis, diagnosis, and intervention strategies. J. Vet. Diagn. Invest. 19, 591–615.

Opriessnig, T., Patterson, A.R., Jones, D.E., Juhan, N.M., Meng, X.J., Halbur, P.G., 2009. Limited susceptibility of three different mouse (*Mus musculus*) lines to porcine circovirus-2 infection and associated lesions. Can. J. Vet. Res. 73, 81–86.

Patterson, A.R., Opriessnig, T., 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). Anim. Health Res. Rev. 11, 217–234.

Pinheiro, A.L.B.C., Bulos, L.H.S., Onofre, T.S., de Paula Gabardo, M., de Carvalho, O.V., Fausto, M.C., Guedes, R.M.C., de Almeida, M.R., Silva Júnior, A., 2013. Verification of natural infection of peridomestic rodents by PCV2 on commercial swine farms. Res. Vet. Sci. 94, 764–768.

Quintana, J., Balasch, M., Segalés, J., Calsamiglia, M., Rodríguez-Arrioja, G.M., Plana-Durán, J., Domingo, M., 2002. Experimental inoculation of porcine circoviruses type 1 (PCV1) and type 2 (PCV2) in rabbits and mice. Vet. Res. 33, 229–237.

Rose, N., Opriessnig, T., Grasland, B., Jestin, A., 2012. Epidemiology and transmission of porcine circovirus type 2 (PCV2). Virus Res. 164, 78–89.

Sambrook, J., Fitsch, E.F., Maniatis, T. (Eds.), 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor New York, pp. 1-65–1-69.

Vincent, I.E., Carrasco, C.P., Herrmann, B., Meeha, B.M., 2003. Dendritic cells harbor infectious porcine circovirus Type 2 in the absence of apparent cell modulation or replication of the virus. J. Virol. 77, 13288–13300.

Yang, Z., Habib, M., Shuai, J., Fang, W., 2007. Detection of PCV2 DNA by SYBR Green I-based quantitative PCR. J. Zhejiang Uni. Sci. B 8, 162–169.