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Development and validation of TaqMan probe-based real-time polymerase chain reaction for detection of *Porcine Circovirus 2*[#]

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Abstract

Pigs are under constant threat from many infectious diseases and post-weaning multisystemic syndrome (PWMS) is one among them. The syndrome is caused by the Porcine Circovirus 2 (PCV2) which belongs to the Circoviridae family. Because the symptoms of PCV2 infection and other porcine infectious illnesses, especially porcine reproductive and respiratory syndrome (PRRS) overlap, diagnosis of the former based on clinical indicators could be challenging. A study was conducted to develop a TaqMan real-time polymerase chain reaction (PCR) for the detection of PCV2 genotypes prevalent in Kerala. Primers and TaqMan probes based on the ORF2 nucleotide sequences of the PCV2 prevalent in Kerala were designed. On testing, it was observed that the TaqMan real-time PCR was not able to detect the PCV2genotypes prevalent in Kerala. However, the designed primers (but not the probe) were able to detect these genotypes. Hence, another TaqMan assay specific for detection of 2d was designed as that genotype was predominant in Kerala. The detection limit estimated using the cloned template was found to be 310 copies of the viral genome. The assay was more sensitive in detecting the virus compared to conventional PCR.

Keywords: *PCV2, ORF2, TaqMan real-time PCR*

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Porcine circovirus 2 (PCV2), a single stranded DNA virus, belongs to the *Circoviridae* family and is classified as a member of the genus *Circovirus.* The virus was discovered for the first time in a Canadian swine herd in 1991. Since then, it has been suggested that PCV2 is the cause of post-weaning multi-systemic wasting syndrome (PMWS) (Cheung, 2003). Growth retardation, pale skin, dyspnoea, enlargement of inguinal lymph nodes and on rare occasions jaundice and diarrhoea are the clinical features of this disease in pigs. In addition to PMWS, PCV2 is also associated with congenital tremors, proliferative and necrotising pneumonia, reproductive failure, porcine dermatitis and nephropathy syndrome (PDNS) and diseases of the central nervous system (Harding, 2004; Vijayaragavan, 2021b). PCV2 infection has a significant negative economic impact on the swine sector globally (Grau-Roma *et al*., 2014). Despite the relatively modest mortality (4–20%), impacted farms might have morbidity rates of up to 50–60% (Segalés *et al*., 2005). However, the incidence of PMWS has drastically declined after immunisation was introduced in 2006 (da Silva *et al*., 2014).

In India and Kerala, the incidence of PCV2 infection in pigs has been reported since 2016 (Aishwarya, 2016) and at least three genotypes of the virus have been recorded in the state (Vijayaraghavan, 2021a). The existence of multiple genotypes of a virus makes its detection challenging. This paper reports the standardisation of a TaqMan real-time PCR for the detection of the genotypes of PCV2 prevalent in Kerala.

Materials and methods

Collection of samples

A total of 40 tissue samples (tonsils, mesenteric lymph node, lung, liver, and spleen) were collected from pigs brought for post-mortem to the Department of Veterinary Pathology, College of Veterinary and Animal Sciences, Pookode. The samples came from pigs that had died while exhibiting any of the PCV2 infection signs, including respiratory disease, gradual wasting, thriftiness, rough hair coat, polypnea, dyspnoea, pallor, diarrhoea, icterus *etc*. The tissue samples were placed in sterile screw-capped polypropylene vials and were processed immediately or stored at-80 °C until processing.

Molecular confirmation of PCV2 in clinical samples

Total DNA was extracted from the tissue samples using DNeasy Blood and Tissue Kit (QIAGEN, Germany) following the manufacturer's protocol. The extracted DNA was kept at -20 °C till further processing.

Detection of PCV2 using conventional PCR using ORF2-specific primers

Presence of PCV2 in the samples was tested by polymerase chain reaction (PCR), employing primers specific to the ORF2 region (coding for Cap protein) of the virus (Ellis *et al*., 1999). The 25 µL reaction mix consisted of 12.5 µL of 2X Emerald Amp GT PCR Mastermix containing Taq polymerase (TaKaRa), 1µL each of 10 pmol PCV2 forward and reverse primers, 2 µL of DNA, and rest nuclease-free water (NFW). The cycling conditions for amplification of PCV2 were 95 ºC for 2 min (initial denaturation), 35 cycles of 94 ºC for 30 seconds (denaturation), 55 ºC for 45 seconds (annealing), and 72 ºC for 45 seconds (polymerisation) followed by single cycle at 72 ºC for 5 min (final extension). The PCR products were electrophoresed in 1.5 % agarose gels containing ethidium bromide and the gel was visualised in a gel documentation system.

