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Development of a droplet digital polymerase chain reaction for sensitive and simultaneous identification of porcine circovirus type 2 and 3

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ABSTRACT

In this study, a sensitive assay for simultaneous detection of porcine circovirus type 2 (PCV2) and type 3 (PCV3) was established using droplet digital PCR (ddPCR). Pairs of primers and probes were specifically designed to amplify PCV2 and PCV3. Specificity of the assay was confirmed by the failure of amplification of DNA of other relevant viruses. The detection limit for ddPCR was 2 copies/μL for PCV2 and 1 copy/μL for PCV3, a 3- and 10-fold greater sensitivity than TaqMan real-time PCR, respectively. Both methods showed a high degree of linearity, although TaqMan real-time PCR showed less sensitivity than ddPCR for clinical detection. In summary, the results demonstrated that the established ddPCR is an effective alternative method for the precise simultaneous quantification of PCV2 and PCV3 in clinical samples, especially in detecting lower concentrations of co-infected samples.

Porcine circovirus (PCV) is a small, non-enveloped virus that contains a circular, single-stranded DNA genome and belongs to the family Circoviridae, genus Circovirus (Bexton et al., 2015). Three PCV major species have been identified as PCV type 1 (PCV1), PCV type 2 (PCV2) and a newly identified PCV type 3 (PCV3) (Ellis, 2014 ; Phan et al., 2016). PCV1 is non-pathogenic to pigs, while PCV2 was a globally important pathogen that caused PCV-associated diseases in growing pigs, including systemic infection, porcine dermatitis and nephropathy syndrome, reproductive failure, and respiratory and enteric manifestations (Ellis, 2014). Recently, PCV3, with a genomic size of about 2000 bp, was reported as the etiologic agent of porcine dermatitis, nephropathy syndrome, and reproductive failure and caused cardiac and multisystemic inflammation (Phan et al., 2016). Since it was first identified in 2016, the co-infection of PCV2 with PCV3 has been frequently reported in clinical samples of diseased pigs (Ku et al., 2017). Currently, PCV2 and PCV3 were tested separately using PCR or enzyme-linked immunosorbent assay (ELISA) methods in routine laboratory detection, which is less sensitive and can be time-consuming. It is more convenient to develop a sensitive and rapid method to detect PCV2 and PCV3 simultaneously.

Recently, a duplex real-time PCR method has been developed to detect PCV2 and PCV3 simultaneously (Li et al., 2018). However, the real-time PCR based method depends on standard curve for quantitative detection and the accuracy of quantification is easily affected by PCR inhibitors (Zhao et al., 2011). To simplify the operation steps and improve the efficiency, a droplet digital (ddPCR) reaction system was established in this study to simultaneously detect PCV2 and PCV3 and the optimization of time, specificity and sensitivity was certified.

The primers and probes were designed for real-time PCR using Primer Premier 3.0. The primers and probes set designed were as follow: PCV2-F1 (5′-CGGGAGTGGTAGGAGAA GG-3′); PCV2-R1 (5′-GTTGAATTCTGGCCCTGCT-3′); and PCV2-probe (5′-FAM-ATG GCGG GAGGAGTAGTTTACATA- BHQ1-3′); PCV3-F1 (5′-GGTTCCAACGGAAA TGA CGTT-3′); PCV3-R1 (‘-GCCCACAGCTGGCACATAC-3′); and PCV3-probe (5′-VIC- ATGGTGGAGTATTTCTT-MGB-3′). The primers and the probes were synthesized by Sangon Biotech (Shanghai). The plasmid standard was constructed by inserting the complete sequence of ORF2 (openreading frame 2, GenBank No.KC823059.1) of PCV2 and ORF2 (GenBank No. KY778776) of PCV3 into commercial vector pUC57 (Sangon Biotech, Shanghai), according to the manufacturer’s instructions, and was then transformed into *Escherichia coli* DH5α cells. A NanoVue Plus (NanoDrop products, USA) was used to quantify the concentration of purified recombinant plasmid, which was then serially diluted. Dilutions and plasmids were stored at −20 °C and −70 °C, respectively.

