



PCV2 load in preweaned piglets affected with porcine circovirus 2 associated diseases

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Received: 27 December 2014; Accepted: 8 March 2015

ABSTRACT

Porcine circovirus associated diseases (PCVAD) caused by Porcine circovirus type 2 (PCV2) are responsible for heavy losses to the global swine industry in recent decades. PCV2 viral load varies significantly between diseased and subclinically infected pigs. Real time PCR is a highly sensitive technique enabling simultaneous amplification and quantification of specific nucleic acid sequences. Using this technique, the distribution and absolute quantification of PCV2 in naturally infected preweaned piglets was investigated. The viral load in different organs was in the range of (32.92×10^8 copies/ μ g DNA to 1.14×10^8 copies/ μ g DNA). Results indicate that in PCVAD affected preweaned pigs, lymphoid organs contain the highest viral loads, while intestine and brain the lowest viral loads. This preliminary data on virus load in the lymphoid organs of preweaned piglets will help differentiate mere PCV2 infection and clinical PCVAD.

Key words: Pigs, Porcine circovirus 2, Real-time polymerase chain reaction, Virus load

Porcine circovirus (PCV), a small, non-enveloped, single-stranded DNA virus with a covalently closed circular genome (Tischer *et al.* 1982) is considered to be the primary etiological agent that is responsible for the causation of several varied clinical manifestations in affected pigs, and the first of them to be recognized was termed as postweaning multisystemic wasting syndrome (PMWS) but the term porcine circovirus 2 associated diseases (PCVAD) is now accepted as a more appropriate term that encompasses all the syndromes caused by porcine circovirus type 2 (PCV2). PCV2 infection is widespread in swine population and many healthy pigs are positive for PCV2-specific DNA without necessarily being affected by PCVAD (Larochelle *et al.* 2003). Real-time quantitative PCR is an excellent diagnostic tool with high sensitivity and specificity within a wider time span (Hansen *et al.* 2010) and it reduces the chances of PCR contamination that occurs when products are examined in gels (Zhao *et al.* 2010). Correlation between severity of the clinical disease and type of syndrome manifested with virus load in serum or tissue of pigs with PCVAD has been observed (Olvera *et al.* 2004). Increasing viral load appears to be a critical step in the development of severe PCVAD (Harding *et al.* 2008). So, viral load estimation will be useful to determine active infection, and study virus–host interaction (Mackay *et al.* 2002). Quantitative PCR (qPCR)

is considered to be a reliable technique to diagnose PMWS on a population, although not on an individual basis (Grau-Roma *et al.* 2009, Harding *et al.* 2008).

Marked variation in qPCR detection limits has been shown among laboratories (Harding *et al.* 2009; Hjulager *et al.* 2009). Moreover, most studies have estimated viral load in serum with a view that such information may be useful for clinical diagnosis of PMWS but the results varied significantly between countries, type of sample used and laboratories that conducted the test. A study conducted in Spain found the virus load was $10^{6.93}$ copies/ml serum (Fort *et al.* 2007), while in Canada, it was $10^{4.7}$ copies/ml serum (Harding *et al.* 2008). A subsequent study observed higher cut-off values in Spain ($10^{6.21}$ viral copies/ml serum) and Denmark ($10^{7.43}$ viral copies/ml serum) (Grau-Roma *et al.* 2009). Though, virus load of 10^7 in serum/plasma and in tissue samples have been correlated with clinical disease, Brunborg *et al.* (2010) found that 18 of 20 pigs with a viral load above 10^7 per ml serum did not develop clinical PMWS or other PCV2 associated clinical signs. In consequence, the potential diagnostic PCV2 load threshold is strongly dependent on the laboratory and particular technique used (Segales 2012).

Quantification of virus load in PMWS affected and non-affected pigs or in stillborn piglets have been reported, but reports on PCV2 load in preweaned piglets are few. Keeping these points in view, the present study was carried out to quantify PCV-2 load in various organs of infected preweaned piglets and to determine the organs that are most suited for detection of viral DNA as an indicator of PCVAD.

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MATERIALS AND METHODS

Samples for the present study consisted of tissue samples of dead preweaned piglets (below 6 weeks of age) presented for post-mortem examination to the Division of Pathology, IVRI, Izatnagar; between August 2013 to May 2014. A detailed necropsy examination was conducted on dead piglets and gross pathological alterations were recorded. Tissue samples from tonsil, lungs, heart, liver, spleen, kidneys, stomach, duodenum, ileum, colon, mesenteric and other carcass lymph nodes and brain from 86 pigs were collected on ice and 10% neutral buffered saline. Formalin-fixed tissues were processed for preparation of routine H&E stained slides following standard procedures. Extraction of DNA from different tissue samples was done by DNA tissue mini kit method. The quality and quantity of extracted DNA was assessed by spectrophotometry.

