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Replication kinetics of different porcine circovirus 2 strains in PK-15 cells, fetal cardiomyocytes and macrophages

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Summary. In this *in vitro* study, the replication kinetics of porcine circovirus type 2 (PCV2) in porcine alveolar macrophages (PAM) and fetal cardiomyocytes (FCM), two target cells *in vivo*, was compared with that in PK-15 cells. Cultures were inoculated with either the postweaning multisystemic wasting syndrome (PMWS)-associated strain Stoon-1010 or the abortion-associated strain 1121. Viral proteins were visualized and virus production was determined. In PK-15 cells, the capsid protein was expressed between 6 and 12 hours post inoculation (hpi), it relocated to the nucleus between 12 and 24 hpi. At that time, Rep protein was also detected in the nucleus. This sequence of events also occurred in FCM and PAM but nuclear localized antigens appeared later (48 hpi) and in a lower percentage of cells. In PAM, clear differences in susceptibility were seen between pigs. In PAM from two out of five tested pigs, nuclear localized antigens were not detected, whereas in PAM from three other pigs they were seen in up to 20% of the antigen-positive cells. Virus production was observed in PK-15 but not in PAM or FCM cultures. In a second study, the replication kinetics of seven different PCV2 strains were compared in PK-15 cells. It was shown that the two abortion-associated strains had a different replication kinetics in comparison with PMWS or porcine dermatitis and nephropathy syndrome associated strains. With the abortion-associated strains, a higher number of infected cells was observed at 24 hpi and the percentage of infected cells with nuclear localised antigens was lower compared to that of other strains.

Introduction

The genus *Circovirus* in the family *Circoviridae* contains 2 porcine viruses: porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2). PCV1 was first described by Tischer et al. [25] as a contaminant in the porcine kidney cell line PK-15. Experimental inoculations with this virus in susceptible pigs did not result in clinical signs or pathological lesions [2, 26] and as a result, PCV1 is generally recognised to be non-pathogenic. PCV2 was first isolated in 1997 from a piglet affected by the postweaning multisystemic wasting syndrome (PMWS) [7]. More recently, this virus was also detected in aborted and stillborn foetuses [12, 31]. Experimental inoculations with PCV2 strain Stoon-1010 which was isolated from a case of PMWS [7] and with PCV2 strain 1121, isolated from aborted fetuses [17] have confirmed the crucial role of PCV2 in the reproduction of both syndromes [3, 21].

The circovirus structure is characterised by its relative simplicity. The 1.8 kb single-stranded genome consists of a single circular ambisense DNA molecule [25]. Transcripts of 2 open reading frames have been characterized from PCV1-infected cells. Open reading frame 1 (ORF1), situated on the viral DNA strand, is transcribed into two collinear transcripts: Rep and Rep' [14, 24]. Both proteins form a complex that is involved in the replication of the virion ssDNA replication [15]. Open reading frame 2 (ORF2), situated on the complementary DNA strand, codes for the capsid protein [19] which has a molecular weight of 30–35 kDa [5, 19]. In order to express the capsid protein, it is believed that the complementary strand has to be synthesized through the intermediate double-stranded replication form (RF) as described for geminiviruses [9, 28]. Since circoviruses do not code for their own DNA-polymerase for the production of the double-stranded RF, they depend on a host DNA-polymerase to complete replication of their genome. The requirement for host DNA-polymerases accounts for the observation that PCV1 requires PK-15 cells in the S-phase of the mitosis cycle to complete its infectious cycle [27].

Immunophenotyping of the target cell of PCV2-replication *in vivo* has shown that the susceptible cell population in the pig depends on the stage of development of the host at the time of infection. In *in utero* inoculated fetuses, infected cells were identified as cardiomyocytes, hepatocytes and macrophages during early gestation and mainly macrophages towards the end of gestation [22]. Postnatally, in the majority of pigs showing low or moderate PCV2-replication the infected cells were demonstrated to be macrophages ($SWC3^+$/$sialoadhesin^+$). In contrast in the low percentage of pigs showing high PCV2 replication, infiltrating monocytes ($SWC3^+$/$sialoadhesin^-$) were also positive for PCV2 antigens [23].

In the continuous cell line PK-15, a productive infection has been demonstrated, starting with detection of viral antigens in the cytoplasm and the nucleus of infected cells at 18 hours post inoculation (hpi) and a release of progeny virus at 32 hpi [5]. This replication cycle differs with *in vitro* observations in PCV2-inoculated porcine monocytes and macrophages in which PCV2 capsid protein was detected in the cytoplasm but for which no evidence of further virus replication was found [1, 8]. In the absence of any evidence of active virus replication,

it was presumed that the presence of capsid protein inside the cell was due to accumulation of viral antigens derived from the inoculum rather than from expression upon transcription of the virus' genome.

Although the genetic similarity between PCV2 strains from cases of PMWS or abortion is high (>95%), differences in biological properties between strains may still exist. Small genetic differences may have important consequences in tropism of viruses as has been described for closely related viruses such as transmissible gastroenteritis virus and porcine respiratory coronavirus [20]. Consequently, although a spectrum of closely related PCV2 genotypes have been characterized from a wide range of clinical syndromes, little information is available regarding possible differences in pathogenicity or tropism.

