



RESEARCH ARTICLE

Unusual outcome of *in utero* infection and subsequent postnatal super-infection with different PCV2b strains

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VC2002, isolated from postweaning multisystemic wasting syndrome (PMWS)-affected pig, is a mixture of two porcine circovirus genotype 2b (PCV2b) viruses, K2 and K39. Preliminary experiments disclosed short-term adverse effects of K39, but not K2, on porcine fetuses. These findings led to the hypothesis that infection of immuno-incompetent fetuses with K2 confers a status of immunotolerance, and postnatal super-infection with K39 triggers PMWS. To explore this hypothesis, nine 55-day-old fetuses were inoculated *in utero* (three with K2-10^{4.3}TCID₅₀, three with K39-10^{4.3}TCID₅₀ and three with medium), and foeto-pathogenicity examined. At 21 days post-inoculation (dpi), K2 did not induce pathology, whereas pathological effects of K39 were evident. Twenty-four 45-day-old fetuses were subsequently inoculated to examine the long-term effect of K2, including six with K2-high dose-10^{4.3}TCID₅₀, six with K2-low dose-10^{2.3}TCID₅₀ and 12 mock-inoculated controls. Both doses resulted in five mummified fetuses and one live-born piglet each (69dpi). K2 was recovered from all mummies. K2 and K2-specific antibodies were not detected in serum of the two live-born piglets at birth, indicating full control of K2 infection. The K2-low dose-infected piglet was immunostimulated at day 2, but not the K2-high dose-infected piglet. Both non-stimulated and stimulated K2-infected piglets were super-inoculated with K39 at day 6 or 8 (taken as 0 days post super-inoculation). Low viral replication was observed in the non-stimulated K2-K39 piglet (up to 10^{3.3} TCID₅₀/g; identified as K39). In contrast, viral replication was extremely high in the stimulated K2-K39 piglet (up to 10^{5.6}TCID₅₀/g) and identified as K2, indicating that K2 infection is controlled during foetal life, but emerges after birth upon immunostimulation. However, none of the piglets showed any signs of PMWS.

KEYWORDS PCV2; immunotolerance; PMWS; porcine fetuses

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INTRODUCTION

Porcine circovirus 2 (PCV2) is the causative agent of postweaning multisystemic wasting syndrome (PMWS), a multifactorial disease that causes severe growth retardation, wasting and increased mortality in weaned pigs (Segalés J, 2012). PCV2 has additionally been isolated from aborted, mummified foetuses and nonviable neonatal piglets (Meehan B M, 2001; Brunborg I M, 2007). PCV2 strains are divided into two major genotypes, PCV2a and PCV2b (Segalés J, 2012), with significant antigenic homology between different strains (McNeilly F, 2001).

Several studies have attempted to experimentally reproduce PMWS by inoculating pigs with PCV2, which mainly led to subclinical infections (reviewed by Segalés J, 2012). At present, scientists working in basic and applied research fields, as well as vaccine development, have problems in studying the pathogenesis of PMWS and designing appropriate vaccine strategies due to the absence of a highly reproducible PMWS model.

Recent studies demonstrated that *in utero* PCV2 infection during insemination (Sarli G, 2012) or late gestation (Ha Y, 2008) results in live-born infected piglets highly susceptible to disease upon co-infection with other pathogens or immunostimulation (Ha Y, 2008). Two different PCV2 sequences had been identified in one pig (Lefebvre D J, 2008; Gerber P F, 2013). The PCV2 strain, VC2002, was originally isolated from an inguinal lymph node of a Belgian PMWS-affected pig (Meerts P, 2004). Our group subsequently discovered that VC2002 was a mixture of two PCV2b viruses, K2 and K39, displaying 94% identity at the nucleotide and amino acid levels (Lefebvre D J, 2008). Preliminary analysis of K2 and K39 in porcine foetuses inoculated at 55 days of gestation revealed different biological characteristics (Saha D and Nauwynck HJ, unpublished results). Notably, K39 induced pathol-

ogy in foetuses collected at 21 days post-inoculation (76 days of gestation), whereas no pathology was evident in K2-infected foetuses. This finding prompted the hypothesis that *in vivo* infection of embryos/foetuses with the comparatively less foetopathogenic PCV2, K2, at early stages of gestation (before the development of immunocompetence) confers a status of immunotolerance at birth. Postnatal super-infection of such immunotolerant animals with the highly foetopathogenic PCV2, K39, should therefore cause disease and no recognition by the immune system.