Genotyping of PCV2

Eleven representative positive samples were subjected to PCR for amplification of the complete genome of the virus using published primers (Anoopraj *et al*., 2015) that amplified three overlapping fragments of sizes 661, 765, and 676 bp. After the run, visualisation of the PCR product was carried out as described previously. The PCR amplicons were gel extracted using the GeneJET gel extraction kit (Thermo Scientific, USA) and sequenced at M/s AgriGenome Labs Private Limited, Kakkanad, Kochi, Kerala. The sequences were confirmed by BLAST and the complete genome was assembled using Emboss Merger (https://www.

bioinformatics.nl/cgi-bin/emboss/merger). The genotyping of the isolates was carried out by phylogenetic analysis as described bv Franzo and Segalés (2018).

Primer designing for TaqMan real-time PCR

The sequences obtained during the study and all the sequences of Kerala isolates of PCV2 available in GenBank were aligned using ClustalW program of MEGA X software (Kumar *et al*., 2018). Primers and probes were designed from the conserved ORF2 region of the aligned sequences using the online Oligo Architect software (www.sigmaaldrich.com). Initially, a set of primers and a probe were designed to detect all the genotypes of PCV2 prevalent in Kerala. Later a forward primer and probe were designed to detect the predominant genotype of PCV2 in Kerala (2d genotype). The details of the primer and probe sequences are given in table 1.

Standardisation of TaqMan real-time PCR

Conventional gradient PCR was conducted using real-time PCR primers (Table 1, Set 1) with an annealing temperature range of 52 to 62 ºC. The optimum concentration of primers and probe were arrived at by checkerboard titration. The 25 µL reaction mixture comprised of 12.5 µL EmeraldAmp GT PCR Master mix (2X) (TaKaRa), 1 µL each of primers (10 pmol), 2 µL of DNA, and rest NFW to make up the volume. The cycling conditions were 95 ºC for 2 min (initial denaturation), 35 cycles of 94 ºC for 1 min (denaturation), gradient from 52 to 62 ºC for 30 sec (annealing), and 72 ºC for 1 min (polymerisation) followed by single cycle at 72 ºC for 5 min (final extension). Representative PCV2b, 2d and 2h samples were used to test the primers. All the products were electrophoresed in two percent agarose gels with ethidium bromide and visualised using a gel documentation system.

The 20 µL TaqMan real-time PCR master mix contained 10 µL of 2X TaqMan^{R} Universal Master Mix II, with uracil-DNA glycosylase (UNG), (Applied Biosystems, USA), 4 µL, 1 µL and 2 µL of 10 pmol forward primer, reverse primer and TaqMan probe respectively, 2 µL of template DNA and rest NFW. The samples were run in a Step One plus real-time PCR machine (Applied Biosystems, Singapore) programmed to record the fluorescence of the reporter dye FAM. The cycling conditions were 50 ºC for 2 min (activation of UNG enzyme), 95 ºC for 10 min (denaturation of UNG enzyme and activation of polymerase), 40 cycles of 95 ºC for 15 sec (denaturation) and 57 ºC for 30 sec (annealing and extension).

The Set 2 primers and probe were tested in conventional and TaqMan real-time PCR as described earlier except that 0.5 µL (5 nmol) of primers and probes were used in real-time TaqMan PCR.

