Virus Research 165 (2012) 90–94

Contents lists available at SciVerse ScienceDirect

Virus Research

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

Short communication

Naked PCV-2 cloned genomic DNA is infectious by mucosal (intratracheal or oro-nasal) inoculation

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ARTICLE INFO

Article history:
Received 17 November 2011
Received in revised form 5 January 2012
Accepted 5 January 2012
Available online 14 January 2012

Keywords:
Viral genome
Mucosal inoculation
Porcine circovirus

ABSTRACT

Porcine circovirus type 2 (PCV-2) is involved in several diseases named porcine circovirus-associated diseases and is transmitted by oro-faecal route. In this study we inoculated porcine-circovirus free piglets by mucosal routes (intratracheal or oro-nasal routes) with a plasmid carrying two copies of PCV-2 genomic DNA and compared the results to the intramuscular route. We observed that this PCV-2 naked DNA serves as template for viral replication and infectious PCV-2 particles are detected in the whole body after parenteral (intramuscular) or mucosal (intratracheal or oro-nasal) delivery. These results suggest that PCV-2 genome could play a role in *in vivo* transmission.

Porcine circoviruses are small, non-enveloped viruses with a circular single-stranded DNA. Porcine circovirus type 1 (PCV-1) was discovered as a contaminant of porcine kidney cell lines (PK15 ATCC CCL33) but is considered non-pathogenic in pigs. Recently, it has been reported that full-length PCV-1 DNA contaminated a human rotavirus vaccine, Rotarix, administered to infants by oral route (Baylis et al., 2011; McClenahan et al., 2011; Victoria et al., 2010). On the other hand, porcine circovirus type 2 (PCV-2) is the causal agent of post-weaning multisystemic wasting syndrome (PMWS) and is associated with several diseases designated by porcine circovirus-associated diseases. Typical PMWS clinical signs are lethargy associated with growth retardation, skin pallor, hyperthermia and enlargement of inguinal lymph nodes. At necropsy, the main gross lesions consist of hypertrophy of lymph nodes. The histological lesions include lymphoid depletion, interstitial pneumonia and hepatitis (Gillespie et al., 2009). Although PCV-2 infection in herd is highly prevalent, co-factors as bacteria, virus and immunostimulation are often required to trigger PMWS (Madec et al., 2008). However, PMWS-affected pigs showed higher PCV-2 viral loads than non-affected pigs. The detection of PCV-2 genome in nasal, oral and faecal swabs demonstrates that the virus is probably shed through respiratory, oral secretions and faeces (Segales et al., 2005; Shibata et al., 2003). Moreover, the horizontal transmission of PCV-2 by direct or indirect contact has been confirmed experimentally (Andraud et al., 2008, 2009; Dupont et al., 2009). Airborne transmission is likely to occur since the viral genome was detected in aerosols in swine herds (Verreault et al., 2010). Molecular PCV-2 genomic DNA clones were developed to study the role of PCV-2 and of co-factors in PMWS pathogenesis and to develop PCV-2 vaccines. Two genomic clones of PCV-2 isolates from Europe and North America have been constructed to investigate if such genomic clones are infectious in pigs and could be used. Both were infectious and caused sub-clinical infection when injected in the liver and/or lymph nodes (Fenaux et al., 2003), by intramuscular or intraperitoneal routes (Roca et al., 2004). Using PCV-2 genomic DNA clone and an immunostimulation known to trigger PMWS, PMWS was reproduced in PCV-free pigs after inoculation by the intramuscular route (Grasland et al., 2005). Moreover PCV-1 naked DNA was found infectious *in vitro* (McClenahan et al., 2011). *In vivo* PCV-1 cloned genomic DNA led to the production of infectious viruses when injected intramuscularly as PCV-2 cloned genomic DNA (Fenaux et al., 2003). Since the PCV-2 may be shed through respiratory, oral secretions and faeces, we investigate in this study if PCV-2 cloned genomic DNA, previously described (GenBank accession number AF201311), is also infectious by mucosal routes, *i.e.* intratracheal or oro-nasal routes and compare the efficacy of these routes to the well-known intramuscular one.

