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Original article

Experimental inoculation of porcine circoviruses type 1 (PCV1) and type 2 (PCV2) in rabbits and mice

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Abstract – The objective of this work was to investigate the susceptibility of rabbits and mice experimentally inoculated with porcine circoviruses type 1 (PCV1) and type 2 (PCV2) to infection and development of disease and/or lesions. Forty six New Zealand rabbits and 50 ICR-CD1 mice were both divided into two groups comprising PCV1 and PCV2 inoculated animals, and a third group inoculated with non-infected cell culture medium. Rabbits were inoculated intranasally while mice were inoculated intraperitoneally. Clinical signs and body weights were recorded at the start of the experiment and at necropsy. Animals were bled, euthanised and necropsied at days 0, 3, 7, 10, 14 and 20 post-inoculation and samples were collected for histopathological, serological, in situ hybridisation and PCR analysis. No clinical signs or gross and microscopic lesions compatible with PCV2 infections such as those seen in pigs were observed. No presence of PCV2 nucleic acid was detected in rabbits and mice by in situ hybridisation. Only one mouse inoculated with PCV1 seroconverted on day 20 PI. PCV1 and PCV2 genome was detected in serum by PCR in mice inoculated with each porcine circovirus, while rabbits were negative for both viral types. These studies indicated that porcine circoviruses did not cause any disease or microscopic lesions in inoculated rabbits and mice during the experimental period. However, intraperitoneally inoculated mice might have harboured PCV2 in circulation without evidence of viral replication.

rabbit / mice / porcine circovirus / polymerase chain reaction / in situ hybridisation

Résumé – Inoculation expérimentale de circovirus de type 1 (PCV1) et de type 2 (PCV2) chez le lapin et la souris. Le but de ce travail était d'étudier la sensibilité à l'infection et au développement de la maladie et/ou de lésions chez des lapins et des souris inoculés expérimentalement avec des circovirus porcins de type 1 (PCV1) et 2 (PCV2). Deux groupes de 46 lapins de Nouvelle-Zélande et 50 souris ICR-CD1 ont été

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chacun divisés en deux groupes comprenant des animaux inoculés avec PCV1 et PCV2, et un troisième groupe inoculé avec un milieu de culture non-infecté. Les lapins ont été inoculés par voie intranasale et les souris par voie intrapéritonéale. Les signes cliniques et le poids des animaux ont été enregistrés au début de l'expérience et au moment de l'autopsie. Aux jours 0, 3, 7, 10, 14 et 20 après inoculation, le sang des animaux a été prélevé, puis les animaux ont été euthanasiés et ont subi une autopsie ; des échantillons ont été prélevés pour des analyses histopathologique, sérologique, d'hybridation in situ et de PCR. Aucun signe clinique ni lésion macro– ou microscopique correspondant à ceux observés chez les porcs infectés par PCV2 n'ont été détectés. Aucune présence d'acide nucléique de PCV2 n'a été détectée chez les lapins et les souris par hybridation in situ. Seule une souris inoculée par PCV1 est devenue séropositive au jour 20 post-inoculation. Les génomes de PCV1 et PCV2 ont été détectés par PCR dans le sérum de souris inoculées par chacun des circovirus, alors que les lapins sont restés négatifs pour chacun des types. Ces études indiquent que les circovirus porcins n'ont provoqué aucune maladie ou lésion microscopique chez les lapins et les souris inoculés durant toute la durée de l'expérimentation. Cependant, les souris inoculées expérimentalement pourraient avoir hébergé du virus PCV2 dans la circulation sanguine sans signe de réplication virale.

lapin / souris / circovirus porcin / réaction en chaîne par polymerase / hybridation in situ

1. INTRODUCTION

Porcine circovirus type 2 (PCV2) infection is strongly associated to the postweaning multisystemic wasting syndrome (PMWS) in pigs [3]. PMWS was first identified in Canada in 1991 and was characterised by progressive weight loss, pallor of skin, lymphadenopathy and jaundice, affecting pigs older than 5 weeks of age [5, 9]. Gross lesions in affected pigs include lymph node hyperplasia and non-collapsed, rubbery lungs, as the most frequent findings. Microscopically, lymphocyte depletion and histiocytic infiltration with or without multinucleate giant cells and amphophilic intracytoplasmic inclusion bodies in lymphoid tissues, and interstitial pneumonia, hepatitis and nephritis are usually observed [18].