Cloning and sequencing of the PCR product

The 676 bp amplicons generated by one of the primer pairs used to amplify complete genome of PCV2 was purified from the gel and cloned into pJET1.2/blunt cloning vector using the CloneJET PCR cloning kit (Thermo

Table 1. Primer pairs and TaqMan probe sequences designed for the detection of PCV2

Set	Primer Name	Sequence 5'-3'	Target region/ Gene	Product size (bp)
1	PCV2 RT Ker F	TCCASTGCTGTTATTCTA	ORF2	81
	PCV2 PKD Taq - RP	GGGAGGAGTAGTTTACATA		
	PCV2 Ker Probe	6FAM-TGTAMCAAAGGCCA MWGCCCTVAC-BHQ1		
2	PCV2 PKD Taq - FP	TCCACTGCTGTTATTCTA		
	PCV2 PKD Taq – RP	GGGAGGAGTAGTTTACATA		
	PCV2 PKD TaqMan probe	6FAM-TGTAACAAAGGCCAATGCCCTAAC-BHQ1		

Scientific, USA) as per the manufacturer's protocol. This region was cloned as it contained the target sequence of the real-time PCR primers and probe. Competent cells (*E. coli* DH5α) were prepared and transformed with the ligated plasmid as described previously (Sambrook and Russell, 2001). The presence of the insert was confirmed in the transformed colonies by vector primers as per the manufacturer's protocol. The recombinant plasmid was extracted by using QIAprep® Spin Miniprep Kit (QIAGEN, Germany) and the concentration was estimated by Qubit 4 Fluorometer.

Sensitivity of TaqMan Real-Time PCR based on copy number

Serial 10-fold dilutions (10^{-1} to 10^{-10}) of the purified plasmid were prepared and it was used to determine the sensitivity of TaqMan real-time PCR based on copy number. Serial dilutions were made in NFW. The reactions were put in duplicate as described earlier with 2 µL of 10^{-1} to 10^{-10} dilution of plasmid DNA as a template. The cycling conditions described previously were followed. The highest dilution of plasmid DNA which could be detected in the real-time PCR was determined from the C_t values obtained. The copy number of the recombinant plasmid was estimated using the formula: No. of copies = (amount x 6.022×10^{23})/ length of plasmid with insert x 1 x 10^9 x 650). This calculation was based on the assumption that the average weight of a base pair (bp) is 650 Daltons *i.e*., one mole of a bp weighs 650 g, and that the molecular weight of any double-stranded DNA template can be estimated by taking the product of its length (in bp) and 650. The inverse of the molecular weight is the number of moles of template present in one gram of material. Using Avogadro's number, 6.022×10^{23} molecules/mole, the number of molecules of the template per gram can be calculated:

Mol/g (x) molecules/mol = molecules/g.

Finally, the number of molecules or number of copies of the template in the sample was estimated by multiplying by $1x10^9$ to convert to ng and then multiplying by the amount of template (in ng).

Statistical analysis

The sensitivity, specificity, and accuracy of the TaqMan Real-Time PCR were compared with the ORF2-based PCR and were calculated using the following formula:

Sensitivity : a / (a+c)

Specificity : d / (b+d)

Accuracy : a+d / (a+b+c+d)

a is the number of samples positive by both i.e., test to be compared and gold standard test, b is the number of samples positive by standard test whereas negative by test to be compared, c is the number of samples negative by standard test and positive by test to be compared and d is the number of samples negative by both.

The results obtained from the test were analysed for the percentage of agreement with ORF2-based PCR with the use of Kappa statistics using SPSS version 24.0 software. Kappa statistics are decimal measures of agreement between two tests, especially in the absence of a standard, and are defined as kappa or κ.

Results and discussion

PCV2 infection is reported to be present in every major swine producing country in the world and the number of cases of porcine circovirus associated disease (PCVAD) is rapidly increasing (Rajesh *et al*., 2020). Infection with PCV2 is clinically characterised by wasting, paleness of the skin, respiratory distress, and occasionally, diarrhoea and jaundice in the late nursery and fattening pigs at usually 4 to 16 weeks of age (Segalés *et al.*, 2005). Besides these, the virus, in association with other porcine viruses such as PRRSV, increases the severity of clinical symptoms of PCV2 in pigs (Sinha *et al*., 2011).