The standard plasmid DNA was constructed by cloning a 676 bp PCR fragment of PCV2 ORF2 into the pTZ57/RT vector according to the manufacturer's instructions. The plasmid was propagated in *E. coli* DH-5 α cells, purified and subsequently quantified by spectrophotometry. For confirmation of the recombinant plasmid for presence of the 676 bp amplicon, conventional PCR using the following forward (5'-AGGAGGGCGTTCTGACTG-3') and reverse (5'-GACTCCCGCTCTCCAACAAG-3') primers was used. The PCR reaction mixture comprised of 10x Dream Taq buffer 2.5 μ l, 10 mM dNTPs 0.5 μ l, Dream Taq enzyme 0.13 μ l, 0.8 μ l forward primer, 0.8 μ l reverse primer and 3 μ l template. The cycling condition was as follows: An initial denaturation step (3 min at 95°C) followed by 40 cycles each comprising 30 sec at 95°C, 30 sec at 59°C, 60 sec at 72°C and a final elongation step for 7 min at 72°C. The expected size PCR amplicon was checked by 1.5% agarose gel electrophoresis. Recombinant clones were stored at -20°C.

Quantitative TaqMan PCR assay was performed using real-time PCR machine and commercial reagents as per manufacturer's recommendation. The following primers and probe designed from an Indian isolate of PCV2 (PCV2Izn-218-13; Accession No. KJ729074) were used in the assay: Forward primer: PCVSKF-5'-TTTCCTTCTCCAGCGGTAAC-3'; Reverse primer: PCVSKR- 5'-TACAGCGCACTTCTTTTCGTT-3' and TaqManProbe: PCVSKP-5'-/56-FAM/TCTTGGCCA/ZEN/GATCCTCCGCC/31ABKFQ/-3. The real-time PCR was carried in 25 μ l volume in individual, special flat tubes with good inter-locking cap, designed exclusively for the Smart Cycler by Cepheid, USA. The reaction mixture contained following ingredients: Kapa qPCR Probe Fast Buffer (10 μ l), Primer-PCVSKF (10 μ M; 0.8 μ l), Primer-PCV2SKR (10 μ M; 0.8 μ l), Probe-PCV2SKP (10 μ M; 1.0 μ l) and template DNA (1 μ g) and rest nuclease free water to prepare 25 μ l reaction mixtures. In each run, appropriate positive control and no template control (NTC) were included. The cycling condition was as follows: An initial denaturation step 30 sec at 95°C was followed by 40 cycles each comprising denaturation 3 sec at 95°C, annealing 20 sec at 53°C, extension 7 sec at 72°C.

For preparation of the standard curve, serial dilutions of 10^9 to 10^1 copies of the recombinant plasmid were used by plotting the logarithm of the plasmid copy number against the measured Ct values. Each assay was performed in duplicate. The standard curve generated had a linear correlation of $R^2 = 0.994$, regression curve with $Y = -0.214x + 11$ and the PCR efficiency was more than 100%. All analysis reports were generated by the inbuilt Software.

RESULTS AND DISCUSSION

Out of 86 preweaned piglets tested, tissue specimens from 6 piglets were found positive for PCV2 genome by conventional PCR and quantitative real-time PCR (TaqMan) assay. Quantification of tissue viral loads in positive test samples was effected by extrapolation from the standard curve and expressed in terms of initial copy number in logarithmic notations. The established real-time PCR assay for PCV2 DNA quantification was found to be in the range of 10^1 to 10^9 with excellent linearity. In this study PCV2 load was quantified in tonsil, mesenteric lymph node, spleen, lungs, liver, heart, kidneys, intestine and brain. The exact copy numbers of PCV2 DNA in different organs/tissues from preweaned piglets are summarized in Table 1. The average viral load in different organs of preweaned piglets was in the range of (32.9×10^8 copies/ μ g DNA to 1.14×10^8 copies/ μ g DNA) which is very high indicating systemic infection. Comparison of PCV2 load in all these organs revealed viral load in decreasing order: mesenteric lymph node, spleen, tonsil, lungs, heart, liver, kidneys, intestine and brain in terms of earliest Ct and its corresponding initial template value. Previous studies that estimated the virus load in serum of pigs affected with postweaning multi-systemic wasting syndrome (PMWS) showed significant variation between countries and laboratories that conducted the test. A study conducted in Spain found the virus load to be $10^{6.93}$ copies/mL (Fort *et al.* 2007), while in Canada, it was $10^{4.7}$ copies/mL serum (Harding *et al.* 2008). A subsequent study observed higher cut-off values in Spain ($10^{6.21}$ viral copies/mL serum) and Denmark ($10^{7.43}$ viral copies/mL serum) (Grau-Roma *et al.* 2009).