The aims of this study were (a) to examine PCV2 replication kinetics in porcine fetal cardiomyocytes and porcine alveolar macrophages, two major target cells *in vivo* and to compare it with replication kinetics in PK-15 cells and (b) to investigate if differences in replication kinetics exist between PCV2 strains.

Material and methods

Viruses and cells

Seven PCV2 strains were enclosed in this study. The origin and passage history of these strains are shown in Table 1.

PCV negative PK-15 cells were seeded on glass coverslips (BELLCO®, Vineland USA) at 150,000 cells per 3.15 cm^2 in minimal essential medium (MEM) with Earle's salts (GIBCO BRL®, Grand Island, USA) supplemented with 5% fetal bovine serum (FBS), 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cells were maintained at 37 °C in the presence of 5% CO_2 for 24 h to obtain a 50% confluent monolayer.

Fetal cardiomyocytes (FCM) were obtained by trypsinisation of heart muscle tissue of foetuses at 75 to 100 days of gestation. Fetal hearts were collected and washed in PBS. The heart muscle tissue was minced and incubated for 5 min in PBS containing trypsin (Sigma, Bornem, Belgium) (2.5 mg/ml) at 37 °C. Trypsinized cells were collected, filtered, cooled to 4 °C in FBS and centrifuged at 300×g for 10 min. The cell pellet was resuspended in

Table 1. Origin and passage history of porcine circovirus 2 strains used in this study

Strain	Origin		Passage level in PK-15 cells
	isolated from...	country [reference]	
Stoon-1010	PMWS affected piglet	Canada [7]	25
1121	aborted fetuses	Canada [17]	21
1103	aborted fetuses	Canada [17]	20
48285	PMWS affected piglet	France [17]	16
VC2002	PMWS affected piglet	Belgium [18]	16
1206	PMWS affected piglet	Belgium	23
1147	PDNS affected piglet	UK [17]	15

PMWS: postweaning multisystemic wasting syndrome
PDNS: porcine dermatitis and nephropathy syndrome

MEM with Hank's salts (GIBCO BRL®, Grand Island, USA) with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. The cells were then seeded onto coverslips and incubated at 37 °C. After 24 hours incubation, medium was replaced by MEM with 10% FBS, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Cardiomyocytes were incubated at 37 °C in the presence of 5% CO_2 until the culture reached 50% confluency. Cardiomyocytes were identified by immunofluorescence stainings with monoclonal antibodies directed against desmin [10] (DAKO Diagnostics, Glostrup, Denmark). Cell cultures from fetal heart tissue contained 90–100% desmin-positive cells showing mitosis in culture.

Porcine alveolar macrophages (PAM) were isolated from lungs of 4-week-old conventional piglets as previously described [11]. Briefly, the lungs were flushed with cold phosphate-buffered saline (PBS). The washing fluid was centrifuged and cells were resuspended in cooled RPMI 1640 medium (GIBCO BRL®, Grand Island, USA) supplemented with 10% dimethyl sulfoxide (DMSO), 30% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin frozen and stored in liquid nitrogen. For subsequent experimentation, PAM were thawed and seeded at 1×10^6 cells per glass coverslip. After 2 h incubation in RPMI 1640 medium supplemented with 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 1% non-essential amino acids 100× (GIBCO BRL®, Grand Island, USA) and 1 mM sodium pyruvate at 37 °C in the presence of 5% CO_2, coverslips were washed to remove non-adherent cells and 1.5 ml of the earlier mentioned medium supplemented with 10% FBS was added. Batches of PAM derived from five different piglets were used in this study. Cells in lung washing fluids were found to contain >95% macrophages, using the monoclonal antibodies SWC3 and 41D3 directed against cells of the monocyte/macrophage lineage and against differentiated macrophages respectively [4, 29].

Inoculation

Cultures of PK-15 cells, FCM or PAM were inoculated with $10^{4.2}$ $TCID_{50}$ of the respective strains, suspended in 0.5 ml MEM (PK-15, FCM) or RPMI (PAM). After 1 h incubation at 37 °C in the presence of 5% CO_2, cell cultures were washed once with medium and further incubated in fresh medium. A mock-inoculated control was included in each experiment.

Fixation and stainings

At 0, 6, 12, 24, 36, 48 and 72 hpi (cell cultures inoculated with reference strains 1010 and 1121) or 0, 12, 24, 48 and 72 hpi (cell cultures inoculated with other strains), the supernatant of inoculated cultures was collected, centrifuged at 15000×g for 10 min and stored at −70 °C until virus titration. Cell cultures on coverslips were fixed in acetone at −20 °C for 20 min, dried and stored at −20 °C.

PCV2 antigen-positive cells were stained by subsequent incubation with biotinylated, purified monospecific porcine polyclonal antibodies against PCV2 [22] and streptavidin-FITC (Molecular Probes, Leiden, The Netherlands). The total number of PCV2 antigen-positive cells was determined by counting the positive cells present in a total of 50,000 to 100,000 cells, depending on the confluency of the culture. The positive cells were further sub-divided into two fractions: cells with exclusive cytoplasmic localisation of antigens and cells with nuclear and cytoplasmic localisation of antigens. All experiments were repeated three times and mean values were calculated. To confirm that a positive signal was due to expression of viral proteins and not due to viral antigens taken up by the cell, PK-15 cells and PAM were inoculated with UV-treated strain Stoon-1010 and stained as described above.

