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SHORT COMMUNICATION

Inter-laboratory and inter-assay comparison on two real-time PCR techniques for quantification of PCV2 nucleic acid extracted from field samples.

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Keywords: porcine circovirus type 2 (PCV2), postweaning multisystemic wasting syndrome (PMWS), quantitative real-time PCR, inter-laboratory comparison.

Abstract

Several real-time PCR assays for quantification of PCV2 DNA (qPCR) have been described in the literature, and different in-house assays are being used by laboratories around the world. A general threshold of 10^7 copies of PCV2 per ml serum for PMWS diagnosis has been suggested. However, neither inter-laboratory nor inter-assay comparisons have been published so far. In the present study, two different qPCR probe assays used routinely in two laboratories were compared on DNA extracted from serum, nasal and rectal swabs. Results showed a significant linear association between the assays ($p<0.0001$), and a systematic difference of 1.4 $\log_{10}$ copies of PCV2 per ml of sample ($p<0.0001$). This difference indicated that the assay from laboratory 1 yielded a higher output than the one from laboratory 2. Results also showed that there was no linear association between the amount of PCV2 DNA and the amount of total DNA, neither in nasal ($p=0.86$) nor in rectal ($p=0.78$) swabs, suggesting that normalizing of PCV2 DNA load in swab samples to total DNA concentration is not suitable. The present exploratory study highlights the need for the performance of ring trials on qPCV2 protocols between laboratories. Meanwhile, the proposed thresholds for PMWS diagnosis should only be considered reliable for each particular laboratory and each particular assay.

Introduction

Porcine circovirus type 2 (PCV2) belongs to the *Circoviridae* family, genus *circovirus*, and is a small, non-enveloped, single stranded ambisense circular DNA virus (Meehan et al., 1998). PCV2 is considered the essential infectious agent of postweaning multisystemic wasting syndrome (PMWS), a global disease of great economic impact (Segalés et al, 2005a). Most if not all pigs become infected during their life, but only a proportion of them develop PMWS (Segalés et al., 2005a). Therefore, the exclusive detection of PCV2 antibodies and/or viral DNA in serum or other samples, is non-diagnostic for PMWS, indicating only current or past infection with PCV2 (Krakowka et al., 2005). Hence, diagnosis of PMWS requires clinical signs compatible with PMWS (mainly wasting and respiratory distress), presence of characteristic PMWS microscopic lesions in lymphoid tissues (lymphoid depletion and granulomatous infiltration) and detection of moderate to high amounts of PCV2 within those lesions (Segalés et al. , 2005a).

Several quantitative PCR (qPCR) methods for detection of PCV2 nucleic acid have been developed in different laboratories, targeting either ORF1 (Ladekjaer-Mikkelsen et al., 2002) or ORF2 genes (Brunborg et al., 2004; Olvera et al., 2004). A threshold of 10^7 PCV2 DNA copies per ml serum has been suggested to establish a PMWS diagnosis (Olvera et al., 2004). However, it is well known that many factors may influence the performance of PCR assays and that the results using different assays may vary, even when identical template DNA is tested (Bustin, 2002).

Although published qPCR methods to detect and quantify PCV2 have been evaluated separately, to date, no inter-laboratory or inter-assay comparisons have been published.

The present study compares the performance of two different PCV2 qPCR assays used routinely in two different laboratories: one located in Denmark (laboratory 1), and another located in Spain (laboratory 2). Comparison was performed on the same DNA samples extracted from serum and nasal and rectal swabs collected from pigs from PMWS affected farms. The usefulness of normalizing PCV2 DNA load in swab samples to the total DNA present in each sample, in order to avoid possible PCV2 load variation due to sampling procedure, was also evaluated.