The real-time PCR analysis was performed using the ABI QuantStudio 6 Flex real-time PCR System (ABI, USA). The standard curve was generated using standard plasmid DNA of 10-fold serial

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dilutions. The real-time PCR reactions comprised 10 μL of AceQ qPCR Probe Master Mix (Vazyme, China), each primer and probe at final concentrations of 400 nM and 200 nM, 1 μL of each template, and double-distilled water to complete a reaction volume of 20 μL. Amplification programs were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 10 s, and 60 °C for 34 s. Standard curves constructed for serial dilutions of plasmid DNA were included in every real-time PCR run to produce quantitative results instead of raw Cq values. A no template control and standard curve were included in all runs and every sample was measured in triplicate.

The ddPCR analysis was performed using a Bio-Rad QX200™ Droplet Digital™ PCR system (Bio-Rad, USA). According to the manufacturer's instructions, the optimized ddPCR reaction mixtures included 10 μL of 2× ddPCR Supermix for Probes (No dUTP) (Bio-Rad, USA), each primer and probe at final concentrations of 800 nM and 400 nM, 1 μL of each template, and double-distilled water to complete a reaction volume of 20 μL. No template controls were used as negative controls. The thermocycling protocol was: initial denaturation at 95 °C for 10 min, then 40 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 60 s (temperature ramp 2 °C/s) and, finally, incubation at 98 °C for 10 min and storage at 4 °C. The copy number of each sample was reported automatically by QuantaSoft™ version 1.7 (Bio-Rad, USA) after set a threshold between positive and negative fluorescent signals. No optimization of ddPCR was necessary with respect to real-time PCR annealing temperature or probe concentration.

The specificity of real-time PCR and ddPCR were assessed with purified genomic DNA from PCV2, PCV3, porcine parvovirus (PPV), classical swine fever virus (CSFV), and porcine reproductive and respiratory syndrome virus (PRRSV), 15 reference *A. pleuropneumoniae* strains (serotype 1–15), 15 reference *Haemophilus parasuis* (serotype 1–15) and *Streptococcus suis* serotype 2 which were obtained from Yongshun biological pharmaceutical Co. Ltd. (Guangdong, China). Recombinant plasmid pUC57-PCV2 and pUC57-PCV3 were used as the positive, respectively. All trials identified the target strains and positive samples correctly without generating false-positive or false-negative results, thereby confirming assay specificity.

To evaluate the sensitivity of real-time PCR and ddPCR, serial dilutions of pUC57-PCV2 and pUC57-PCV3 were used as templates (Table 1). Three replicates with three lowest concentrations of pUC57-PCV2 and pUC57-PCV3 were run for determination of limit of detection (LoD). The LoD was determined as the lowest template concentration when 95% positive samples can be detected. For comparison, copy numbers were calculated for real-time PCR based on the concentration calculated by standard curve using a calculator (available at http://scienceprimer.com/copy-number-calculator-for-realtime-pcr). The results showed that the LoD was 6 copies/μL for PCV2 and 10 copies/μL for PCV3 by real-time PCR, respectively. In contrast, the LoD was 2 copies/μL for PCV2 and 1 copy/μL for PCV3 by ddPCR, respectively (Table 1, Fig. 1). The results suggested that the sensitivity of ddPCR assay was observed to increase at least 3 times than real-time PCR assay.

Table 1
Sensitivity and efficiency of the real-time PCR and ddPCR assays obtained by series of 10-fold and 2-fold dilutions of the plasmid DNA.

Concentration of plasmids[a] (copies/μL)		Real-time PCR (Mean Cq value)		ddPCR (Mean concentration, copies/uL)[b]	
PCV2	PCV3	PCV2	PCV3	PCV2	PCV3
9.850×10^6	9.846×10^6	17.4	18.8	ND	ND
1.640×10^5	1.720×10^5	22.5	22.3	12659	14236
1.281×10^4	1.872×10^4	26.9	25.7	1225	1419
1.275×10^3	1.871×10^3	29.4	29.2	136	136
1.33×10^2	1.873×10^2	31.6	32.9	16	15
92	96	34.9	36.3	8	8
23	36	38.7	38.4	3	4
17	9	ND	ND	2	1

Cq = quantification cycle; ND = not detected.
[a] Concentration based on 10-fold and 2-fold serial dilutions of plasmids.
[b] Concentration based on ddPCR detection.

Linearity over the dynamic range was determined by the R^2 calculated on the mean value of target copy numbers measured in the replicated dilution series for ddPCR and real-time PCR. TaqMan assays with real-time PCR (R^2 = 0.9613 for PCV2, and 0.9659 for PCV3) and ddPCR (R^2 = 0.9987 for PCV2, and 0.9959 for PCV3) showed good linearity in the range of quantification (Fig. 2).