Western blot analysis of the porcine polyclonal antibodies showed that the antibodies detected a 35 kbp protein which corresponds to the molecular size of the PCV2 capsid protein.

No specific bands could be detected in the region of the Rep or Rep′ protein, indicating that the polyclonal antibodies only recognized the capsid protein. This result was confirmed by performing double immunofluorescence stainings using the biotinylated porcine polyclonal antibodies and mouse monoclonal antibodies (Mab) directed against PCV2 proteins (F210 directed against Rep protein and F217 or F190 against capsid protein) [16] on PCV2-infected PK-15 cells. Mab F217 positive signals always colocalized with polyclonal antibody positive signals, indicating that both antibodies recognised the same protein. In contrast, some cells positive for F210 were not stained by the polyclonal antibodies, indicating that the polyclonal antibodies did not recognise the Rep protein. These findings correspond with the results in a previous study where it was shown that porcine hyperimmune serum only reacted with capsid protein [5].

Expression kinetics of both known PCV2 proteins (capsid and Rep protein) were assessed using Mab F210 (directed against Rep protein) and F217 or F190 (against capsid protein). Bound Mab were visualised with goat-anti-mouse-FITC or goat-anti-mouse-Texas Red (Molecular Probes, Leiden, The Netherlands). Double immunofluorescence stainings were performed to detect both viral proteins and their colocalization in PK-15 cells. Inoculated cell cultures were incubated with Mab F210 (IgG_1) against Rep protein and F190 (IgG_{2b}) against capsid protein. Bound Mab were visualised with isotype specific secondary antibodies (Serotec, Oxford, United Kingdom). Hoechst 33342 (Molecular Probes, Oregon, USA) at a concentration of 10 μg/ml was used in all immunofluorescence stainings to visualize the nucleus. Viral antigen positive cells were counted by fluorescence microscopy. Digital images were made using a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Heidelberg, Germany).

Virus titration of culture supernatant

Progeny virus titres in culture supernatants were determined by inoculation of 10-fold dilution series on PK-15 cells. After 72 h of incubation at 37 °C in the presence of 5% CO_2 supernatant was removed, cells were fixed in 10% paraformaldehyde in PBS and viral antigens were detected using an immunoperoxidase monolayer assay (IPMA), as described elsewhere [21].

Results

PCV2 replication kinetics of reference strains Stoon-1010 and 1121 in PK-15 cells

Considerable differences were observed between replication kinetics of strains Stoon-1010 and 1121 (Fig. 1). After inoculation with strain Stoon-1010, cells with viral antigens in the cytoplasm were detected starting from 12 hpi using the polyclonal antibodies and the numbers of positive cells increased in time. At 24 hpi, 0.3% of the cells expressed viral antigens, a maximum of 1.8% viral antigen positive cells was reached at 48 hpi. Starting from 24 hpi, nuclear localized viral antigens were seen in up to 25% of the positive cells. Inoculation with strain 1121 resulted in viral antigen-positive cells detectable from 6 hpi and rapidly increasing in time to 1.8% at 24 hpi and a maximum of 3.0% at 48 hpi. Starting from 12 hpi, nuclear localised viral antigens were seen in 1–5% of the positive cells. Foci of infected neighbouring cells were seen starting from 36 hpi with both strains. Inoculation with UV-inactivated PCV2 resulted in the absence of a signal after staining with the polyclonal or monoclonal antibodies. The changes of antigen expression patterns in Stoon-1010 inoculated PK-15 are shown in Fig. 2.

