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Letter

Development of a fluorescent probe hydrolysis-insulated isothermal PCR for rapid and sensitive on-site detection of African swine fever virus

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Dear Editor,

African swine fever virus (ASFV), the sole member of the family *Asfarviridae*, is the causative agent of African swine fever (ASF), a viral disease that leads to high mortality in domestic pigs. Since firstly identified in Kenya in the 1920s, ASFV has been prevalent in Africa, Europe, and Russian Federation (Sanchez et al., 2019). Recently, ASFV was introduced to Asian countries including China, Mongolia, Vietnam, Cambodia, Laos and South Korea, which lead to huge economic losses to local pig industries (Li and Tian, 2018; Gaudreault and Richt, 2019). The first case of ASFV in China was reported in August 2018 (Zhou et al., 2018). Since then, hundreds of cases in most provinces were officially announced and more than 1 million pigs were slaughtered under the strict stamping-out policy (Miao et al., 2019).

Currently, the World Organisation for Animal Health (OIE) recommends conventional and real-time PCRs as the commonly used assays to detect virus DNA in clinical samples (Luo et al., 2017). However, these molecular methods require expensive devices and well-trained personnel and can only be performed in the well-equipped laboratories. Therefore, a low-cost, sensitive and user-friendly method is urgently required for onsite detection of virus in the field. Insulated isothermal PCR (iiPCR) has been recently described to detect some veterinary viruses with the equivalent of sensitivity and specificity of real-time PCR (Lung et al., 2016; Ambagala et al., 2017; Zhang et al., 2019). Based on Rayleigh-Bénard convection PCR (Tsai et al., 2012), spontaneous fluid convection is triggered in a capillary tube heated within a thermally baffled device, where the denaturation, annealing, and extension of PCR are automatically completed passing sequentially through different temperature zones in a capillary tube. Fluorescence signals produced by probe hydrolysis during the reaction are collected and converted to Signal $_{after}$/Signal $_{before}$ (S/N) ratios by a data processing module. Finally, the positive or negative results are displayed on the device screen, which is user-friendly enough to perform by untrained personnel.

In this study, we developed a simple and rapid iiPCR for ASFV field surveillance targeting a highly conserved region of *B646L* gene, which only takes 25 minutes and the sensitivity is equivalent to that of real-time PCR in the laboratory. The sequences of forward and reverse primers were 5′-ATT TCA TTA ATG ACT CCT GG-3′ and 5′- TAC TAT CAG GCC CCC TCT-3' , respectively. The sequence of probe was 5'-[FAM]-ATA AAC CAT GGT TTA AAG-3′-BHQ1. The primers and probe were synthetized by Shanghai BIOLIGO Biotechnology Co. Ltd, China. For iiPCR setup, 35 μL PCR reaction mix (Luoyang Putai Biotechnology Co., Ltd, China) including 0.15 μmol/L primers, 0.05 μmol/L probe was added to individual PCR tubes. Five microliter template (diluted plasmid or extracted nucleic acid) and 10 μL liquid paraffin wax were added before a briefly spun in a mini centrifuge. The PCR tubes were put into the reaction chamber in the iiPCR analyzer (Beijing Wantai Biological Pharmacy Enterprise Co., Ltd., China) and run on the preset program, which top and bottom temperatures were set as 58 °C and 95 °C, respectively (Fig. 1). A threshold was formulated as mean S/N ratio of non-template blank reactions +5 standard deviations (SD) as previously described (Tsai et al., 2012). The mean and SD of S/N ratios from 20 blank ASFV iiPCR reactions were 1.02 and 0.08, respectively, resulting in a threshold of 1.42. The results were calculated with defaulted S/N thresholds and displayed as "+" or "–" for positive and negative samples.