Up to now, no PCV2 infection has been consistently detected in the mammal species tested (sheep, cattle and human beings, [4, 6]) other than swine. However, Nayar et al. [15] suggested that PCV2 can also infect bovids; PCV2 was demonstrated by PCR but tested tissues were negative by immunohistochemistry. Furthermore, a recent publication has shown the presence of very mild lymphoid lesions (enlargement of germinal follicles and very mild lymphocyte depletion) associated to PCV2 infection in BALB/c mice [11]. Porcine circovirus type 1 (PCV1) serological studies in non-porcine species have also led to controversial results. Tischer et al. [21] detected antibodies to PCV1 in human beings, cattle and mice, while other workers did not find any evidence of PCV1 infection in other species (sheep, cattle, turkeys, chickens, human beings, mice, rabbits, goats and ducks) but swine [1]; PCV1 has also been demonstrated as non-pathogenic for swine [2, 20]. However, at present, no association has been established between infection of both porcine circoviruses and non-porcine species. Therefore, the infectivity of PCV1 and PCV2 in species other than swine should be further investigated in order to determine the potentiality of these other species to support viral replication, to transmit the virus and to act as reservoirs of porcine circoviruses.

The purpose of the present work was to evaluate if other non-porcine species such as rabbits and mice were susceptible to PCV1 and PCV2 infection, and if they could develop lesions and/or clinical signs associated with these viral infections.

2. MATERIALS AND METHODS

2.1. Animals

Forty-six New Zealand 1.8–2 kg female rabbits, and 50 ICR-CD1 20–30 g mice of both genders were used for the experiments. Twenty-four additional mice from the same source were also purchased to perform serological studies for PCV1 and PCV2 in this mice strain before the beginning of the experiment. The health status of the animals was determined upon arrival. Rabbits and mice were divided into three groups and housed in three different pens in a biosafety level 3 isolation building. For each treatment group, the rabbits and mice shared the same room, but the mice were kept in cages. The animals were allowed to have an acclimatisation period of 7 days before the start of the experiment. This study met the standards of the Guide of the Care and Use of Laboratory Animals and the study protocol was approved by the *Comitè Ètic d'Experimentació Animal de Fort Dodge Veterinaria S.A.,* Girona, Spain (Ethic Committee of Animal Experimentation of Fort Dodge Veterinaria, S.A., Girona, Spain) and the *Comissió d'Experimentació Animal del Departament d'Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya*, Barcelona, Spain (Committee of Animal Experimentation of Agriculture, Livestock and Fisheries Department of the Catalan Government, Barcelona, Spain).

2.2. Viral inocula

PCV1 was obtained from the supernatant of the ATCC CCL-33 cell line, which is persistently infected with this virus. The supernatant of 4-day-old cultures (passage 10) was recovered and clarified by centrifugation at 650 × *g*, and further ultracentrifuged at 100 000 × *g*. The viral pellet was resuspended in Dulbecco minimum essential medium (DMEM), titrated in PK-15 cells free of PCV1, aliquoted and frozen at –80 ºC until use.

PCV2 was obtained from a PCV2-persistently infected cell line supernatant. The swine kidney (SK) cell line, demonstrated to be free of porcine pathogens, was inoculated with a tissue homogenate from a pig affected PMWS. This inoculum was tested by PCR or the immunoperoxidase monolayer assay on cell culture to confirm its freedom of PRRSV, PPV, ADV, TGEV, PRCV, SIV and PCV1. These cells were passaged, the supernatant of passage 4 was recovered and clarified by centrifugation at 650 × *g* and further ultracentrifuged at 100 000 × *g* (this methodology did not allow to separate free PCV2 genomic DNA from the PCV2 virions). The viral pellet was resuspended in DMEM, titrated in non-infected SK cells, aliquoted and frozen at –80 ºC until use.

2.3. Experimental design

The rabbits were divided into 3 groups. One group of sixteen rabbits was intranasally inoculated with 10^4 $TCID_{50}$ of PCV2 per animal (in 0.2 mL of DMEM). The second group of 16 rabbits was intranasally inoculated with $10^{2.75}$ $TCID_{50}$ of PCV1 per animal (with the same inoculum volume). Finally, the third group of 14 rabbits was intranasally inoculated with 0.2 mL of DMEM and was kept as the negative control.

A group of 24 mice was bled and euthanised before any inoculation procedures. The remaining 50 mice were also divided into 3 groups. One group of eighteen mice was inoculated intraperitoneally with 10^3 $TCID_{50}$ of PCV2 per animal (in 0.1 mL of DMEM). The second group of 18 mice was inoculated intraperitoneally with $10^{1.75}$ $TCID_{50}$ of PCV1 per animal (with the same inoculum volume). Finally, the third group of 14 mice was inoculated intraperitoneally with 0.1 mL of DMEM and kept as the negative control.