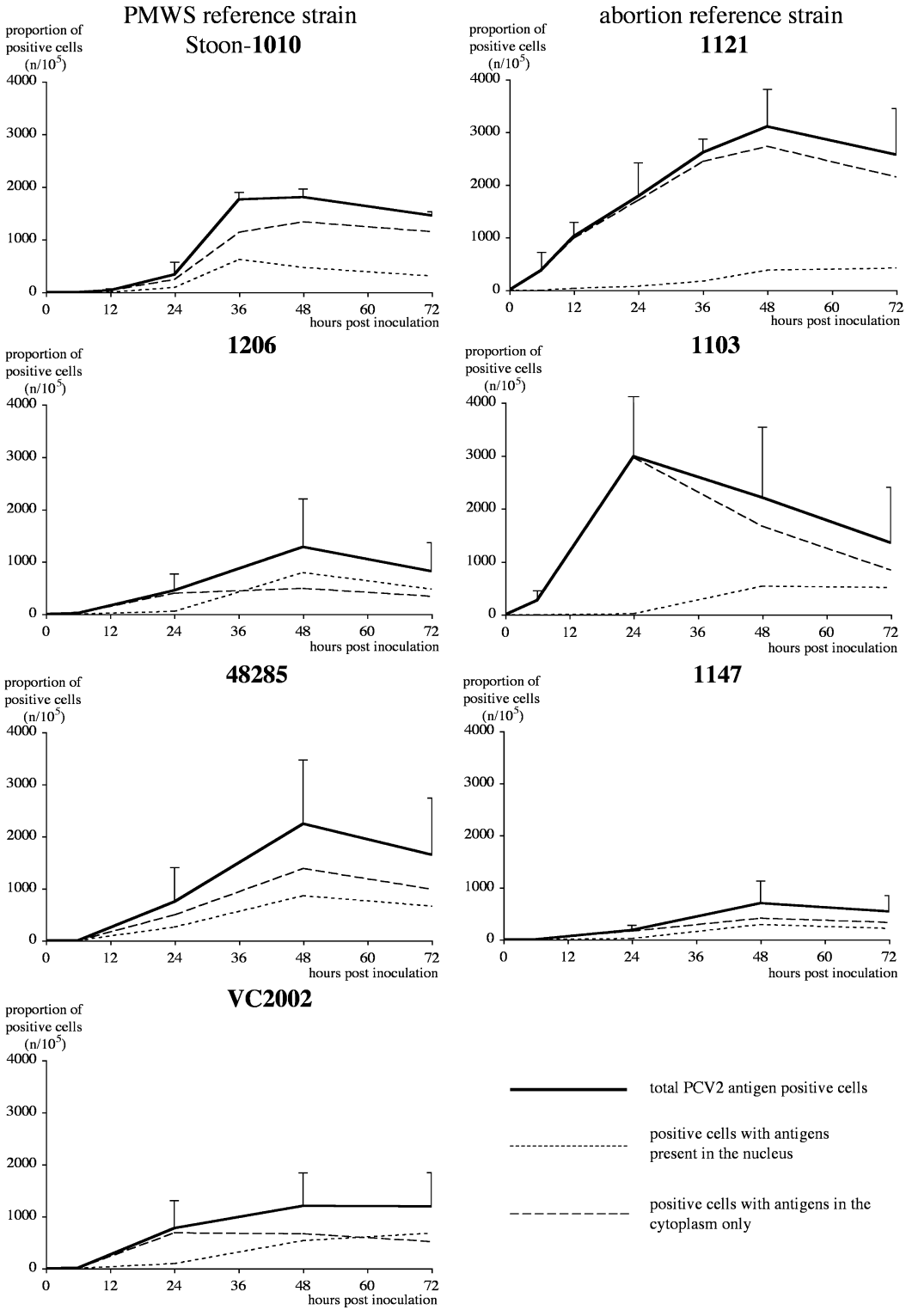

Fig. 1. Number of porcine circovirus 2 antigen-positive PK-15 cells and antigen expression pattern at different time points after inoculation with strains Stoon-1010, 1121, 48285, 1103, 1206, 1147 and VC2002

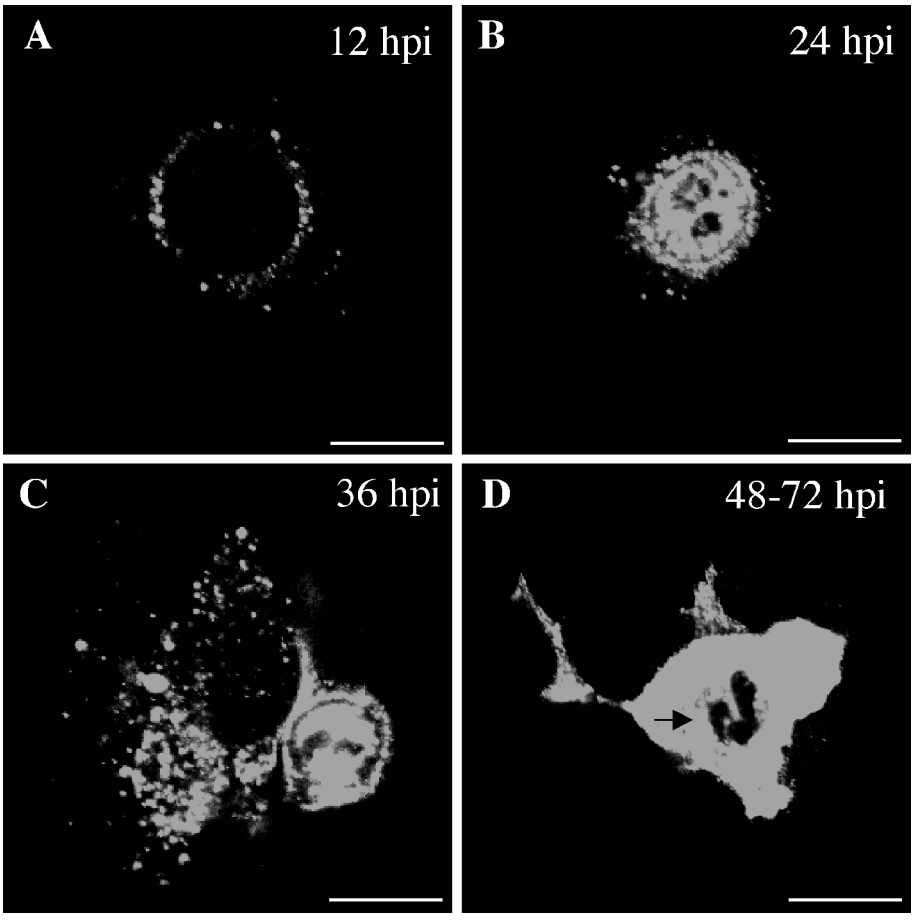

Fig. 2. Changes in antigen expression patterns in porcine circovirus 2 infected PK-15 cells. Evolution in antigen expression pattern in Stoon-1010 inoculated PK-15 cell cultures, stained with polyclonal anti-PCV2 antibodies. **A**: PCV2 antigens in the perinuclear region of the cytoplasm at the early stage of infection (12 hpi). **B**: expression of PCV2 antigens in cytoplasm and nucleus (24 hpi). **C**: foci of infected cells (36 hpi). **D**: irregularly shaped cell with intense viral antigen positive cytoplasm and a viral antigen negative and deformed nucleus (arrow) (48–72 hpi). Bar = 30 μm