Material and methods

Ninety-nine samples randomly collected from pigs of different ages (1 to 21 weeks of age) from PMWS affected farms located in Spain and Denmark were studied. Specifically, 33 serum samples, and 33 nasal and 33 rectal swabs were analyzed. For each type of sample, 16 out of 33 were obtained from Danish and 17 out of 33 from Spanish pigs. Danish (Kirudan A/S, Denmark) and Spanish (Collection swab, Eurotubo, Rubí, Spain) obtained swabs were placed in tubes containing 2 and 1 ml of phosphate-buffered saline (PBS) solution, respectively. Pigs were bled at the cranial cava vein and 5-10 ml of blood was collected in vacuum glass tubes (Vacutainer®, Becton-Dickinson, Meylan Cedex, France). Blood samples were allowed clotting at 4ºC or room temperature and then centrifuged at 200 g for 10 minutes at 4ºC. All samples were kept at -80ºC until their use. DNA from Danish samples was extracted in laboratory 1 and DNA from Spanish samples was extracted in laboratory 2. In laboratory 1, DNA was extracted from 200 µl of serum, nasal swab (QIAamp DNA Mini Kit, Qiagen® GmbH, Germany) or rectal swab PBS solutions (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany), and eluted in 200 µl elution buffer. In laboratory 2, DNA was extracted from 200 µl of serum or 300 µl of nasal swab PBS solution (Nucleospin®

Blood, Macherey-Nagel, GmbH & Co KG, Düren, Germany), and eluted in 100 µl buffer, whereas DNA was extracted from 300 µl of rectal swab PBS solution (QIAamp DNA Stool Mini Kit, Qiagen® GmbH, Germany) and eluted in 200 µl buffer. Extracted DNA was divided into two tubes, one for analysis in each laboratory. Samples were sent between laboratories on dry ice. Thus, both laboratories analysed all 99 samples using their own qPCR assay described below.

The assay of laboratory 1 used Primer Probe Energy Transfer chemistry and the primers and PCV2 probe targeting ORF 1 described previously (Ladekjaer-Mikkelsen et al., 2002). PCR reactions containing 3 µl DNA, 1 x PCR Gold Buffer (Applied Biosystems), 2.6 mM $MgCl_2$ (Applied Biosystems), 0.2 mM (each) dNTP mix, 100 nM forward primer, 300 nM reverse primer, 500 nM PCV2 probe, and 0.05 units/µl AmpliTaq Gold DNA Polymerase (Applied Biosystems) in 25 µl PCR reactions were run on Rotorgene 3000 PCR machines (Corbett Research). PCR cycling was: 95 °C 10 min; 45 cycles of 95 °C 15 s, 60 °C 40 s, 75 °C 20 s; 75 °C 5 min; 95 °C 30 s; 45 °C 1 min; followed by melt curve analysis: ramping from 45 °C to 99 °C, rising by 1 °C per step. Fluorescence data were collected at the annealing step of the PCR amplification and at each step during the melt curve analysis. Samples were analysed in duplicate, including in each separate PCR run: negative PCR controls (Nuclease-free water, Amresco) and standards representing 10^7, 10^6, 10^4, and 10^2 plasmid copies of PCV2 per reaction, respectively. Standards were prepared as a dilution series in 100 ng/µl yeast tRNA (Ambion) of a PCR-product cloned into pCR2.1-TOPO vector (Invitrogen), the DNA concentration of which was determined spectrophotometrically (ND-1000 v.3.1, NanoDrop Technologies, Inc., USA).Only replicates having a standard deviation (SD) less than 0.30 were considered. The correlation coefficient (R^2) of standard curves was

always >0.99 and the PCR assays showed a PCR efficiency of 90-100% measured from the standards. The quantification range of the assay was $3.3x10^4 – 3.3 x10^9$ copies of PCV2 per ml of sample material.

Laboratory 2 used a previously described TaqMan PCR method using primers and probe targeting specifically the PCV2 ORF2 gene (Olvera et al., 2004) with the only exception that an internal control was not included.

Intra-assay and inter-assay variability (Martell et al., 1999) was established by calculating the coefficient of variation (CV) percentage of the cycle threshold (Ct) values of each PCV2 standard dilution of the different independent PCR runs used in each laboratory (5 runs in laboratory 1 and 6 runs in laboratory 2).