In order to evaluate the reproducibility of ddPCR, a serial diluted pUC57-PCV2 and pUC57-PCV3 was prepared and was repeated for three times. The coefficient of variation (CV) was calculated to reflect the reproducibility between runs. For PCV2, the CVs of intra-assay and inter-assay ranged between 0.34% and 4.00%, 0.48% and 4.97%, respectively (Table S1). For PCV3, The CVs of the intra-assay and inter-assay ranged between 0.08% and 4.00%, 0.18% and 4.00%, respectively, indicating a good reproducibility of the ddPCR assay (Table S2). The CVs of the intra-assay and inter-assay for PCV2 and PCV3 by real-time PCR also indicated a good reproducibility (Tables S3 and S4).

Three hundred clinical tissue samples (livers, lungs, kidneys, spleens and lymph nodes) were obtained from ten pig farms supplied by Institute of Animal Sciences, Guangdong Academy of Agricultural Sciences. In these samples, 30 were PCV2-positive and PCV3-negative, 20 were PCV3-positive and PCV2-negative confirmed by PCR and sequencing, 250 were suspected PCV2 or PCV3 infected samples. Total DNA was extracted using RNA/DNA extraction kit (Magen, China) following the manufacturer's recommendations and analyzed by both real-time PCR and ddPCR within the optimized conditions. For 50 PCV2 or PCV3 positive clinical samples, both real-time PCR and ddPCR assays showed 100% positive and did not show any false positive results. For 250 clinical samples, both methods detected 210 PCV2-positive samples and 2 PCV3-positive samples, and did not show any false positive results for 35 negative samples (Tables 2a and 2b). However, for 3 co-infected samples, real-time PCR detected only PCV2, whereas ddPCR correctly identified PCV2 and PCV3. The positive samples were further confirmed by ELISA, PCR and sequencing. The negative samples were further confirmed by ELISA. The ddPCR assay showed higher positive predictive value compared to the real-time PCR assay, indicating that ddPCR is a more effective method for suspected PCV2 and PCV3 mixed infected clinical samples diagnose. Kappa statistics were used to determine the agreement between ddPCR and TaqMan real-time PCR. The kappa statistic was 1 and 0.930 for PCV2 and PCV3 (with 95% confidence intervals), respectively, indicating almost perfect agreement between ddPCR and TaqMan real-time PCR.

According to previous reports, several PCR based methods have been established for detecting PCV, including a ddPCR assay for detecting PCV2 (Zhao et al., 2015), SYBR green-based and TaqMan-based real-time PCR assays for detecting PCV3 (Chen et al., 2018; Wang et al., 2017), a LAMP assay for detecting PCV3 (Zheng et al., 2018) and duplex real-time PCR for detect PCV2 and PCV3 simultaneously (Li et al., 2018). Compared with these reported PCR methods, ddPCR has been confirmed to be more sensitive for low copy number quantification (Zhao et al., 2011), less susceptible to PCR inhibitors, and thus can detect target DNA in complex environments (Zhao et al., 2015). However, when the template was higher than 100,000 copies, the ddPCR droplets become completely saturated which indicating that this new technique would be more practical applied to vaccinated animals. In contrast, real-time PCR exhibited a wider range of detection.

In this study, we established a ddPCR method for the simultaneous identification of PCV2 and PCV3. The ddPCR system developed in the present study was observed to be specific and the detection limit of ddPCR was about at least 3 times lower than real-time PCR. The linear regression correlation coefficients measured using either ddPCR or TaqMan real-time PCR showed good R^2 values. There were 3 mixed