The aim of the present study was to examine the above theory by generating PCV2-immunotolerant pigs via intra-uterine inoculation of K2 into immuno-incompetent porcine foetuses and analysing the outcome of postnatal super-infection with K39 after birth.

MATERIALS AND METHODS

Viruses and cells

Two different PK-15-adapted PCV2b strains, K2 (EF990645) and K39 (EF990646) (Lefebvre D J, 2008), were used. PCV-negative PK-15 cells were grown in minimal essential medium (MEM) containing Earle's salts and GlutaMAX™-I (MEM+GlutaMAX™-I, Gibco, Grand Island, USA) and supplemented with 5% foetal calf serum (FCS), 100 U/mL penicillin, 0.1 mg/mL streptomycin and 0.1 mg/mL kanamycin. Cell cultures were maintained at 37°C in the presence of 5% CO₂ (Saha D, 2010).

Short-term outcome (21 days post inoculation/76 days of gestation)

At 55 days of gestation, three conventional PCV2-seropositive Landrace sows (S1, S2 and S3) were subjected to laparotomy as described earlier by Saha D (2010) (Table 1). Three foetuses of each sow were inoculated: one with K2-10^{4.3} TCID₅₀, one with K39-

Table 1. Virus replication in different foetal organs after intra-foetal inoculation with K2 or K39 at 55 days of gestation, collected at 21 days post-inoculation

Sow no.	Foetus no.*	PCV2 strain	PCV2 replication																	
			Heart		Lungs		Spleen		Liver		Kidneys		Thymus		Tonsils		Ileum		Cerebrum	
			VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF	VT	IIF
S1	L1	K2	6.3	900	3.0	253	5.7	NA	5.2	10353	2.7	587	5.6	NA	3.7	NA	4.1	NA	2.0	10
S2	R3	K2	5.5	976	4.7	2363	6.3	23803	5.3	12110	5.0	2890	5.8	7073	4.8	3416	4.3	3850	2.5	13
S3	L2	K2	4.0	580	3.5	1260	5.6	8320	5.0	2337	2.5	880	4.8	10383	4.1	1297	4.8	670	2.5	17
S1	L2	K39	6.0	3550	2.5	223	4.5	1160	2.8	1337	<1.7	10	3.0	11883	3.8	13	1.8	63	<1.7	20
S2	L2	K39	6.0	4027	2.2	3	3.5	80	3.9	1297	2.1	27	3.0	30	2.8	37	3.0	10	<1.7	0
S3	L3	K39	5.8	4350	3.0	20	3.8	163	4.3	1137	2.7	3	4.3	NA	2.8	NA	1.8	NA	2.5	0

*L = left horn; R = right horn. Numbering of foetuses is in sequence from ovary to cervix.

VT = Virus titres (log₁₀TCID₅₀/g); IIF = Indirect immunofluorescence staining (number of PCV2-positive cells /10 mm²); NA = not available

$10^{4.3}$ TCID₅₀ and one with PK-15 cell culture medium. Inoculation and operative procedures were performed according to the above report (Saha D, 2010). Sows were housed separately, and clinical signs monitored as exactly as described in Saha D (2010). At 21 days post inoculation (dpi), sows were humanely euthanized. All foetuses were examined for gross lesions. Foetal tissue samples were obtained for histopathological examination, virus titration and indirect immunofluorescence analyses (Saha D, 2010). Serum and abdominal fluid from all foetuses and pre-serum (before laparotomy) and post-serum (at the time of euthanasia) of sows were collected.