Table I. Days of necropsy and number of rabbits and mice euthanised during the experiment.

	Postinoculation days					
	0	3	7	10	14	20
PCV1	–	3	3	3	3R/4M	4R/5M
PCV2	–	3	3	3	3R/4M	4R/5M
Control	2	2	2	2	2	4

R: rabbits; M: mice.

Both rabbits and mice were observed daily to detect possible clinical signs. All animals were weighed at the start of the experiment and at the day of necropsy. Animals were euthanised following the schedule shown in Table I. Blood was collected from the euthanised animals, and a complete necropsy was performed. Tissue samples were fixed by immersion in 10% buffered formalin for a histopathological evaluation, as well as to perform in situ hybridisation to detect PCV2.

2.4. Histopathology and in situ hybridisation (ISH)

Lymphoid organs (cecal tonsil, spleen, thymus, lymph nodes), lung, liver, kidney, myocardium, pancreas, and intestines were collected from the rabbits. The thymus, lung, liver, kidney, myocardium and intestines were collected from mice. Tissues were paraffin-embedded after a 24-hour fixation period; sections were cut at 3–4 μm thickness and stained with haematoxylin and eosin. An in situ hybridisation technique to detect PCV2 was performed on all formalin-fixed, paraffin-embedded tissues using a previously described protocol and oligonucleotide probe [19].

2.5. Serology to PCV1 and PCV2

Detection of antibodies to PCV1 and PCV2 was performed using an immunoperoxidase monolayer assay (IPMA) technique [17].

2.6. Polymerase chain reaction (PCR)

Viral DNA was extracted from bacterial and viral cultures, and from rabbit and mice serum using the Qiagen DNA blood kit (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. DNA from rabbit tissue samples (lung, spleen and cecal tonsil) was extracted using the Qiagen DNA mini kit (Qiagen Inc., Valencia, CA, USA).

A PCR technique for PCV1 and PCV2 detection was performed. The primers and conditions used for PCV1 amplification have been previously described [16]. The previously established PCR technique for PCV2 yielded amplification of several unspecific bands using rabbit and mouse samples. A new PCV2 PCR test was set up to detect viremia in rabbits and mice. PCV2 primers were designed based on the sequence from the Genbank Accession Number AF027217 [8]. The forward primer (5'-GCCAGTTCGTCACCCTTTC-3') was located between genomic positions 940 and 958 (located in PCV2 ORF1). The reverse primer (5'-CTCCCGCACCTTCGGATAT-3') was located between positions 1578 and 1596 (located in PCV2 ORF2). PCV2 specific primers amplified a 657 bp DNA fragment. The optimised PCR reaction mixture contained 200 nM dNTPs, 1.5 mM MgCl, 1 × PCR buffer, 500 nM of each primer and 1.25 U of Taq polymerase (Ecogen S.R.L., Barcelona, Spain). Reaction conditions were the following: initial penetration at 95 ºC for 3 min, followed by 35 cycles of

95 ºC during 30 s, primer annealing (58 ºC for PCV1 and 64 ºC for PCV2) for 1 min, initial extension at 72 ºC for 2 min, and a final extension of 72 ºC for 7 min. The specificity of the primers was tested adding extracted nucleic acids from several viral and bacterial swine pathogens (*Actinobacillus pleuropneumoniae, Mycoplasma hyorhinis, Mycoplasma hyopneumoniae, Haemophilus parasuis, Steptococcus suis, Pasteurella multocida* type D, *Bordetella bronchiseptica*, swine influenza virus H1N1, porcine reproductive and respiratory syndrome virus, Aujeszky's disease virus, porcine parvovirus and transmissible gastroenteritis virus). The sensitivity of the PCV2 PCR was tested using DNA extracted from the PCV2 isolate used later on to inoculate the animals, titrated in pfu/mL in SK cells. The amplified products were run in a 2% agarose gel and visualised by staining with 0.5 μg/mL of ethidium bromide.

2.7. Statistical analysis

Mean values of weights of each group of rabbits and mice were compared by ANOVA using the Epitable program from the EpiInfo 6.04 package.