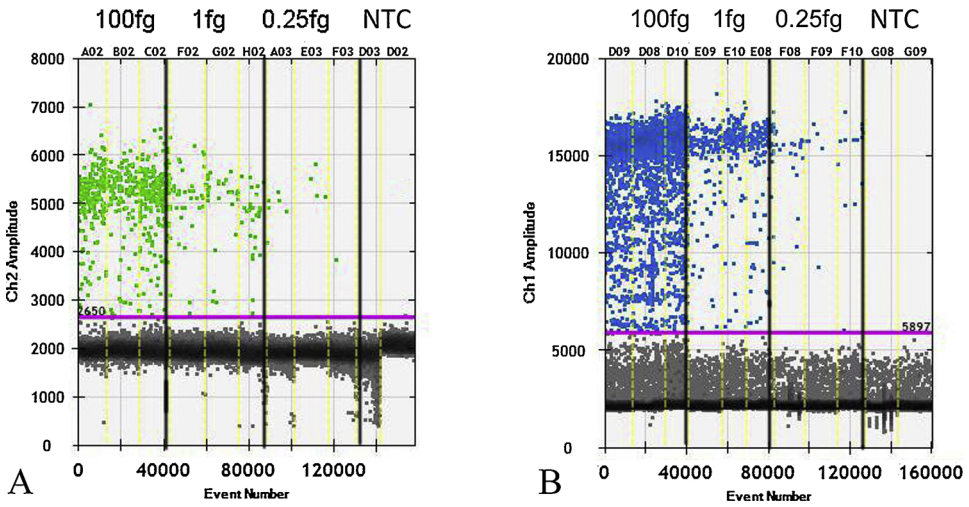

Fig. 1. Example of ddPCR (1D Droplet Plots) by using dilutions of recombinant pUC57-PCV2 (A) and pUC57-PCV3 (B). Six replicates were run with the lowest concentrations. NTC = negative template control.

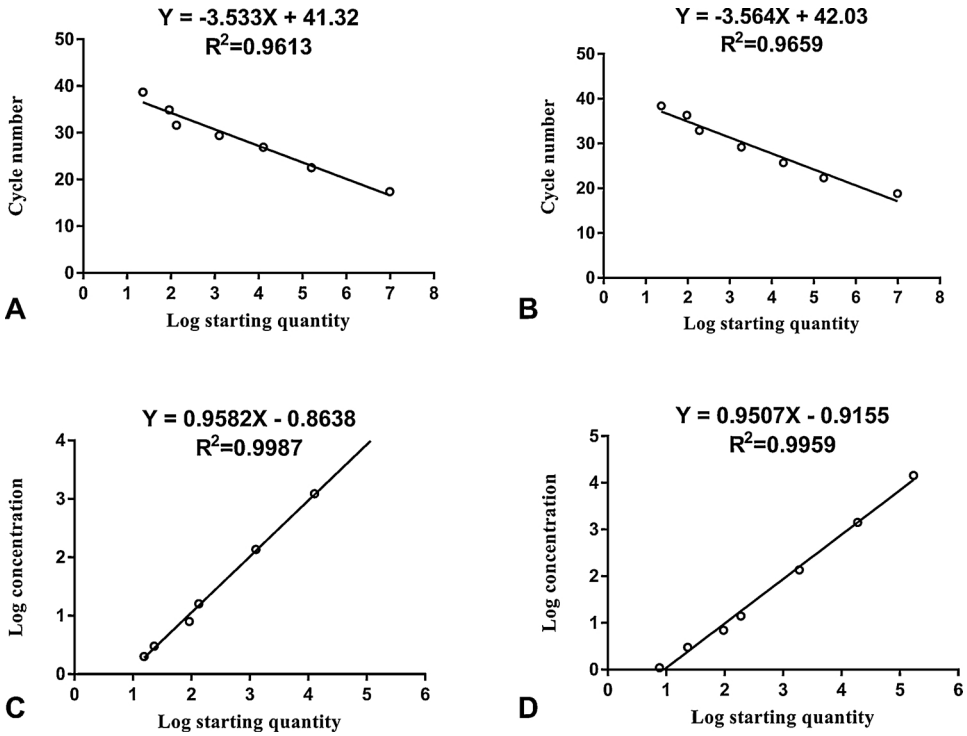

Fig. 2. Quantification of serially diluted recombinant pUC57-PCV2 and pUC57-PCV3 by ddPCR and real-time PCR. A and B, standard curves of pUC57-PCV2 and pUC57-PCV3 constructed by real-time PCR, respectively. The quantification correlation was obtained by plotting the quantification cycle value against the Log starting concentration. C and D, standard curves of pUC57-PCV2 and pUC57-PCV3 constructed by ddPCR, respectively. The quantification correlation of ddPCR was obtained by plotting the Log absolute concentration against the Log starting concentration.

Table 2a
Performance of ddPCR and real-time PCR assay for detection of PCV2 in clinical samples.

