Comparison of porcine circovirus type 2 load in serum quantified by a real time PCR in postweaning multisystemic wasting syndrome and porcine dermatitis and nephropathy syndrome naturally affected pigs

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Received 10 September 2003; received in revised form 3 December 2003; accepted 4 December 2003

Abstract

Postweaning multisystemic wasting syndrome (PMWS) diagnosis is based on the presence of characteristic histopathological lymphoid lesions and porcine circovirus type 2 (PCV2) within these lesions. Previous studies indicate that PCV2 load is higher in PMWS affected than in PCV2 infected, healthy pigs. On the other hand, PCV2 has been suggested to play a role in porcine dermatitis and nephropathy syndrome (PDNS) pathogenesis. This study describes a new TaqMan® real time PCR assay and its use to quantify viral load in serum samples. Serum viral loads were related with different degrees of PMWS characteristic lesions and PDNS cases. DNA extracted from serum samples from 75 animals with mild, moderate and severe PMWS lesions and 12 animals with PDNS was used as template. PCV2 DNA was quantified in 69 of 75 PMWS cases and in 11 of 12 PDNS cases. Significant differences in PCV2 load were observed between animals with severe, moderate and mild PMWS lesions, although variability within each group was high, probably due to heterogeneity in disease progression. These results suggest that high viral load is a major feature of PMWS affected pigs. PDNS affected animals had lower PCV2 loads. No significant differences in viral load were found between animals suffering from PDNS and animals with mild PMWS characteristic lesions, which were unaffected clinically.

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Keywords: PCV2; PMWS; PDNS; Real time PCR; Histopathological lesions; Viral load

1. Introduction

Porcine circovirus type 2 (PCV2) is a ubiquitous virus, member of the Circoviridae family. This un-enveloped, single-stranded circular DNA virus is considered the etiological agent of postweaning multisystemic wasting syndrome (PMWS) of pigs (Allan et al., 1999; Rosell et al., 1999). The presence of compatible clinical signs, characteristic histopathological lesions in lymphoid tissues, together with the detection of PCV2 within these lesions are the criteria to establish a PMWS diagnosis (Segalés and Domingo, 2002).

PCV2 infection is widespread and may occur subclinically. There is a high percentage of PCV2 seropositive pigs in the swine population (Rodriguez-Arrijo et al., 2000) and viral DNA has been detected in pigs from PMWS affected and non-affected farms (Calsamiglia et al., 2002; Larochelle et al., 2003; Sibila et al., 2004). Therefore, the detection of PCV2 antibodies or viral DNA in serum samples is non-diagnostic of PMWS, and only indicates infection. However, several studies have shown that PCV2 DNA levels in serum are higher in PMWS affected pigs than in healthy pigs (Rosell et al., 1999; Liu et al., 2000; Meehan et al., 2001; Quintana et al., 2001; Ladekjær-Mikkelsen et al., 2002; Rovira et al., 2002), suggesting that viral quantitation could be indicative of clinical disease. PCV2 has also been suggested to play a role in the pathogenesis of porcine dermatitis and nephropathy syndrome (PDNS) (Rosell et al., 2000; Choi and Chae, 2001). The most characteristic PDNS microscopic lesions are a systemic necrotising vasculitis and fibrinous glomerulitis (Segalés et al., 1998; Drieot et al., 1999), and a systemic type III hypersensitivity has been suggested as the mechanism of lesion development (Helie et al., 1995). PCV2 DNA and PMWS-like microscopic lesions...
have also been found in animals affected of PDNS (Rosell et al., 2000).

PCV2 DNA detection from serum samples is carried out with end-point PCR techniques (Larochelle et al., 1999; Fenaux et al., 2000; Hamel et al., 2000; Mankertz et al., 2000; Kim and Chae, 2001), and viral load has been measured using a competitive PCR (Liu et al., 2000) and real time PCR (Ladekjær-Mikkelsen et al., 2002; Rovira et al., 2002). Real time PCR has the advantage over competitive PCR to be faster, more sensitive and more robust against contamination due to the minimal sample manipulation in closed-tube assays.

Monitoring of viral load is an indicator of active infection, virus-host interaction, and a good marker of disease progression (Mackay et al., 2002), and would provide critical information on the clinical and epidemiological relevance of PCV2 infection. Moreover, use of serum samples to monitor viral load would allow the use of the assay in live animals.