Long-term outcome (69 days post inoculation/114 days of gestation)

Four conventional PCV2-seropositive Landrace sows were subjected to laparotomy on the left side of the abdomen at 45 days of gestation. Six foetuses of each sow were inoculated (three with K2 and three mock-inoculated with PK-15 cell culture medium). In the first two sows (S4 and S5), six foetuses (three for each sow) were inoculated with a K2-high dose- $10^{4.3}$ TCID₅₀/foetus, whereas six foetuses (three for each sow) from the other two sows (S6 and S7) were inoculated with a K2-low dose- $10^{2.3}$ TCID₅₀/foetus (Table 2). The procedures of laparotomy and intra-foetal inoculation of virus or medium were identical to those described previously (Saha D, 2010). At 69 days post inoculation (114 days of gestation), a second laparotomy procedure was carried out on the right side of the abdomen, following which all foetuses were collected (Table 2). Proper care was taken during collection of foetuses to avoid any possibility of PCV2 spread from the virus-inoculated to mock-inoculated foetuses. Each of the collected newborn piglets was housed individually in separate isolators.

Blood was collected at birth from the umbilical cord of K2-inoculated and mock-inoculated piglets. Serum samples of the sows were obtained prior to the first (pre-serum) and second laparotomy (post-serum) procedures.

Super-inoculation of *in utero* K2- or mock-inoculated piglets with PCV2 (K39)

Live-born K2- and mock-inoculated colostrum-deprived, caesarean-derived (CD/CD) piglets from S4 and S5 or S6 and S7 were oronasally super-inoculated with either K39- $10^{4.3}$ TCID₅₀/pig or medium at days 6 and 8, respectively. Piglets from S6 and S7 were vaccinated against parvovirus (Parvoject, Merial, Belgium) on day 2. Piglets were monitored daily for PMWS-associated symptoms (Segalés J, 2012). Half of the left and right inguinal lymph nodes (ILNs) were biopsied at 14 and 21 days post super-inoculation (dpsi), respectively (Meerts P, 2005) and the remaining ILNs collected at euthanasia (28 dpsi). All pigs were bled at 0, 7, 14, 21 and 28 dpsi.

Table 2. Clinical outcome of pigs inoculated intra-foetally with PCV2-K2 or mock-inoculated at 45 days of gestation and collected at 69 days post-inoculation (at birth)

Sow no.	Numbering of pigs at birth	Intra-foetal inoculation with	Dose (log ₁₀ TCID ₅₀)	Clinical outcome at birth (69 dpi)
S4	F1	K2	4.3	live
	F2	K2	4.3	mummified
	F3	K2	4.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live
S5	F1	K2	4.3	mummified
	F2	K2	4.3	mummified
	F3	K2	4.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live
S6*	F1	K2	2.3	live
	F2	K2	2.3	mummified
	F3	K2	2.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live
S7*	F1	K2	2.3	mummified
	F2	K2	2.3	mummified
	F3	K2	2.3	mummified
	F4	mock	-	live
	F5	mock	-	live
	F6	mock	-	live

*live-born pigs from sows S6 and S7 were immunostimulated.

Tissue suspensions (10% or 20% wt/vol) were prepared from collected organs, and PCV2 titres determined. PCV2-positive cell numbers in different organs were estimated using indirect immunofluorescence staining (IIF) (Saha D, 2010). For IIF, anti-PCV2 monoclonal antibodies (mAbs) F190 (1:500), F217 (1:100) (McNeilly F, 2001) or biotin-conjugated anti-PCV2 polyclonal antibodies (pAbs) (1:50) in PBS and fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse pAbs (Molecular Probes, USA) or FITC-labelled streptavidin (1:200) (Molecular Probes) in PBS were used as primary and secondary antibodies, respectively. The number of PCV2-positive cells per 10 mm² of tissue was determined (Saha D, 2010).

To differentiate between K2 and K39, double immunofluorescence staining was performed in ILNs (Saha D, 2011). The mAbs 114C8 (IgG1; reacts with K2 and K39) and 16G12 (IgG2a; reacts with K39) diluted 1:10 (Saha D, 2012a) were used as primary and 1:200 diluted mAbs FITC-labelled goat-anti-mouse IgG1 and goat-anti-mouse IgG2a Alexa-Fluor 594 (Molecular Probes) as secondary antibodies. The number of PCV2-positive

cells was determined as above. DNA was extracted from heart and lung tissue suspensions of PCV2-inoculated and adjacent foetuses (21 dpi) as well as piglet sera (69 dpi) (Saha D, 2010). Amplification and sequencing of the PCV2 capsid gene were performed in keeping with previous reports (Saha D, 2010; Saha D, 2012b).