Detection methods		ddPCR		
		Positive	Negative	Total
Real-time PCR	Positive	243	0	243
	Negative	0	57	57
	Total	243	57	300

Table 2b
Performance of ddPCR and real-time PCR assay for detection of PCV3 in clinical samples.

Detection methods		ddPCR		
		Positive	Negative	Total
Real-time PCR	Positive	22	0	22
	Negative	3	275	278
	Total	25	275	300

infected samples that tested correctly by ddPCR but not with real-time PCR. Taken together, these data suggest that the established ddPCR is an effective alternative method for the precise simultaneous quantification of lower concentrations of PCV2 and PCV3 in clinical vaccinated animals. Therefore, it represents a new strategy to simultaneous quantitative detection of PCV2 and PCV3, and may serve as a basis for the development of alternative rapid diagnostic techniques.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.04.021.

References

Bexton, S., Wiersma, L.C., Getu, S., van Run, P.R., Verjans, G.M., Schipper, D., Schapendonk, C.M., Bodewes, R., Oldroyd, L., Haagmans, B.L., Koopmans, M.M., Smits, S.L., 2015. Detection of circovirus in foxes with meningoencephalitis, United Kingdom, 2009–2013. Emerg. Infect. Dis. 21, 1205–1208. https://doi.org/10.3201/eid2107.150228.

Chen, G.H., Tang, X.Y., Sun, Y., Zhou, L., Li, D., Bai, Y., Mai, K.J., Li, Y.Y., Wu, Q.W., Ma, J.Y., 2018. Development of a SYBR green-based real-time quantitative PCR assay to detect PCV3 in pigs. J. Virol. Methods 251, 129–132. https://doi.org/10.1016/j.jviromet.2017.10.012.

Ellis, J., 2014. Porcine circovirus: a historical perspective. Vet. Pathol. 51, 315–327. https://doi.org/10.1177/0300985814521245.

Ku, X., Chen, F., Li, P., Wang, Y., Yu, X., Fan, S., Qian, P., Wu, M., He, Q., 2017. Identification and genetic characterization of porcine circovirus type 3 in China. Transbound. Emerg. Dis. 64, 703–708. https://doi.org/10.1111/tbed.12638.

Li, X., Qiao, M., Sun, M., Tian, K., 2018. A duplex real-time PCR assay for the simultaneous detection of porcine circovirus 2 and circovirus 3. Virol. Sin. 33 (2), 181–186. https://doi.org/10.1007/s12250-018-0025-2.

Phan, T.G., Giannitti, F., Rossow, S., Marthaler, D., Knutson, T.P., Li, L., Deng, X., Resende, T., Vannucci, F., Delwart, E., 2016. Detection of a novel circovirus PCV3 in pigs with cardiac and multi–systemic inflammation. Virol. J. 13, 184. https://doi.org/10.1186/s12985-016-0642-z.

Wang, J., Zhang, Y., Wang, J., Liu, L., Pang, X., Yuan, W., 2017. Development of a TaqMan-based real-time PCR assay for the specific detection of porcine circovirus 3. J. Virol. Methods 248, 177–180. https://doi.org/10.1016/j.jviromet.2017.07.007.

Zhao, K., Shi, W., Han, F., Xu, Y., Zhu, L., Zou, Y., Wu, X., Zhu, H., Tan, F., Tao, S., Tang, X., 2011. Specific, simple and rapid detection of porcine circovirus type 2 using the loop–mediated isothermal amplification method. Virol. J. 8, 126. https://doi.org/10.1186/1743-422X-8-126.

Zhao, S., Lin, H., Chen, S., Yang, M., Yan, Q., Wen, C., Hao, Z., Yan, Y., Sun, Y., Hu, J., Chen, Z., Xi, L., 2015. Sensitive detection of porcine vcircovirus–2 by droplet digital polymerase chain reaction. J. Vet. Diagn. Invest. 27 (6), 784–788. https://doi.org/10.1177/1040638715608358.

Zheng, S., Wu, X., Shi, J., Peng, Z., Gao, M., Xin, C., Liu, Y., Wang, S., Xu, S., Han, H., Yu, J., Sun, W., Cong, X., Li, J., Wang, J., 2018. Rapid specific and visible detection of porcine circovirus type 3 using loop–mediated isothermal amplification (LAMP). Transbound. Emerg. Dis. 65 (3), 597–601. https://doi.org/10.1111/tbed.12835.