Table 1. Average Ct (dR) and quantity (copies) values of TaqMan Q PCR on different tissue samples of preweaned piglets

Organ	Average ct value/ μ g DNA	Average quantities copies/ μ g DNA
Mesenteric lymph node	16.76	32.92e +008
Tonsil	17.67	20.37e +008
Spleen	17.13	26.59e+008
Lungs	20.38	5.37e +008
Heart	18.99	10.65e +008
Intestine	23.52	1.14e +008
Liver	20.35	5.44e +008
Kidney	22.91	1.55e +008
Brain	27.73	Less than 1.14e+008

In another previous study conducted in Norway, viral load in the mesenteric lymph nodes and serum/plasma from healthy and PMWS affected animals differed significantly. While healthy pigs had viral load lesser than 10^6 PCV2 genomes /ml serum or 500 ng tissue sample, all clinically sick PMWS pigs had PCV2 loads above 10^7 in both serum/plasma and in tissue samples. Furthermore, the estimated viral load in tissue samples from PMWS pigs was related to the immunohistochemical findings, with especially lymph nodes, ileum, and tonsil giving both high viral load, and a high degree of staining by immunohistochemistry (Brunborg *et al.* 2004). In a farm showing PCV2 associated reproductive failure in Denmark, comparative assessment of the diagnostic value of immunohistochemistry (IHC) and quantification of PCV2 DNA by real-time PCR revealed that all the IHC positive heart specimens from foetuses had PCR values above 10^8 PCV2 genomes per 500 ng DNA and most IHC negative foetuses had values below it (Hansen *et al.* 2010). Though, virus load of 10^7 in serum/plasma and in tissue samples have been correlated with clinical disease, in one study, 18 of 20 pigs with a viral load above 10^7 per ml serum did not develop clinical PMWS or other PCV2 associated clinical signs (Brunborg *et al.* 2010). It may be noted that pigs with high viral load of PCV2 may mount a protective immune response to the infection, and do not necessarily develop PMWS (Grau-Roma *et al.* 2009).

The higher virus load observed in the present study may be accounted for by the relatively younger age group of pigs sampled (preweaning vs postweaning), nature of the samples (tissue vs serum) tested and sensitivity of the real-time PCR assay. The quantitative real-time PCR assay detected highest viral load in the mesenteric lymph nodes (32.92×10^8 copies/ μ g DNA) followed by spleen (26.59×10^8 copies/ μ g DNA), and tonsil (20.37×10^8 copies/ μ g DNA). Similar observations of high viral load in lymph nodes (mesenteric, mandibular, superficial inguinal) and tonsil in PCVAD affected pigs (Brunborg *et al.* 2004) and highest viral load in the mesenteric lymph nodes of PCV2 infected pigs showing diarrhoea (Zlotowsky *et al.* 2010) corroborate our observations. Apparently, lymphoid organs are the main target organs for virus replication and pathogenesis and hence the best samples for PCV2 detection and quantification.

In our study, PCVAD affected preweaned piglets showed high virus load in the heart, which was only next to the lymphoid organs and higher than levels found in various visceral organs and brain. This is in accordance with a previous report that detected relatively lower virus load in brain and higher viral load in heart with lesions of myocarditis. In fetuses, myocardium is the primary organ for virus replication (Brunborg *et al.* 2007). The virus load in the lungs of the preweaned piglets examined in the present study had lower virus load than heart but was higher than the levels detected in liver, kidney and intestines. Interstitial pneumonia is a frequent finding in PCVAD affected pigs but the lesions probably develop as a result of the recruitment of acute inflammatory and mononuclear

phagocytic cells, related to the expression of cytokines and chemokines from macrophages infected with PCV-2 (Chang *et al.* 2005), and does not occur as a direct result of viral replication in lung tissues (Silva *et al.* 2011).

In conclusion, the quantitative real-time PCR (TaqMan) assay established in this study can be used as a tool for screening PCV2 infected herds and aid in the study of pathogenesis of PCVAD. Quantification of PCV2 load in various organs of PCVAD affected preweaning piglets indicated highest viral loads in the lymphoid organs. The preferred specimens for diagnostic purposes are mesenteric lymph nodes, spleen and tonsil. The study also established for the first time that the virus load in PCVAD affected preweaned piglets of India is comparatively higher than previously recorded virus loads in cases of PMWS.

ACKNOWLEDGEMENT

The authors thank Department of Biotechnology, Govt. of India, for their financial support and acknowledge the Director, Indian Veterinary Research Institute for providing facilities to conduct this research work.

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