PCR results from both assays were expressed as the number of PCV2 copies per ml serum, or nasal swab/rectal swab suspensions. Data were $\log_{10}$ transformed prior to statistical analysis, because of the highly skewed distribution of PCV2 copies per ml of sample. Descriptive statistics, agreement test, paired t-test and linear regression were conducted using SAS, version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Agreement (Bland and Altman, 1986) and linear regression plots were constructed by SPSS, version 15.0.1 (SPSS, Inc. Headquarters, USA) to further illustrate the agreement of the two assays and to assess bias. Moreover, to investigate the PCV2 DNA concentration measurements of the plasmid standards as a source of inter-assay variability, the DNA concentration of 7 randomly selected DNA samples was determined by spectrophotometry in laboratory 1 (ND-1000 v.3.1, NanoDrop Technologies, Inc., USA) and laboratory 2 (BioPhotometer Eppendorf®. Hamburg, Germany).

Possible mismatches between the primers and probes used and PCV2 sequences from NCBI nucleotide database (http://www.ncbi.nlm.nih.gov) were assessed by comparison with the previously described alignment focusing on marker positions, the nucleotide changes characteristic of each clade described in Olvera et al., 2007.

In order to evaluate the usefulness of normalizing PCV2 DNA load in swab samples by the total DNA present in each sample, a second set of rectal and nasal swabs was analysed in laboratory 2. Nasal (n=625) and rectal (n=625) swab samples collected from 120 pigs aged between 1 and 21 weeks and coming from Spanish PMWS affected farms, were processed and analysed by PCV2 qPCR as described above for laboratory 2. In addition, total DNA was assessed from all the 1250 DNA extracts by fluorometry (Fluoroscan Ascent1 FL, Thermo Labsystem, Helsinki, Finland), using Picogreen staining (PicoGreen1 dsDNA Quantitation Reagent Kit, Molecular Probes, Eugene, USA). Thus, the viral concentrations in swabs were also expressed as the mean $\log_{10}$ viral DNA copy number per total amount of DNA present in the sample. Regression analyses were performed to assess whether there was a linear association between the results expressed as $\log_{10}$ viral DNA copy numbers per ml PBS and $\log_{10}$ viral DNA copy numbers per µg total DNA, as well as to compare the association between the total amount of DNA and copies of PCV2 per ml present in each sample.

Results

Mean and SD of the quantification of PCV2 DNA in the three different types of samples by both assays are summarized in table 1. Obtained results comprised a wide spectrum

of PCV2 loads, from negative samples to 10^{11} copies PCV2 per ml sample. Considering the 62 samples that were positive by both tests, a significant association between the assays was observed by linear regression ($p<0.0001$), with a slope close to one (0.979) and an intercept of 1.505 (figure 1). The mean (±SD) difference between the assays was 1.4±0.6 Log_{10} copies of PCV2 per ml, which was significant in the paired t-test ($p<0.0001$). This difference indicated that the assay from laboratory 1 generally yielded a higher output than the one from laboratory 2. Similar results were obtained when linear regression and paired t-tests were performed for serum, nasal and rectal swabs separately (data not shown).

In order to further characterise the significant difference and compare results from the two assays, an agreement plot of the differences in PCV2 copy number between the assays against their mean was constructed (figure 2). Normality plot of the differences between the assays showed an apparently normal distribution (figure not shown). However, a Shapiro-wilk test for normality showed that the differences were not normally distributed ($p=0.0107$, skewness=-0.15, kurtosis=1.96), probably due to a high peakness of the distribution. When a linear regression of the differences in PCV2 copy number between the assays and their mean was assessed (figure 2), a non-significant slope was found ($p=0.405$). Moreover, when a linear regression of the absolute values of the centred difference between the assays and their mean was assessed, a non-significant slope was found ($p=0.3195$). Both regression analyses indicated that the difference between the values of the two assays is independent of the virus count. The mean difference of 1.4 Log_{10} copies of PCV2 per ml, with limits of agreement being 0.2 to 2.6 Log_{10} copies of PCV2 per ml (figure 2), showed that the results from laboratory 1 were systematically higher than those from laboratory 2.