3. RESULTS

3.1. Clinical evaluation

All the animals remained clinically normal during the experimental period. Weight gains were not significantly different between PCV1 and PCV2 inoculated and control groups for both rabbits and mice. Four rabbits were found dead on days 2 (control group), 6 (the PCV1 inoculated group) and 2 (one from the PCV1 inoculated group and another from the PCV2 inoculated group) postinoculation (PI). Only one mouse (control group) was found dead on day 3 PI.

3.2. Macroscopic and microscopic lesions

Macroscopic lesions were only observed in dead rabbits. These consisted of severe fibrino-purulent pericarditis and pleuritis. No other gross lesions were detected in the rest of the rabbits. No macroscopic lesions were observed in mice, not even in the animal found dead at 3 days PI.

No microscopic lesions compatible with PCV2 infection in pigs (lymphoid depletion, interstitial pneumonia, nephritis or hepatitis) were detected in rabbits and mice. None of the tissues examined presented PCV2 nucleic acid by ISH at any time of the experiment.

3.3. PCV1 and PCV2 serology

All rabbits were seronegative throughout all the experimental period. The 24 euthanised mice at 0 days PI were also seronegative to porcine circoviruses. Only one mouse inoculated with PCV1 had a very low titre (1:20) on day 20 PI whereas the rest of the animals were seronegative to both PCV1 and PCV2 during the experimental period.

3.4. PCV2 PCR specificity and sensitivity

None of the tested swine pathogens besides the porcine circovirus type 2 reacted with the designed primers. Serial dilutions of the PCV2 culture were positive up to a dilution of 10^{-4} pfu/mL (Fig. 1).

3.5. PCV1 and PCV2 PCR results in control and inoculated animals

PCR results are summarised in Table II. A PCV1 204 nucleotide fragment was amplified from the serum of various mice inoculated with PCV1 that were euthanised between days 3 and 20 PI. No PCV1 amplification was detected in the serum from the mice of control or PCV2 inoculated groups.

Table II. PCR positive results on serum samples of mice for PCV1 and PCV2.

	Postinoculation days					
	0	3	7	10	14	20
PCV1 PCR in PCV1 infected animals	–	2/3[a]	1/3	0/3	3/4	1/5
PCV2 PCR in PCV2 infected animals	–	1/3	3/3	3/3	4/4	0/5
Control	0/2	0/2	0/2	0/2	0/2	0/4

[a] Number of animals with a positive PCR result in serum/number of euthanised animals.

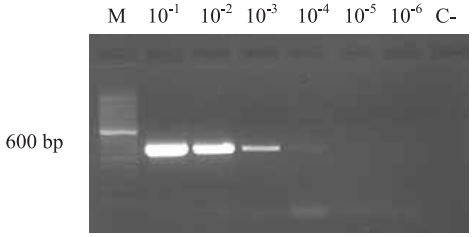

Figure 1. Sensitivity of PCV2 specific PCR. A PCV2 culture containing 10^{-1} PFU was tenfold serially diluted, its DNA extracted and the PCR performed with each dilution sample. M: 100 bp marker (Ecogen, Barcelona, Spain). C-: Negative control (double distilled water).

A PCV2 specific 657 nucleotide fragment was amplified in some of the mice inoculated with PCV2 that were euthanised between days 3 and 14 PI. No PCV2 amplification was detected in the serum from the mice of control or PCV1 inoculated groups. All PCR analyses from rabbit sera and tissues yielded negative results for both porcine circoviruses.

4. DISCUSSION

To date very little information has been published on the host range of PCV, but it is well known that non-porcine species, such as rats and mice, can be host reservoirs for some swine pathogens such as *Leptospira* spp., *Salmonella* spp. and *Brachyspira* spp. and that many pig farms are infested with wild rodents [10]. Therefore, since porcine circovirus infection is widespread in the pig population, mice can potentially act as a reservoir of PCV2 and PCV1. Also, the present experiment was designed to determine if non-porcine species, such as rodents (mice) and rabbits, were able to get infection and disease with porcine circoviruses (potential model of PMWS-like disease reproduction).

The present study showed that PCV1 and PCV2 genomes were found in the serum of intraperitoneally inoculated mice at different times of the experimental period. However, no microscopic lesions similar to those observed in swines suffering from PMWS or PCV2 nucleic acid by ISH were detected in the studied tissues of mice. Consequently, no conclusive demonstration of viral replication of PCV2 was achieved in ICR-CD1 mice. Since no other detection system for PCV1 is available besides PCR and serology, replication of PCV1 in ICR-CD1 mice could not be ruled out.