Mab F217 and F210 were used to detect respectively PCV2 capsid and Rep proteins in Stoon-1010 and 1121 inoculated PK-15 cells. Capsid protein was the first viral protein detected. In early stages, the protein was only seen in the perinuclear region of the cytoplasm. Starting from 12 hpi in 1121-inoculated and 24 hpi in Stoon-1010 inoculated cells, the capsid protein was also detected in the nucleus of infected cells. Starting from 48 hpi, irregularly shaped cells with condensed nuclei were seen. In these cells, the nucleus was negative for capsid protein while an intense signal was observed in the cytoplasm. During the early stages of cytoplasmic PCV2 capsid protein expression, Rep protein was not detected in infected cells. At 12 hpi in 1121 inoculated cultures and at 24 hpi in Stoon-1010 inoculated cultures, Rep protein was clearly apparent in the nucleus

of infected cells. Rep protein was never detected in cytoplasm of infected cells except in low amounts in irregularly shaped cells starting from 48 hpi. Double IF stainings with monoclonal antibodies F210 and F190 showed that all cells with nuclear localized capsid protein, also contained Rep protein in their nucleus. In contrast a low fraction of cells (<5% of cells with nuclear localized PCV2 antigens) was found to contain only Rep protein in their nucleus (Fig. 3).

Virus titres in culture supernatants at different stages of infection are shown in Fig. 4. A time-dependent rise of titres was seen starting from 36 hpi for both

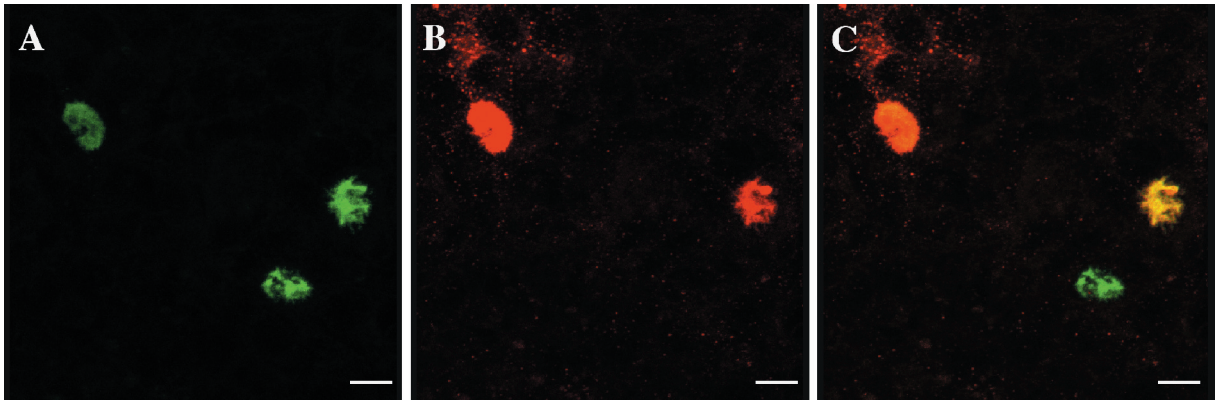

Fig. 3. Expression of rep and capsid protein in porcine circovirus 2 infected PK-15 cells at 72 hpi. **A**: PCV2 rep protein is detected in the nucleus of 3 infected PK-15 cells. **B**: PCV2 capsid protein is detected in the nucleus of 2 infected PK-15 cells and in the cytoplasm of 2 other infected cells. **C**: merged image of both stainings showing 2 cells with both capsid and Rep proteins in the nucleus and one cell with only Rep protein in the nucleus. Bar = 30 μm

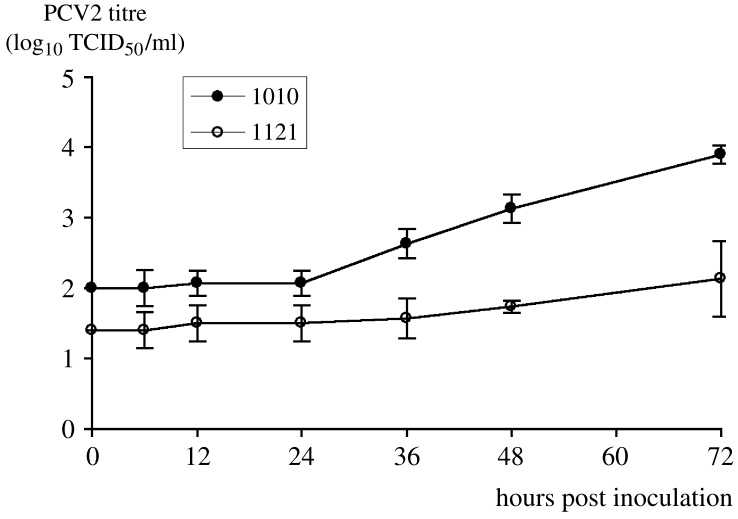

Fig. 4. Evolution in porcine circovirus 2 titres in supernatant of PK-15 cell cultures inoculated with strains Stoon-1010 or 1121. Mean values and standard deviations are shown

PCV2 strains, with a maximum titre of 2.1 $\log_{10}$ $TCID_{50}$ for strain 1121 and 3.9 $\log_{10}$ $TCID_{50}$ for strain Stoon-1010.