Twenty-five out of the 99 samples analysed were positive for PCV2 in laboratory 1 but negative in laboratory 2 (table 1). All these samples contained levels of PCV2 DNA which, taking into account the systematic difference between the assays, would be below or close to the limit of detection of the assay used in laboratory 2 (10^4 copies of PCV2 per ml). Twelve samples gave negative results in both laboratories. Since negative results by qPCR mean that the amount of PCV2 is either zero or below the detection limit of the respective technique, negative samples were excluded from the statistical analysis described above.

The average intra-assay CV was 0.79% for laboratory 1 and 0.67 % for laboratory 2. The average inter-assay CV was 1.73% in laboratory 1 (between 5 PCR runs used to obtain present results) and 1.36% in laboratory 2 (between the 6 PCR runs used).

Significant linear association ($p<0.0001$) with a high coefficient of correlation ($R^2=0.998$) was found between total DNA concentration measurements of the seven DNA samples analysed by both laboratories. Moreover, no bias was observed between measurements from both laboratories when agreement plot was constructed (data not shown).

One of the targeting nucleotides of the forward primer used in laboratory 1 contained a described marker position at genome position 737. The primers and probes used in laboratory 2 all annealed outside described marker positions.

In the second set of studied samples, 237 and 147 out of 625 nasal and 625 rectal swabs, respectively, gave positive PCV2 qPCR results. Total DNA mean±SD in nasal and

rectal swab DNA extracts was 7.9±17.2 and 0.7±0.13 µg DNA per ml of sample material (PBS), respectively. A significant association between the expression of copies of PCV2 per ml PBS and copies of PCV2 per µg total DNA, was observed by linear regression for both nasal ($p<0.0001$) and rectal swabs ($p<0.0001$). However, linear regression of copies of PCV2 per ml PBS versus total amount of DNA per ml PBS, from the studied swabs with positive PCV2 qPCR, showed that there was no association between parameters, neither when all swabs were considered together ($p=0.32$) nor separately (nasal swabs: $p=0.86$, rectal swabs: $p=0.78$). Therefore, although a significant correlation between viral DNA copy numbers per ml PBS and per µg total DNA was observed, no association between the viral load and the total DNA concentration of samples was detected.

Discussion

PMWS diagnosis requires the necropsy of suspicious animals and the access to histopathological expertise, which is not available in all diagnostic laboratories worldwide. On the other hand, qPCR is a very attractive methodology for laboratory diagnosis of infectious diseases, because it could be carried out on live animals on easily accessible material such as serum samples and/or faecal and rectal swabs, and could provide rapid results and precise quantification in an easy-to-use platform (Bustin et al., 2002). However, the lack of harmonisation among PCV2 qPCR methods raises the question whether results obtained by different laboratories are comparable and thereby whether a theoretical general threshold is valid for all methods.

The inter-laboratory assay comparison performed in this study showed a close relative association between viral loads measured by both laboratories in all three types of

samples studied. However, a bias of 1.4 Log_{10} copies PCV2 per ml was observed, with laboratory 1 constantly yielding a higher output than laboratory 2. The laboratory 1 assay was shown to have a lower detection level than the one used in laboratory 2, being able to detect PCV2 loads of 3-4 log_{10} copies of PCV2 per ml in samples that gave negative results in the laboratory 2 assay. Taking the 1,4 Log_{10} copies PCV2 per ml bias into account, results of 3-4 log_{10} copies of PCV2 per ml in laboratory 1 corresponds to results of 2-3 log_{10} copies of PCV2 per ml in laboratory 2. This is below the detection limit of the assay used in laboratory 2, explaining why more samples are considered negative by laboratory 2 than by laboratory 1. There are many possible factors that could produce variation in the results between assays and laboratories (Bustin, 2002). In the present exploratory study, comparison was performed on exchanged aliquots of DNA extracts, ruling out any cause related to pre-PCR procedures. Also, differences in the actual concentration of plasmid DNA in the standards, which potentially could introduce a systematic bias in the results as the one observed, were excluded. Two different qPCR platforms (ABI PRISM® 7000 and Rotorgene® 3000) were used, but previous studies reported similar accuracy and performance for different platforms using the same assay (Nitsche et al., 1999). Variation of assays may also rely on the nature of the design of the assays, such as length of amplicon or primer and probe design to conserved regions. However, both assays evaluated in this study seem to meet the criteria for well designed qPCR assays: amplicon lengths are below 200 bp as generally recommended (Qvarnstrom et al., 2005). It is known that even few or only one mismatch in the primers can reduce the PCR efficiency, and if in fluorescent probes, may reduce or eliminate the strength of the fluorescence signal (Ratcliff et al., 2007). Although PCV2 ORF1 is known to be more conserved than ORF2 PCV2 (de Boisseson et al., 2004), primers and probes used in the assay targeting ORF2 performed