Of the 40 samples tested, 20 (50 %) were positive for PCV2 as evidenced by 481 bp amplicons. The nucleotide sequences on BLAST analysis were confirmed to be of PCV2. On phylogenetic analysis, eight samples were found to contain PCV2d, 2 samples contained 2h and one sample was found to contain PCV

Fig. 1. Agarose gel showing the 81 bp amplicons generated by conventional gradient PCR by primer set 1

Lane 1 and 9: 100 bp ladder

Lane 2-5: Clinical sample (PCV2d) (Annealing temperatures 53, 55, 57 and 60 respectively)

Lane 7, 8, 10, 11: Clinical sample (PCV2b) (Annealing temperatures 53, 55, 57 and 60 respectively)

Lane 13-16: Clinical sample (PCV2h) (Annealing temperatures 53, 55, 57 and 60 respectively)

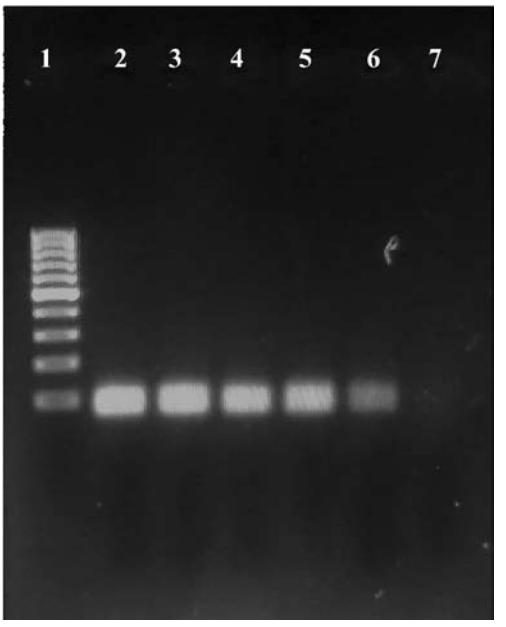

Fig. 2. Agarose gel showing the 81 bp amplicons generated by conventional gradient PCR by primer set 2 for PCV2d

Lane 1- 100 bp ladder; Lane 2-52°C; Lane 3-55°C;

Lane 4-56°C; Lane 5-58°C; Lane 6-60°C;

Lane 7-62°C;

2b. The existence of these genotypes in Kerala has been reported previously (Vijayaraghavan, 2021) and in India, the genotypes 2a, 2b, 2d, 2f, 2g, and 2h are commonly detected (Bhattacharjee *et al.*, 2021). Franzo and Segalés, (2018) reported eight different genotypes after carrying out phylogenetic analysis of the ORF2 sequences of PCV2 namely, PCV2a, PCV2b, PCV2c, PCV2d, PCV2e, PCV2f, PCV2g, and PCV2h.

Larochelle *et al.* (2002) studied the phylogenetic relationship of the Canadian isolates of PCV2 based on ORF1 and ORF2 genes and reported that ORF2 had greater nucleotide variation than ORF1. The standardisation of TaqMan real-time PCR was carried out using the primers and probe which were designed from the ORF2 region of the aligned sequences. In conventional PCR, the real-time PCR primers (Set 1) amplified the expected 81 bp fragment of ORF2 of PCV2d, 2d and 2h (Figure 1). The degeneracy was introduced in forward primer (S= C/G) and the probe (i.e., M= A/C; W= A/T; V= A/C/G) to make them sensitive to all the genotypes of PCV2 reported in Kerala until now. However, the probe was not able to detect any of these genotypes as evidenced by absence of amplification in the real-time PCR reaction. This might be due to the high degree of degeneracy in the designed probe.

The modified forward primer and the reverse primer (Set 2) were able to detect PCV2d in conventional PCR (Fig. 2). The sensitivity of TaqMan real-time PCR was estimated by using the recombinant plasmid with PCV2 insert and it was confirmed by subjecting it to PCR using vector primers. The concentration of the recombinant plasmid was estimated to be 6.18 ng/µL, using a Qubit 4 Fluorometer. The

Table 2. Comparison of results of conventional PCR and TaqMan real-time PCR

Conventional PCR	Real-time PCR	
	Positive	Negative
20 Positive	19	1
20 Negative	4	16