Evolution of the number of FCM and PAM expressing viral antigens upon PCV2 inoculation

The replication patterns of strains Stoon-1010 and 1121 in PAM and FCM are shown in Fig. 5.

In FCM, a similar pattern was seen as in PK-15 cells although the percentage of viral antigen positive cells was approximately 10 times lower. In Stoon-1010 inoculated cell cultures, positive cells were detected from 24 hpi resulting in a limited number of cells expressing viral antigens in the nucleus at 48 hpi. Cell cultures inoculated with strain 1121 showed cytoplasmic positive cells starting from 6 hpi and nuclear positive cells starting from 36 hpi. For both strains, the number of positive cells was still increasing with both strains at 72 hpi.

In PAM of one piglet, the number of viral antigen-positive cells increased in a time-dependent way from 0.03% at 12 hpi to 0.25% at 72 hpi with strain Stoon-1010 and from 0.22% at 6 hpi to 1.1% at 72 hpi with strain 1121. Up to 48 hpi, antigens were exclusively localized in the cytoplasm and the signal increased in intensity with time. The first nuclear localisation of viral antigens in PAM of this piglet was observed at 48 hpi (Stoon-1010) or at 72 hpi (1121). At 72 hpi, in Stoon-1010 inoculated cultures, 0.05% of the cells showed nuclear localized antigens (20% of the total number of positive cells) and in 1121 inoculated cultures, 0.03% of the cells showed nuclear localized antigens (3% of the total number of positive cells). The results obtained in this batch of PAM are shown in Fig. 5. Nuclear PCV2 antigen positive cells were confirmed to be macrophages by double immunofluorescence stainings with monoclonal antibodies F210 for the PCV2 Rep protein and SWC3 and 41D3 as shown in Fig. 6. Of particular significance the number of PAM showing nuclear localisation of PCV2 antigens varied strongly between batches of PAM derived from different piglets. In two batches of PAM, cells exclusively contained PCV2 antigens in their cytoplasm and nuclear localised PCV2 antigens were never seen. In two other batches nuclear antigens were rarely seen ($<1\%$) in the antigen positive cells, while in one batch up to 20% of the total PCV2 positive PAM contained antigens in the nucleus. Inoculation with UV-inactivated PCV2 resulted in the absence of a signal after staining with the polyclonal or monoclonal antibodies.

Supernatants of FCM and PAM cultures were titrated but no significant rise of the virus titre was detected.

Comparison of replication kinetics of different PCV2 strains in PK-15

Replication kinetics of five additional strains are shown in Fig. 1. Four strains (1206, 48285, VC2002, 1147) showed an evolution in number of viral antigen positive cells and in intracellular distribution of antigens similar to strain Stoon-1010,