by laboratory 2, did not contain any of the marker positions previously identified (Olvera et al., 2007), whereas one of the primers used in laboratory 1 did contain one of the described marker positions. This suggests that the higher heterogeneity of ORF2 could not explain the differences observed between the laboratories. Finally, other potential factors might influence the difference in qPCR performance observed in the present study. On one hand, the studied assays used different primers and probes chemistry. On the other hand, different PCV2 DNA regions (ORF1 and ORF2) may have different topology, offering different accessibility to the DNA polymerase.

Results showed a significant linear association between $\log_{10}$ viral DNA copy numbers per ml PBS and $\log_{10}$ and viral DNA copy numbers per µg total DNA, indicating that such normalization would not substantially change the relative results obtained between different swabs collected in the same way. This is due to the fact that the mean and the SD of total DNA present in nasal and rectal swabs was relatively low compared to the amount of PCV2. However, results also showed that there was no association between the amounts of total and PCV2 DNA present in nasal and rectal swabs. This suggests that normalizing PCV2 DNA load in swab samples to the total DNA concentration is not suitable for the expression of the amount of PCV2 present in swab samples. Thus, if swabs are processed similarly within a study, expression as copies of PCV2 per ml or copies of PCV2 per swab would be more adequate for relative comparison of results. It may always be associated with a certain degree of error if results from swab samples collected in different ways are being compared.

The present exploratory study provides the basis for the need of performing ring trials on PCV2 qPCR protocols between laboratories, towards a harmonization of the

methodology and towards the full understanding of possible different outputs between laboratories. Faced with the lack of ring trials and standardization procedures, proposed thresholds for PMWS diagnosis (Brunborg et al., 2004; Olvera et al., 2004; Segalés et al., 2005b) should be interpreted with caution, being only considered reliable for each particular laboratory and each particular assay.

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Figure 1. Linear regression of 62 positive samples by both laboratories. All the results are expressed as $\log_{10}$ copies of PCV2 per ml sample.

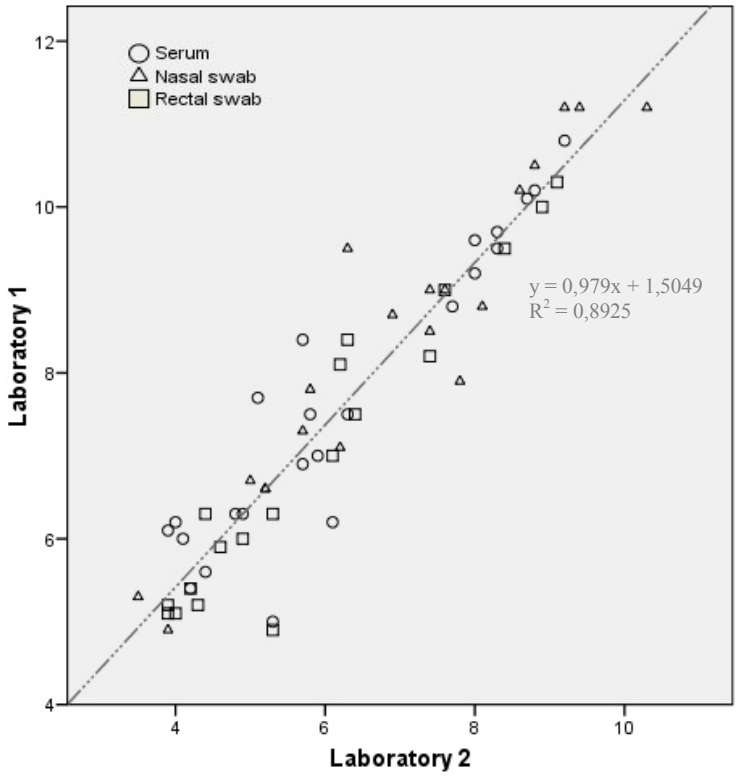

Figure 2. Agreement plot including the 62 positive samples by both assays. All the results are expressed as $\log_{10}$ copies of PCV2 per ml sample.