The aims of the present paper are: (1) to develop a real time PCR that offers two advantages over quantitative PCR methods described previously; it works under universal conditions (ABI Prism 7000 User’s Guide, Applied Biosystems, Foster City, CA, USA); it includes an internal amplification control to detect false negative results due to the presence of PCR inhibitors, and (2) to use the assay to quantify serum viral loads from pigs with several degrees of PMWS characteristic lymphoid lesions and PDNS cases, and correlate viral load with lesions.

2. Materials and methods

2.1. Animals

Eighty-seven conventional pigs submitted to the Veterinary Pathology Diagnostic Service at the Veterinary School of Barcelona from 1997 to 2002 were used in the study. Animals ranging from 1 to 5 months of age were selected based on the presence of different degrees of PMWS microscopic lesions in lymphoid tissues, PCV2 detection by in situ hybridisation (Rosell et al., 1999) and PDNS cases.

PMWS affected animals were divided into three categories of lesion severity: mild ($n = 27$), moderate ($n = 24$) and severe ($n = 24$). Twelve animals diagnosed as PDNS cases (Rosell et al., 2000) were also included. Of the PDNS affected animals two had no PMWS characteristic lesions, seven had mild PMWS lesions and three had moderate PMWS lesions.

2.2. Quantitative real time PCR

2.2.1. Primer and probe design

GenBank entry AF465211 was used for primer and probe design. The Cap gene region (corresponding to nucleotides 1033–1734 bp of the whole PCV2 genome) (Nawagitgul et al., 2000; Mankertz and Hillenbrand, 2001; Cheung, 2003) was chosen for primer and probe design, since it has a lower nucleotide homology with porcine circovirus type 1 (PCV1) than ORF1 (~65%) (Liu et al., 2001). Primer forward (PCV2F), reverse (PCV2R) and probe (PCV2S) (Table 1) were designed using Primer Express v.1.5 software (ABI Prism 7000 User’s Guide, Applied Biosystems, Foster City, CA, USA). Primers and probe were selected to work under Universal conditions (ABI Prism 7000 User’s Guide, Applied Biosystems, Foster City, CA, USA). Probe, located 1 bp from PCV2R, was labelled at 5' with FAM® (6-carboxyfluorescein) and at 3’ with TAMRA® (6-carboxytetramethylrhodamine).

As endogenous internal control (IC), pre-optimised 18S rDNA primers and probe were used (ABI Prism 7000 User’s Guide, Applied Biosystems, Foster City, CA, USA). The probe was labelled 5’ with VIC™ and 3’ with MGB™ (Minor Groove Binder). FAM™ and VIC™ have different fluorescence emission wavelengths (518 and 552 nm, respectively) allowing the performance of the two amplifications in multiplex PCR reaction.

2.2.2. PCV2 TaqMan real time PCR standard

A PCV2 genome cloned in pCR2.1 plasmid (Invitrogen, Carlsbad, CA, USA) and transformed in Escherichia coli competent cells (kindly provided by Dr. Annette Mankertz) was used as a quantitation standard. The plasmid was purified using a commercial kit (Qiaprep Spin Miniprep Kit, Qiagen, Valencia, CA, USA), and quantified by spectrophotometry. PCV2 plasmid was mixed with swine DNA extracted from a PCV2 PCR negative blood sample (Quintana et al., 2001), and both DNAs brought to a final concentration of $10^{11}$ plasmid copies/ml and 20 μg/ml, respectively. Ten-fold dilutions of this mixture (from $10^0$ to $10^5$ PCV2 plasmid copies/ml) were used as standard for PCV2 quantitation of diagnostic samples.

2.2.3. PCV2 TaqMan real time PCR reactions

Real time PCR reactions were optimised based on primer and probe concentration selection criteria, and IC primer limiting criteria (ABI Prism 7000 User’s Manual, Applied Biosystems). The optimised reaction contained 900 nM of

<table>
<thead>
<tr>
<th>Oligo</th>
<th>$T_m$ (°C)</th>
<th>GC%</th>
<th>bp</th>
<th>Sequence (5’ → 3’)</th>
<th>Location in PCV2 genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV2F</td>
<td>60</td>
<td>63</td>
<td>19</td>
<td>CCGAGAAGGCTTTTGGACT</td>
<td>1538 → 1553</td>
</tr>
<tr>
<td>PCV2R</td>
<td>59</td>
<td>55</td>
<td>20</td>
<td>CGCTACCGTGGAGGAA</td>
<td>1633 → 1644</td>
</tr>
<tr>
<td>PCV2S</td>
<td>68</td>
<td>52</td>
<td>25</td>
<td>AATGCGATCTCAAGACCCGCTCT</td>
<td>1612 → 1592</td>
</tr>
</tbody>
</table>