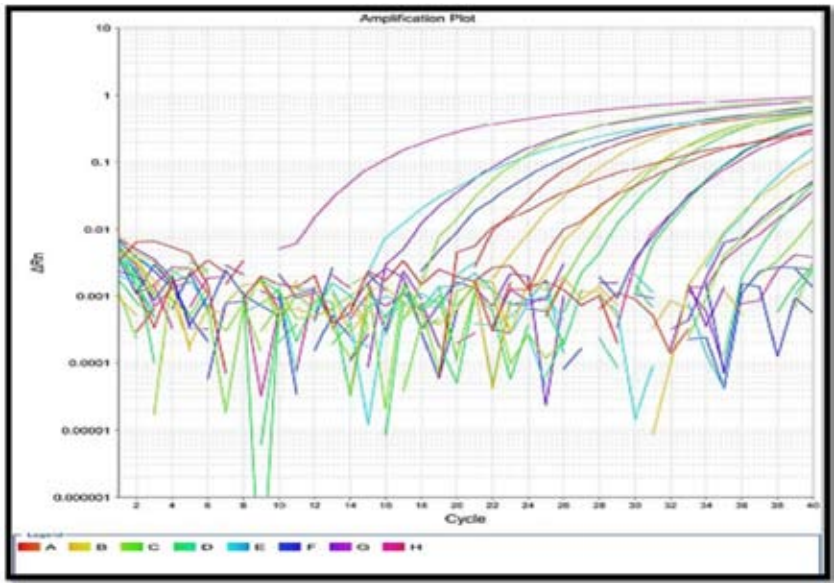

Fig. 3. Amplification plot of clinical samples tested by TaqMan real-time PCR

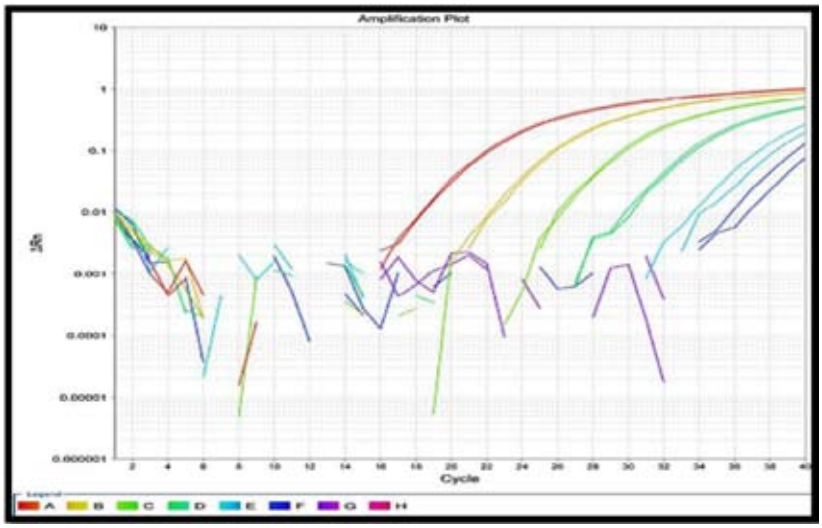

Fig. 4. Determination of limit of detection of TaqMan real-time PCR (Amplification plot)

copy number of the recombinant plasmid was estimated as 1.55x 10^9 copies/µL. The highest dilution that exhibited a Ct value was 10^{-7} which corresponds to a copy number of 155 copies/µL and the detection limit of the assay was estimated to be 310 copies. Slope and R^2 were estimated to be -3.705 and 0.992, respectively. The efficiency of the TaqMan real-time PCR was calculated as 86.153 per cent (Fig. 4 and 5).

The sensitivity, specificity and accuracy of the TaqMan real-time PCR were estimated to be 0.95, 0.80, and 0.875, respectively in