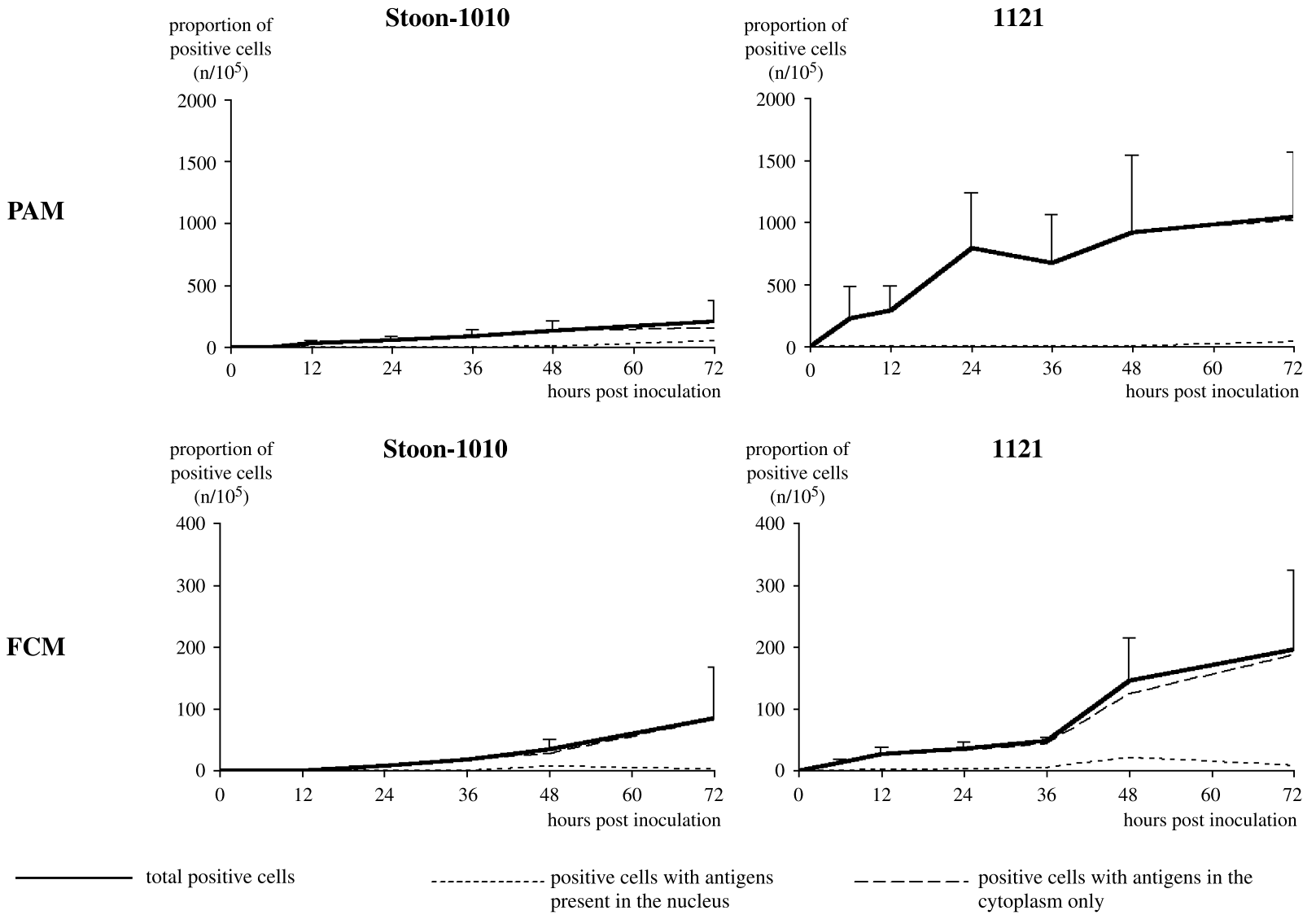

Fig. 5. Evolution of the number of porcine alveolar macrophages and fetal cardiomyocytes expressing porcine circovirus 2 antigens after inoculation with strains Stoon-1010 or 1121. The results in PAM represented in this figure, are the results obtained in one batch of PAM from one pig. In four other batches of PAM from other pigs, a lower fraction or no PCV2 antigen positive cells contained antigens in their nucleus

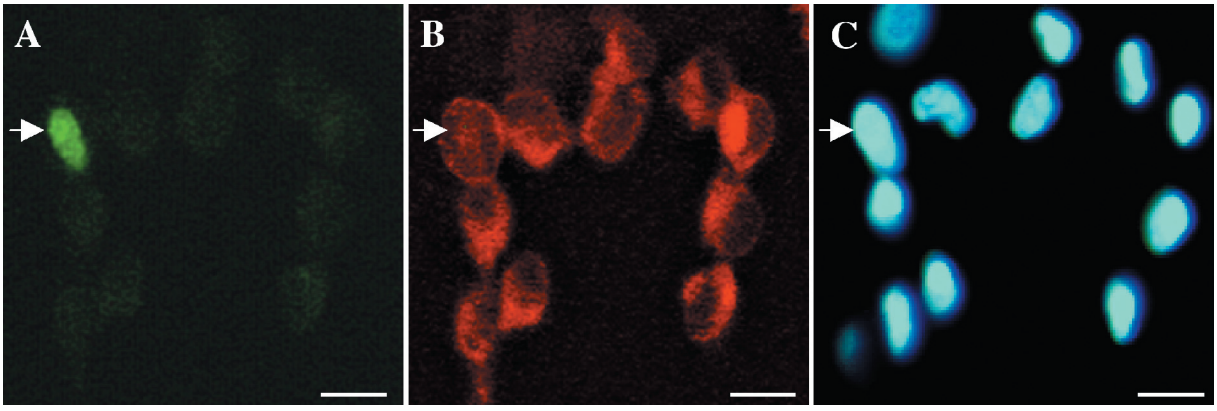

Fig. 6. Porcine circovirus 2 infection in alveolar macrophages. Triple immunofluorescence staining showing nuclear localisation of PCV2 Rep protein in a $SWC3^+$ macrophage. **A**: PCV2 Rep protein, **B**: SWC3, **C**: Hoechst visualizing the nucleus of the cell. Nuclear localisation of PCV2 antigens remained a rare event since the majority of antigen positive PAM only contained antigens in the cytoplasm. Bar = 30 μm

using the polyclonal antibodies. Some variation in the absolute numbers of infected cells was observed. Strain 1103, which is an abortion-strain from Canada, similar to strain 1121, showed similar replication kinetics as strain 1121. A rapid increase of cytoplasmic positive cells was observed during the first 24 hours of the experiment. The percentage of infected cells showing nuclear localization of viral antigens in 1103 infected cell cultures was comparable with 1121 (1% at 24 hpi) infected cell cultures and lower compared to cell cultures inoculated with PMWS or PDNS-associated PCV2 strains (≥10% at 24 hpi).

Discussion

In the present study, new insights were gained in the replication characteristics of PCV2 in PK-15 cells and the natural target cells, alveolar macrophages and fetal cardiomyocytes. Furthermore, indications for biological differences between PCV2 strains were found in the replication kinetics of the strains in PK-15 cells.