In this work, thirty specific-pathogen-free (SPF) piglets, including free of PCV-1 and PCV-2 were used. The piglets weaned at four weeks of age, were housed in four separate SPF-growing rooms with a level 3 of biosecurity. The experiment was carried out in accordance with EU and French regulations on animal experimentation and with national guidelines for animal welfare. The first group of six piglets correspond to the non-inoculated control. The three other groups were composed of eight piglets inoculated with 400 μg of PCV-2 cloned genomic DNA (2 mL) by intramuscular (IM),

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doi:10.1016/j.virusres.2012.01.006

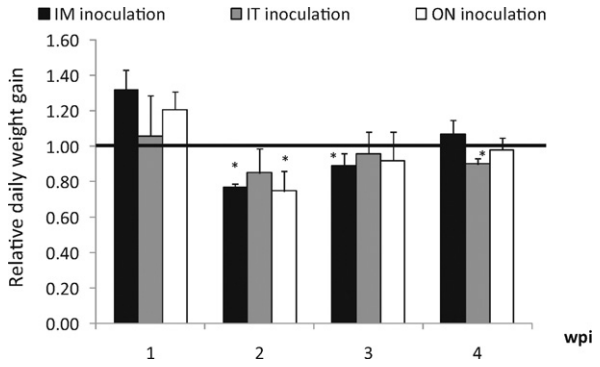

Fig. 1. Impact of the inoculation route of PCV-2 naked genome DNA on the relative daily weight gain assessed for each group by comparison of piglet daily weight gains with those of control piglets. The asterisk represents a significant difference ($p \leq 0.05$) with the control group indicated by a line with a value of 1.

intratracheal (IT) or oro-nasal (ON) (1 mL in the right nostril and 1 mL in the mouth) routes. Rectal temperatures and clinical observations were monitored daily. Weekly, piglets were weighed and blood samples collected. At 13 and 23 days post-inoculation (dpi), one pig in the control group and two in inoculated groups were euthanized by anaesthesia with Nesdonal® (Merial, France) followed by bleeding. The trial ended at 34 dpi and the remaining animals were slaughtered. At necropsy, macroscopic lesions were examined and a sample of tracheobronchial (TB) and inguinal (ING) lymph nodes (LN), tonsils, lung and liver were fixed in 10% buffered formalin (Sigma–Aldrich, MO, USA) for histopathological examinations as previously described (Grasland et al., 2005). Additional tissue samples were collected and stored at −80 °C for quantitative PCR and immuno-peroxidase monolayer assay (IPMA).

No hyperthermia was recorded in all piglets throughout the experiment (data not shown). A significant decrease of the relative daily weight gain was observed in animals inoculated by IM and ON routes in the second week post-inoculation ($p \leq 0.05$ with the non-parametric *U*-test of Mann and Whitney used throughout this study) (Fig. 1). In the third week, the relative daily weight gain of IM inoculated piglets was still significantly lower than that of the control group ($p \leq 0.05$). One week later, it was significantly lower for the IT inoculated group compared to the control group. At necropsy, no macro- and micro-lesions were detected in all control piglets. At 13 dpi, despite the absence of macrolesions, mild-to-severe lymphoid depletion was noticed in TB and ING LN and mild-to-moderate interstitial pneumonia in lungs in the three PCV-2 DNA-inoculated groups. At 23 and 34 dpi, whatever the route of inoculation used, only the TB LN were slightly enlarged (1/2 piglet per group at 23 dpi and 3/4 piglets at 34 dpi). At 23 dpi, the animals inoculated by IM and IT routes showed the same microlesions than earlier and in the ON group, the lesions were less serious. At 34 dpi, microlesions consisting of a mild-to-moderate lymphoid depletion in TB and ING LN and of a mild interstitial pneumonia in lungs were observed in IM and IT inoculated animals. The animals of the ON group presented only mild lymphocyte depletion in TB and ING LN. Moreover, PCV-2 capsid protein was detected in the organs of the IM, IT and ON groups at 23 and 34 dpi by immunohistochemistry as already described (Allemandou et al., 2011).