$T_m$ is calculated by the neighbour joining method (Primer Express v.1.5 software).
each primer, 150 nM of PCV2S, 0.4 μl of IC kit, 12.5 μl of TaqMan Universal Master Mix (which includes ampliTaq gold DNA polymerase, dNTP’s with UTP, MgCl₂, ROX, Amperase UNG and buffers) and 2.5 μl of template. Autoclaved nanopure water was added to bring the final volume to 25 μl. Amplification was carried out under Universal Cycling conditions (10 min at 95 °C, 2 min at 50 °C and 40 cycles of 15 s at 95 °C, 1 min at 60 °C).

2.2.4. Sensitivity tests
PCV2 real time PCR sensitivity was tested with quadruplicates of different templates in three different assays: (1) 10-fold dilutions of the PCV2 plasmid used as standard from 10⁹ to 10² copies/ml, (2) 10-fold dilutions of PCV2 cultured in PK15 cells free of PCV1 (10⁻⁴(TCID₅₀/ml) until a dilution of 10⁻⁴(TCID₅₀/ml), (3) PCV2 plasmid mixed with exogenous DNA in 10-fold dilutions. As exogenous DNA, calf thymus DNA (1 μg/ml) and swine DNA (20 μg/ml) were used. DNA from PCV2 culture in PK15 cells was extracted using a commercial kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co., KG Düren, Germany) following manufacturer’s indications.

2.2.5. Specificity test
To test the technique’s specificity, DNA from several bacteria and virus isolated frequently from pigs and two porcine cell lines (Table 2) was extracted with a commercial kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co., KG Düren, Germany) following manufacturer’s protocol, and used as template in quadruplicates.

2.2.6. Diagnostic samples
DNA was extracted from the selected swine sera using a commercial kit (Nucleospin® Blood, Macherey-Nagel GmbH & Co., KG Düren, Germany), and PCV2 DNA quantified with the previously optimised method. Reactions were carried out in 96-well plates including sample and standard triplicates (from 10⁶ to 10² PCV2 plasmid copies/ml and from 20 μg swine DNA/μl to 20 x 10⁻⁶ μg/μl). A negative control was added every four samples, using autoclaved nanopure water as template.

2.3. Data analyses
The ABI Prism 7000 Detection software created a standard curve with the threshold-cycle (Ct) values of the 10-fold dilutions PCV2 plasmid (from 10⁹ to 10² copies/ml). The detection software also calculated the correlation coefficient (R²) of the standard curve, standard deviations of triplicates, and the PCV2 copy number of the samples based on the standard curve.

The reproducibility of the assay was calculated using Ct values of the standard curve of five runs of diagnostic samples. Reproducibility was calculated as the mean coefficient of variation (CV) for Ct of the PCV2 standard inside runs (intrasay) and between (interassay) runs (Martell et al., 1999). The method’s accuracy was measured calculating the mean R² between runs.

PCV2 loads were log₁₀ transformed and an ANOVA test (SAS software, SAS Institute Inc., Cary, NC, USA) was used to correlate PDNS and PMWS lesion levels and PCV2 genome quantification.

3. Results

3.1. Real time TaqMan PCR development

3.1.1. Reaction analysis

After optimisation, only reactions accomplishing the following criteria (ABI Prism 7000 User’s Guide, Applied Biosystems) were accepted: (1) standard deviation of sample triplicates should be under 0.38, (2) standards curves with an accuracy of R² > 0.97, (3) the reaction efficiency, measured as the slope of the standard curve, between 3.2 and 3.7. Also, if any of the negative controls was positive the run was repeated.

3.1.2. Method sensitivity and specificity

The quantitation range of the method was between 10⁹ and 10⁴ PCV2 plasmid copies/ml. At lower and higher dilutions, quantitation was not always reproducible.

Using 10-fold dilutions of DNA extracted from PCV2 culture (10⁻⁴(TCID₅₀/ml), the assay sensitivity was 10⁻⁴(TCID₅₀/ml).