The presence of the PCV genome in the serum of mice could be explained by the possibility that the residual viral genome from the inoculum was still present in the blood stream at the time of necropsy. This fact would not be a surprise since any material inoculated by the intraperitoneal route may reach systemic circulation; the virus would be transported, either after phagocytosis by intraperitoneal macrophages or in suspension in the lymph, towards the cranial

sternal lymph nodes of the ventral thoracic lymphocentrum, and to the lymph nodes of the mediastinal lymphocentrum, prior to systemic dissemination, as has been demonstrated for *Listeria monocytogenes* and Indian ink [14]. However, since the PCV genome was detected in the blood by PCR up to the end of the experiment, the possibility that viral replication occurred at a level that was not detectable by ISH cannot be definitely excluded. A quantitative PCR method to detect porcine circoviruses in serum would have been very useful to confirm the possibility of PCV replication by the increase of PCV nucleic acid amounts on sequential days of study.

Antibodies to PCV1 have been previously demonstrated in mice [21]. Twenty-four mice were euthanised and bled before the starting of the experiment to be sure that the ICR-CD1 mice strain was free of antibodies to PCV1 and PCV2. From all mice, only one PCV1 inoculated mouse showed a very low serological titre to PCV1 at 20 days PI. The absence of an obvious seroconversion could be related to the short duration of the experimental period and antibodies may have appeared later, as previously described [11]. However, it cannot be elucidated whether this positive titre to PCV1 is a seroconversion related to the viral infection or whether it is due to the immunisation of the mouse.

The results of this study are in contrast to those obtained by Kiupel et al. [11], who found some evidence of PCV2 replication in tissues of BALB/c mice. The use of different mice strains, PCV2 isolates, experimental designs and doses of viral inoculation may explain the differences observed in both experiments. Phylogenetic analysis of all PCV2 isolates show the relationship of isolates from Europe, while North American sequences display a separate cluster [13]. This variation may have important implications for PCV2 diagnosis and research [7] and, therefore, it cannot be discarded that the PCV2 isolate variation may account for some of the differences observed between the present results and those from the study of Kiupel et al. [11]. In our study, the inoculum was of relatively low titre and a single inoculation was performed; however, no references were available on the amount of virus adequate to infect non-porcine species, and the titration system used in the study from Kiupel et al. [11] was not comparable with the one used in the present study. Another relative difference between the two experimental studies on PCV2 inoculation is the route of inoculation; Kiupel et al. [11] used a combination of intranasal and intraperitoneal routes while in this study we only used the intraperitoneal route of inoculation. Although the latter is not a likely route for natural infection, it is one of the most frequently used for experimental studies with different infectious agents or in pharmacological toxicology [14].

Previous techniques to detect either PCV1 or PCV2 have been used by our group to study these viral infections in swine [16]. Surprisingly, the PCV2 PCR previously used in swine sera and tissues yielded numerous nonspecific bands when working with rabbit and mice samples. The newly developed PCR technique to detect PCV2 showed high specificity and sensitivity, which was suitable for the use on sera of rabbits and mice of these experiments without any nonspecific amplification.

Rabbits were not susceptible to PCV1 or PCV2 infection under the described experimental conditions. The absence of clinical signs, macro and microscopic lesions, and PCV1 or PCV2 genomes indicated that intranasally inoculated rabbits did not become infected with these viruses. The results obtained are in agreement with other authors' findings [1], in which no serological evidence of PCV1 infection in rabbits was found. However, it cannot be ruled out that other inoculation routes, viral dosages, or lengths of the experiment would have different results concerning porcine circovirus infection in rabbits.

Four rabbits died during the experimental period and all of them had fibrino-purulent pleuritis and pericarditis. Since all rabbits were bled by intracardiac puncture on day 0 of the experiment to confirm its negative serologic status to porcine circoviruses, these lesions were probably the result of this handling and puncturing procedure. Bacteriological analysis was performed in only two of the dead rabbits and in both cases a pure culture of *Pasteurella multocida* was identified (data not shown).

On the contrary, since rabbits and mice were maintained under experimental conditions, they were not exposed to other environmental factors, exogenous microflora, other pathogens and immunostimulators. These other factors seem to play an important role in the development of PMWS in pigs [3, 12] and this effect cannot be discounted for non-porcine species.

In conclusion, the present study indicated that porcine circoviruses did not cause any disease or microscopic lesions in inoculated rabbits and mice after 20 days of an experimental period. Although PCV1 and PCV2 genomes were amplified from serum of mice by PCR, no evidence of viral replication was consistently detected.

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