PCV2-specific Ab titres in serum or abdominal fluids of foetuses were determined with the immuno-peroxidase monolayer assay (IPMA) (Saha D, 2010; Saha D, 2014). Sow antibody titres against PCV2 and PRRSV were assessed with IPMA and against porcine parvovirus (PPV) with the hemagglutination inhibition (HI) test (Saha D, 2010). PCV2-specific IPMA Abs in sera of piglets were collected at birth and 0, 7, 14, 21 and 28 dpi. PCV2-neutralising Abs against K2 or K39 were determined using the classical or sensitive neutralisation assay (Meerts P, 2005; Saha D, 2012c).

RESULTS

Short-term outcome

Evaluation of sows – All three sows remained clinically healthy during the study period. The PCV2-specific IPMA Ab titre was 40,960 in both pre- and post-sera of the sows. PRRSV-specific IPMA and PPV-specific HI Ab titres were <10 and ranged from <8 to 8, respectively, in pre-serum, and identical in post-serum.

Macroscopic lesions – At 21 dpi, K2-inoculated foetuses had a normal external appearance (Figure 1A). No gross lesions were observed in the different organs, except for enlarged spleen in one foetus (S2R3). K39-inoculated foetuses showed oedema, hydrothorax, hydroperitonium, ascites and distended abdomens (Figure 1B).

Microscopic lesions – No histopathological lesions were observed in the different organs of K2-inoculated

foetuses, except one, which displayed infiltration of neutrophils in spleen (S2R3). Histopathological lesions, mainly haemorrhage and infiltration of inflammatory cells, were present in heart, lung, liver, spleen and kidney of K39-inoculated fetuses.

PCV2 replication – In K2- and K39-inoculated foetuses, highest PCV2 titres (\log_{10} TCID₅₀/g) were found in the heart (5.3 ± 1.2 and 5.9 ± 0.1 , respectively). Virus titres in different organs are shown in Table 1.

Moderate to high numbers of PCV2-infected cells/10 mm² tissue (900, 976 and 580 in K2- and 4027, 4350 and 3550 in K39-inoculated foetuses) were estimated in the heart. PCV2-positive cell numbers in different organs are presented in Table 1.

Serology – Both K2- and K39-inoculated foetuses were negative (<10) for PCV2-specific IPMA Ab, except one (inoculated with K39), which developed very low anti-PCV2 titres (40).

Long-term outcome

Evaluation of sows – All four sows remained clinically healthy during the study period. The PCV2-specific IPMA antibody titre was 40,960 in both pre- and post-sera of the sows. PRRSV-specific IPMA and PPV-specific HI antibody titres were <10 and ≤ 8 , respectively, in pre-serum, and identical in post-serum.

Evaluation of piglets at birth – Intra-foetal inoculation of six foetuses each from two sows with K2-high dose- $10^{4.3}$ TCID₅₀ at 45 days of gestation resulted in five mummies and one live-born piglet in both cases. Similar results were obtained with six foetuses each from the other two sows inoculated with K2-low dose- $10^{2.3}$ TCID₅₀ (Table 2). Recovered mummies had variable crown-rump lengths, indicating that foetuses died at different times *in utero* (data not shown). Live-born *in utero* K2- and



Fig.1. Effects of PCV2 replication on 55-day-old fetuses inoculated collected at 21 days post-inoculation. a) K2-inoculated foetus with normal external appearance. b) Subcutaneous oedema and abdominal distension in K39-inoculated foetus.

and K39-inoculated foetuses, K2-positive cells were 4.9 times lower than K39-positive cells. This may be due to the small focal distribution of K2 infection, compared to wide dispersion of K39 infection throughout heart tissue. Although K2-positive cell levels were lower, the number of apoptotic cells in hearts of K2- and K39-inoculated foetuses was similar (data not shown).