None of the bacteria, virus (other than PCV2) and porcine cell lines tested (Table 2) gave any amplification signal. The presence of exogenous DNA had no detectable effect on the reaction signal.

3.1.3. Reproducibility and accuracy

Method reproducibility (Martell et al., 1999) was established by calculating the CV values with the Ct values of each PCV2 standard dilution (from 10⁹ to 10² copies of PCV2 plasmid copies/ml) of five different runs. The average
CV of the technique was of 1.3% intrassay (within runs) and of 4.2% interassay (between runs). Average between runs accuracy ($R^2$) was 0.987.

3.2. Diagnostic samples

PCV2 DNA was detected in all samples tested with a wide range of PCV2 load, which was related with the different PMWS lesion categories (Fig. 1). PCV2 load was significantly higher ($P < 0.05$) in pigs with severe lesions (ranging from $3.9 \times 10^5$ to $1.8 \times 10^{12}$ PCV2 genomes/ml) compared to the group with moderate lesions (ranging from $2 \times 10^3$ to $9.8 \times 10^8$ PCV2 genomes/ml). Besides, both groups had a significantly higher ($P < 0.05$) viral load in serum than the groups with PDNS or mild PMWS lesions. On the other hand, no significant differences ($P > 0.05$) in viral load between pigs with mild PMWS lesions and PDNS lesions were found (with viral loads ranging from $2 \times 10^3$ to $3.9 \times 10^6$ PCV2 genomes/ml and from $2 \times 10^3$ to $8.1 \times 10^3$ PCV2 genomes/ml, respectively).

Seven samples (8%) had $<10^4$ PCV2 genomes/ml and five samples (5%) gave readings of $>10^9$ PCV2 genomes/ml. The seven samples showing $<10^4$ PCV2 genomes/ml were from the following categories: PDNS (one animal), mild PMWS lesions (five animals) and moderate PMWS lesions (one animal). The five samples showing $>10^9$ PCV2 genomes/ml were from the severe PMWS lesion category. These latter values were out of the standard range of viral load meaning they were extrapolations to the standard curve, being therefore less reliable.

The animals from the PDNS group were also classified regarding PMWS microscopic lymphoid lesions. Table 3 shows PCV2 viral loads in serum found in the animals of this group according to the PMWS-like lesions and PCV2 in situ hybridisation result.

<table>
<thead>
<tr>
<th>PMWS-like lymphoid lesions severity</th>
<th>Number of positive pigs</th>
<th>PMWS-like lymphoid lesions severity</th>
<th>Number of negative pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>5</td>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>$&lt;10^4$ to $10^6$</td>
<td></td>
<td>$&lt;10^3$ to $10^6$</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>2</td>
<td>None</td>
<td>2</td>
</tr>
<tr>
<td>$&lt;10^5$ to $10^6$</td>
<td></td>
<td>$&lt;10^3$ to $10^6$</td>
<td></td>
</tr>
</tbody>
</table>

4. Discussion

This study describes a new real time PCR for PCV2 and its evaluation as a diagnostic tool for PMWS. PCR techniques are sensitive, specific and can be performed in poor quality samples with no recoverable virus (Klein, 2002). Real time PCR allows the quantitation of the amount of virus present in a sample, being this technique a useful tool in virology (Mackay et al., 2002). The TaqMan® real time PCR for detection of PCV2 genome is as sensitive as end-point PCR but offers several other advantages over end-point PCR methods: (1) the use of two different fluorescent probes gives higher specificity and allows the development of multiplex reactions (Klein, 2002), (2) a wide dynamic range of detection (5 logarithmic decades) with high precision (Klein, 2002), (3) it is carried out in a close tube being, not only more robust against contamination, but also faster and less labour intensive since no post-PCR steps are required.
ampersand UNG and AmpLiTaq Gold DNA polymerase avoid amplicon contamination and minimise unspecific amplifications, respectively, and (5) the use of an internal control makes the method more robust, detecting false negative results due to the presence of PCR inhibitors in the samples. The 18S rRNA gene (the target gene of the IC kit) is a high copy number housekeeping gene in eukaryotes (several hundreds in mammals) (Lewin, 1994) and can be used as IC without adding artificial mimics to the samples and minimising sample manipulation. PCV2 viral load has been shown to be higher in clinically affected animals than in animals infected subclinically (Liu et al., 2000; Rovira et al., 2002; Ladekjær-Mikkelsen et al., 2002; Quintana et al., 2001; Meehan et al., 2001; Rosell et al., 2000). Our study further supports this observation: PCV2 viral load increases with the severity of PMWS microscopic lesions in lymphoid tissue. Although viral load was significantly different in the three PMWS lesion categories, a high variability within each category existed. The dynamics of viral infection, related with lesion development and recovery overtime is still unknown. It is possible that recovering pigs still have PMWS lesions and decreasing viral loads, and this would explain those cases found in this study (8 of 48 animals) with moderate to severe lesions and low viral loads (under 10^6 PCV2 genomes/ml). On the other hand, the critical viral load to elicit PMWS characteristic lesions is not known. The latter point explains the finding of pigs infected asymptomatically with relatively high viral loads (under 10^6 PCV2 genomes/ml). On the other hand, the critical viral load to elicit PMWS characteristic lesions is not known. The latter point explains the finding of animals infected asymptomatically with relatively high viral loads, still insufficient to cause lesions.