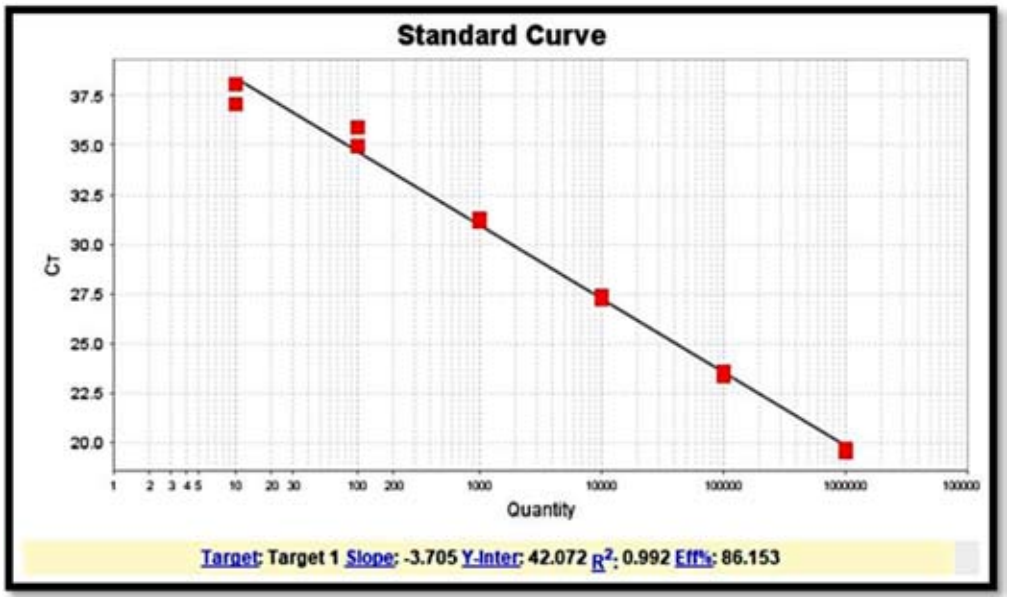

Fig. 5. Determination of limit of detection of TaqMan real-time PCR (Standard curve and estimation of efficiency)

comparison with ORF2-based PCR. The percentage of agreement of the TaqMan real-time PCR based on ORF2 with conventional PCR based on ORF2 was analysed with the results obtained using Kappa statistics. Kappa or κ was estimated to be 0.75 which indicates substantial strength of agreement between the two tests.

TaqMan real-time PCR was carried out to detect the presence of PCV2, using the DNA of 40 (20 positive and 20 negative) samples and their respective Ct values were noted. The Ct values varied from 6.86 to 39.14. Out of 40 samples tested by TaqMan real-time PCR, 23 samples were found to be positive for PCV2. Percentage positivity was estimated to be 57.5 (Fig. 3) which is more when compared to that obtained in ORF2-based PCR. This indicates that TaqMan real-time PCR assay is more sensitive than conventional PCR. The results of TaqMan real-time PCR in comparison to the results of gel-based PCR are given in Table 2.

When compared to conventional PCR, the increased sensitivity and specificity of TaqMan real-time PCR have been reported previously (Klein, 2002). The 5'-3' exonuclease activity of the Taq DNA polymerase cleaves the dual-labelled fluorogenic probe of the TaqMan real-time PCR assay. The assay includes binding both primers and probes specific to their target sequences, eliminating the possibility of nonspecific amplification products and resulting in a more accurate result (Egli *et al*., 2001). There are other reports also on the superior sensitivity of TaqMan Real-time PCR. Zhao *et al*. (2010) and Yuan *et al*. (2014) reported that on comparison of conventional PCR and TaqMan probe-based RT-PCR, the latter had the best detection rate for PCV2. Additionally, TaqMan real-time PCR assay can be employed for detection of viral load and virus quantitation in tissues of pigs with PMWS and PDNS (Olvera *et al.*, 2004).

The ORF2 of PCV2 has a lower nucleotide homology with *Porcine circovirus 1* (PCV1) than ORF1 (~65%) (Liu *et al*., 2001). Thus, this region is best suitable for designing primers and probes for detection of PCV2 (Olvera *et al.,* 2004). The ORF2 is also a reliable phylogenetic and epidemiological marker for PCV2 strains since it was able to reconstruct the same tree as the whole viral genome.

Though the study was not successful in standardising a real-time PCR for the detection of all the PCV2 genotypes prevalent in Kerala, a PCR that could detect all the three genotypes was standardised. Additionally, a TaqMan real-time PCR that could detect PCV2d

was standardised which was found to be more sensitive than conventional PCR for detection of the virus. It is suggested that for diagnosis of diseases caused by viruses having multiple genotypes, more than one diagnostic assay may be employed to attain reliable results.

Conclusion

Porcine circovirus 2 is the causative agent of a variety of disease conditions in pigs causing huge economic losses to the farmers. In the present study, an attempt was made to standardise a TaqMan real-time PCR that is capable of detecting the genotypes of the virus prevalent in pigs in Kerala. While an assay that could detect all the prevalent genotypes could not be standardised, one that could detect PCV2d genotype that is predominant in Kerala was standardised. The TaqMan assay was more sensitive in detecting the PCV2 when compared to conventional PCR.

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Conflict of interest

The authors declare that they have no conflict of interest.

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