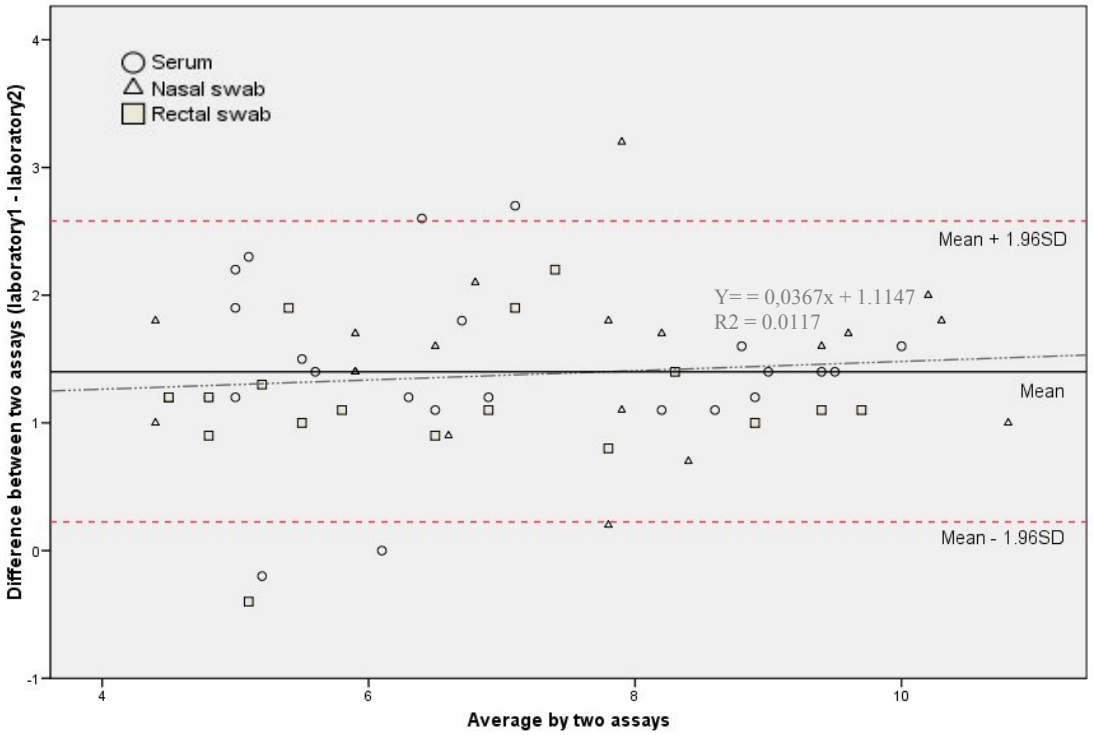

Table 1. Log_{10} copies of PCV2 per ml sample by laboratory 1 and 2 qPCR assays.

	Number of samples (sera; nasal swabs; rectal swabs)	Mean ± SD in laboratory 2 assay (sera; nasal swabs; rectal swabs)	Mean ± SD in laboratory 1 assay (sera; nasal swabs; rectal swabs)
Positive results in both assays	62 (23; 20; 19)	6.3±1.8 (6.2±1.8; 6.9±1.9; 5.9±1.7)	7.7±1,9 (7.7±1.8; 8.4±1.9; 7.0±1.8)
Positive results by laboratory 1 and negative by laboratory 2	25 (6; 9; 10)	Negative	4.6±0.7 (4.6±0.9; 4.7±0.8; 4.7±0.6)
Negative results by both laboratories.	12 (4; 4; 4)	Negative	Negative