PCV2 replication in PK-15 cells was studied as a reference and positive control for the full replication cycle of the virus. The earliest detectable viral protein in infected PK-15 cells was the capsid protein, which was mainly found in the perinuclear region of infected cells during the earliest stages of infection. Absence of immunofluorescence signal after inoculation with UV-inactivated PCV2 demonstrated that the early detected capsid protein was newly produced in the infected cell, rather than derived from captation of viral antigens from the inoculum. ORF2, which codes for the capsid protein, is located on the complementary strand of the virus' genome [19] and can thus only be transcribed from the double stranded replication form. The early expression of capsid protein in PK-15 cells suggests that the complementary strand was formed very shortly after infection of the cell, as has been described for monopartite geminiviruses [6].

Starting from 12 hpi, capsid protein was found in the nucleus of infected cells. At that time point also Rep protein was found in the nucleus of the infected cells. Although Rep protein, like any other protein, has to be produced in the cytoplasm, it was immediately observed in the nucleus of the infected cell, indicative for a strong affinity for the nucleus. At present however, no nuclear localization signal (NLS) has been described for this protein. Double immunofluorescence stainings for both PCV2 capsid and Rep protein showed that most of the cells with nuclear localized PCV2 antigens contained both capsid and Rep. A low percentage of cells contained only Rep protein without expression of capsid (nuclear or cytoplasmic). This indicates that, although the capsid protein is the first viral protein detected in most of the infected cells, Rep protein can occasionally be the first expressed viral protein. The cellular or viral mechanisms that form the basis for this event remain to be elucidated. The fact that capsid protein was never found in the nucleus of an infected cell in the absence of Rep protein, although it can be abundantly present in the cytoplasm of that cell, suggests that the capsid interacts in some way with the Rep protein to cross the nuclear membrane. The inability of the capsid protein to reach the nucleus on itself in our study is surprising since Liu et al. [13] identified a nuclear localisation signal and detected the capsid protein in the nucleus upon transfection.

The rise in virus titre in culture supernatant at 36 hpi coincided with the appearance of the first foci of infected cells. This indicates that the full replication cycle of PCV2 in the PK-15 cell has a duration between 24 and 36 h. These data agree with a previous study in which the first detectable progeny virus was found at 32 hpi [5]. Starting from 48 hpi and increasing in numbers at 72 hpi, detaching cells were observed with capsid protein abundantly expressed in their cytoplasm. Only in these cells, Rep protein was detected in the cytoplasm most likely as a result of leakage from the degrading nucleus. These cells showed irregular formed nuclei with condensation of genomic material, indicating that infection of PK-15 cells with PCV2 probably leads to cell death.

Similar viral protein expression patterns were observed in primary porcine cells and in PK-15 cells. In cardiomyocytes, PCV2 capsid protein and subsequently Rep protein were detected. The low absolute number of infected cells may be the reason for the absence of progeny virus in culture supernatant. Mitotic activity of cardiomyocytes *in vitro* is lower compared to PK-15 cells and this may explain why only 0.2% of the cells were infected. This accurately reflects the situation *in vivo* where it was observed that with further development of the fetus towards parturition, the cardiomyocytes lose their susceptibility for PCV2 infection together with their ability to divide [22].

PCV2 capsid antigens have already been described in the cytoplasm of monocytes and alveolar macrophages [1, 8]. It was, however, questioned if this positive signal was due to transcription of the viral genome or due to accumulation of virus present in the inoculum. In the present study, capsid protein was detected in the cytoplasm of a low percentage of macrophages ($<1\%$). The number of positive cells increased in a time dependent manner. UV-inactivation of the inoculum resulted in absence of the immunofluorescence signal at any time after inoculation,

indicating that the observed signal was due to expression of newly formed viral antigens. Limited numbers of infected macrophages ($SWC3^+/41D3^+$) contained PCV2 antigens in their nucleus. The low number of macrophages showing this nuclear staining is comparable with the *in vivo* situation, where antigens are mainly found in the cytoplasm of cells of the monocyte/macrophage lineage in PCV2 inoculated-piglets [23]. Macrophages with nuclear expression of PCV2 antigens were not described in previous studies [1, 8], which can be explained by the differences in origin of the cells or in the inoculation dose or duration. In the present study, nuclear expression of viral proteins was observed in PAM originating from some specific piglets only. It is unclear if this *in vitro* difference between piglets can form the base of a different clinical outcome of a PCV2 infection *in vivo*.

Replication of PCV2 in macrophages, which are fully differentiated cells, seems contradictory with the fact that the virus depends on the mitosis cycle of PK-15 cells and FCM to replicate in these cell types. The cell cycle dependency of PCV2 to complete its replication cycle is credited to the expression of DNA-polymerase expressed during the S-phase of the cell cycle [27]. Although macrophages do not divide, it has been shown that they can express high DNA polymerase activity in response to damage to their DNA [30], an event which might be misused by PCV2 to complete its replication in these cells.

In this study, biological differences in-between PCV2 strains besides genetical differences were described for the first time. It is not fully understood if the contrasting behaviour of strains 1121 and 1103 in comparison with the other strains can be explained by their origin (abortion cases) or by the fact that the geographical regions where they were isolated, were close to each other. The importance of differences in the *in vivo* replication kinetics is not fully understood and will be the subject of further investigations.

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