Several experimental challenges with a longitudinal monitoring of viral load in serum have been made (Liu et al., 2000; Ladekjær-Mikkelsen et al., 2002; Rovira et al., 2002). In the first study, animals with PMWS had PCV2 mean viral loads of 4.6 × 10^6 PCV2 genomes/ml of serum (n = 25). In the second study, all infected animals (n = 10) developing the disease reached PCV2 loads up to 10^7 PCV2 genomes/ml of serum 10 days after inoculation. In our study, clinical PMWS correlated with the moderate and severe PMWS-characteristic lymphoid microscopic lesions categories. The fact that animals in the moderate lesion category had mean PCV2 loads of 9 × 10^6 PCV2 genomes/ml serum, and that other studies have also found viral loads in the range of 10^6–10^7 PCV2 genomes/ml in PMWS affected pigs, 10^6 PCV2 genomes/ml of serum could be proposed as the threshold for PMWS diagnosis.

The results obtained in this study indicate that PCV2 viral load in serum could be used for differentiating PMWS from PCV2 subclinical infection. However, two points need to be considered: (1) there is a high variability in viral load within the different lesion groups, although statistically significant differences exist between them, and (2) in a group of pigs, animals at different stages of the disease might coexist. Animals in the initial phases of the disease show more severe lesions, and presumably higher viral loads, than animals in the late stage of the disease (Quintana et al., 2001). Therefore, both the number and the selected animals to be used for PMWS diagnosis in a farm are critical. Diagnosis using TaqMan PCR should be made from serum samples from several animals showing initial signs of wasting.

PDNS affected pigs showing mild to moderate microscopic PMWS-like lesions had low viral loads in concordance with what has been found in PMWS mild lesions category. This fact suggests that, although PCV2 may play a role in PDNS pathogenesis, viral load is not a decisive factor for developing PDNS. In fact, PCV2 DNA has not been detected, or detected in low amounts in the most characteristic PDNS lesions: systemic necrotising vasculitis and fibronodular glomerulitis (Rosell et al., 2000; Choi and Chae, 2001). On the other hand, PCV2 load is not incompatible with PCV2 having a role in PDNS. PDNS has been proposed to be a type III hypersensitivity, where low amounts of antigen can elicit this type of immune response and lesions. However, it cannot be ignored that PCV2 infection in PDNS would be a mere coincidence. The fact of having the same viral load than pigs with mild PMWS lesions, could also suggest that PDNS affected animals are subclinically infected with PCV2 as a high number of pigs of the same age worldwide (Larochelle et al., 2003; Sibila et al., 2004).

In conclusion, real time PCR can be a useful tool to diagnose PMWS outbreaks without having to sacrifice animals. The presence in a sample of animals of high PCV2 loads (>10^7 PCV2 genomes/ml of serum) is suggested as indicating the presence of PMWS lesions. However, a larger number of animals should be analysed to confirm the PCV2 load necessary to diagnose PMWS in a group of animals.

Acknowledgements

This work was partially funded by the Project QLRT-PL-199900307 from Fifth Framework Programme 1998–2002 of the European Commission. We would like to thank Dr. Annette Mankertz, Fort Dodge Veterinaria Spain and Applied Biosystems technical support for their help, and Merche Mora and Eva Huerta for technical support.

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