While K2 did not induce pathology in mid-gestational foetuses, intra-foetal inoculation of a high or low dose of K2 at 45 days of gestation resulted in a negative long-term effect (10 mummies out of 12 K2-inoculated foetuses), confirming the hypothesis that K2 needs longer time to cause foetal damage. In the two *in utero* K2-inoculated live-born piglets, K2 virus and specific antibodies were not detected at birth, indicating that young foetuses had fully controlled K2 infection, possibly through raising an innate immune response. Most cellular components of innate immunity, including natural killer (NK) cells, are found in porcine foetuses before 45 days of gestation (Sinkora M, 2003) but do not show killing activity until activation by microbial pathogens (Sinkora M, 2009). It is possible that NK cells gain their full functional status upon intra-foetal inoculation of PCV2 at 45 days of gestation, which eventually restricts PCV2 replication in foetuses. Wrong inoculations can be excluded, since we have demonstrated 100% success in performing transuterine, intra-foetal inoculations in the past (Saha D, 2010; Saha D, 2011). The accuracy of intra-foetal inoculation of K2 is further confirmed by recovery of viruses from the mummies.

Postnatal super-inoculation of *in utero* mock-inoculated piglets with K39 resulted in low levels of K39 replication, clearly signifying that K39 has different replication kinetics from foetal to early postnatal life. This finding confirms the results of Sanchez RE (2003). Super-inoculation of *in utero* K2-inoculated piglet (S4F1) with K39 did not result in disease, consistent with the observed low level of viral replication. High PCV2 replication is a prerequisite for the successful development of disease or PMWS (Meerts P, 2006). The late onset of humoral immune response in S4F1 indicates somewhat disturbed immunological response. Serological analysis indicated that viral replication in S4F1 was associated with K39, not K2, in view of the higher anti-K39 Ab levels. Although viral replication was associated with K39 in both K2-K39 (S4F1) and mock-K39 (S4F4 to S4F6) piglets, the S4F1 piglet had exceptionally low anti-K39 titres, compared to mock-K39 piglets. A low anti-K39 titre in S4F1 may indicate a state of partial immunotolerance, which develops due to intra-foetal K2 inoculation at 45 days of gestation. Immunofluorescence staining similarly showed that viral replication in S4F1 was associated with K39, not K2, which was further established by sequencing of the capsid gene (data not

shown). Our results clearly indicate that K2 infection is fully controlled by the young foetus without full priming of the immune response. S6F1, inoculated with a low dose of K2 *in utero*, developed a PCV2-specific immune response early after birth, indicative of re-activation of K2. This may be associated with parvovirus vaccination after birth. Super-inoculation of S6F1 with K39 led to extremely high viral replication, which was subsequently remarkably reduced, probably due to the appearance of K2-neutralising antibodies. Serological analysis showed higher levels of anti-K2 IPMA Abs than anti-K39 Abs, signifying that viral replication in S6F1 is associated with K2, not K39. K2 replication was further confirmed by immunofluorescence staining and sequencing of the capsid gene (data not shown). Based on the results, we suggest that K2 infection is silent during foetal life but activated after birth upon vaccination. Another possibility is that K39 replication occurs in S6F1, but at a very low level in the period before sampling of the lymph nodes (14 dpi).

None of the piglets (except S6F1 at 28 dpi) developed neutralising Abs. The appearance of neutralising antibodies against PCV2 could be age-dependent. An earlier study by Meerts and co-workers (2006) showed that young pigs, especially CDCD or specific pathogen free (SPF) piglets, developed no or low-level neutralising antibodies against PCV2. Cytotoxic T lymphocytes (CTL) are thought to play a role in determining the immune responses capable of neutralising PCV2.

In conclusion, our study demonstrated that PCV2 K2 is not pathogenic to porcine foetuses over a short-term period, while K39 displays pathogenicity. However, long-term infection with K2 resulted in the birth of both mummies and viable piglets. Moreover, super-inoculation of live-born K2-infected piglets with K39 did not result in disease. Further studies are necessary to identify PCV2 strains with very low pathogenicity to inoculate foetuses with the aim of producing PCV2-immunotolerant pigs. Work in this context is ongoing.

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COMPLIANCE WITH ETHICS GUIDELINES

All authors declare no competing interests. The animal

experiments described in this manuscript were authorised and supervised by the Ethical and Animal Welfare Committee of the Faculty of Veterinary Medicine of Ghent University, Belgium.

AUTHOR CONTRIBUTIONS

DS and HJN designed the study. DS, HJN, UUK, MG and MV performed the experiments. DS, HJN, DJL and PM analysed data and wrote the manuscript. RD analysed histopathological data. JVD and LH carried out PCR and sequencing.

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