Serum antibodies to PCV-2 were detected using an ELISA test based on the recognition of a recombinant PCV-2 capsid protein/GST fused protein (Blanchard et al., 2003). All the animals of the control group remained PCV-2 seronegative throughout the study (Table 1). Two out of six animals that received PCV-2 DNA by the IM route developed antibodies against the PCV-2 capsid at 20 dpi. At the same time, only one out of six pigs in the IT and ON inoculated groups developed an immune response to the PCV-2 capsid protein. Afterwards, all the IM inoculated pigs seroconverted. At 34 dpi, two out of four animals that received PCV-2 DNA by IT or ON routes were PCV-2 seropositive.

Table 1
PCV-2 seroconversion assessed by PCV-2 ELISA.

Group	Day post-inoculation					
	0	6	13	20	27	34
Number of seropositive pigs						
Control group	0/6	0/6	0/6	0/5	0/4	0/4
Intramuscular group	0/8	0/8	0/8	2/6	4/4	4/4
Intratracheal group	0/8	0/8	0/8	1/6	2/4	2/4
Oro-nasal group	0/8	0/8	0/8	1/6	2/4	2/4

PCV-2 genomic load was assessed by quantitative real-time PCR (in TB, ING and mesenteric LN; tonsil, spleen, ileum, lung and liver) as previously reported (Grasland et al., 2005). No PCV-2 genomic DNA was detected in the organs collected from the negative control piglets throughout the experiment (data not shown). At 13 and 23 dpi, an individual variability in the PCV-2 genomic load was observed for the three inoculated groups (Fig. 2A). For the animals inoculated by IM or IT routes, all the tissues were positive for PCV-2 genomic DNA at 13 dpi with respectively a mean of 2.1×10^{11} and 5.7×10^{10} genomic copies/g of tissue (Fig. 2A). In the ON group, PCV-2 DNA was also found in all the tissues for one piglet but with a lower genomic load than in the two other groups (10^8 copies/g of tissue). For the second ON inoculated animal, PCV-2 DNA was only detected in tonsils and mesenteric LN at a level of 6×10^5 copies/g of tissue. At 23 dpi, all the organs of all pigs were positive for the presence of PCV-2 DNA when the IM or IT routes were used with quite similar individual genomic loads. The PCV-2 DNA quantity raised slightly with a mean of 2.9×10^{11} and 4.2×10^{11} in the IM and IT groups respectively. In the ON group, one piglet showed PCV-2 DNA in all the tested organs with an average of 9.2×10^9 copies/g of tissue and the other one had only four organs positive with 2.2×10^5 copies/g of tissue in average. At 34 dpi, the genomic loads in the three inoculated groups were not significantly different (Fig. 2B). The mean number of PCV-2 DNA copies remained at the same order of magnitude compared to the time before in IM and IT group (5.3×10^{10} and 5.4×10^{10} copies/g of tissue respectively). On the other hand, the genomic loads in the ON group increased with a mean value of 1.2×10^{11} copies/g of tissue which is more or less equivalent to the values of the two other inoculated groups.

The persistence of the PCV-2 clone DNA input in the organs (TB, ING and mesenteric LN; tonsil, spleen, ileum, lung and liver) was evaluated by quantitative real-time PCR with two primers (PCV-2 primer 5′TCCCGCTCTCCAACAAGGTA3′ and pBKS reverse primer 5′TCGATAAGCTTGATATCGAATTCCT3′) and a TaqMan probe (5′ FAM-CCTAGATCTCAGGGACAAC-MGB 3′) overlapping the junction between pBluescript vector and the PCV-2 genome in the PCV-2 DNA clone. The amplification parameters were similar to the PCV-2 quantitative PCR. No plasmid DNA corresponding to input of PCV-2 cloned DNA was detected in all the organs of the control and inoculated pigs throughout the trial. Thus the PCV-2 DNA detected in the organs of inoculated piglets was viral genomic DNA and not input plasmid DNA. This is in agreement with the rapid clearance of plasmid DNA (within 24 h) further to intranasal (IN) (Oh et al., 2001) or IM (Gravier et al., 2007) inoculation of plasmid DNA. Only 14% of the plasmid DNA administered by IN route reached the systemic circulation and are distributed to different organs including LN (Oh et al., 2001) against 10% when administered by IM route (Gravier et al., 2007).

The infectious viral titres were assessed in three lymphoid organs (tonsils, TB and ING LN) by IPMA as already described (Grasland et al., 2005). Purified naked PCV-2 cloned DNA was put