To determine the sensitivity of ASFV iiPCR, 10-fold serial dilutions of a plasmid DNA containing *B646L* gene were used and compared with OIE recommended real-time PCR (Chen et al., 2019). The purified plasmid was quantified by measuring OD_{260} with the spectrophotometer. The concentration was converted into copy numbers using the following

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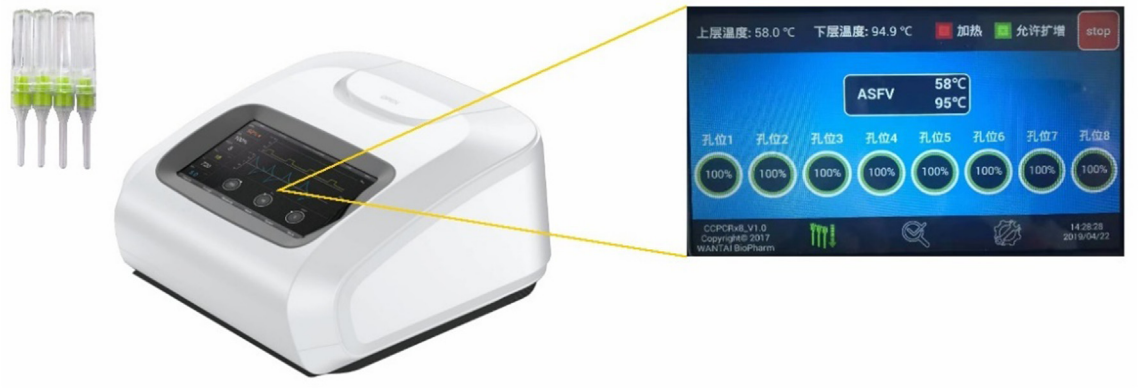

Fig. 1. Illustration of ASFV iiPCR device and PCR tube. The PCR tubes were put into the individual reaction chambers in the compact and portable iiPCR device. The reaction started when pushing the start button on the touch screen and the real-time process of reaction visualized in percentage appeared on the screen until the results shown with "+" for positive sample or "–" for negative sample. The iiPCR could be completed within 25 minutes.

formula: y (copies/μL) = $(6.02 \times 10^{23}) \times$ (x (ng/μL) $\times 10^{-9}$ DNA)/(DNA length $\times$ 660) (Chen et al., 2019). As shown in Table 1, detection limits of iiPCR reached at 10^{-8}-fold dilution with positive results of five repeats. By contrast, limits of detection of real-time PCR reached same dilution with four positive results and one suspected result of five repeats. The one suspected sample was proved to be positive when it was repeated as the manual described. The above results indicated that the established iiPCR had equivalent sensitivity as the OIE recommended real-time PCR.

To assess the specificity of ASFV iiPCR, nucleic acids were extracted from 200 different tissues of pigs including serum, nasal swabs, spleen, kidney, lung, lymph nodes, heart, liver, and brain, which had been collected before 2018 and confirmed ASFV negative using OIE recommended real-time PCR. The S/N values of above samples ranged from 0.96 to 1.07, indicating the good specificity of ASFV iiPCR. Nucleic acids were also extracted from other swine viruses including classical swine fever virus (CSFV) C strain, highly pathogenic porcine reproductive and respiratory syndrome virus (PRRSV) CH-1R, pseudorabies virus (PRV) Bartha-K61, porcine circovirus 2 (PCV2) HH3, porcine epidemic diarrhea virus (PEDV) HN1301, transmissible gastroenteritis virus (TGEV) HN1203, porcine rotavirus (PRoV) HN03 and porcine parvovirus (PPV) HN-2011. The RNAs of these RNA viruses were reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (TaKaRa) before performed ASFV iiPCR. As shown by Table 2, positive signals were only generated from ASFV plasmid sample but not from other swine pathogens tested, which indicated the established ASFV iiPCR has no cross-reactivity with other swine viruses.

Fifty-six DNA samples were isolated from clinical samples including whole blood, sera, heart, lung, spleen, brain, liver, lymph node, tonsil, kidney and nasal swabs of 56 suspected infected pigs. Nucleic acids were extracted using the Ambion®MagMAXTM Total Nucleic Acid Isolation Kit (Applied Biosystems) by ASFV Regional Reference Laboratory in Guangzhou, China. Surveillance protocol was approved by the ethics committee of reference laboratory and clinical samples were inactivated before genomic DNA extraction and detection. Performance of the established ASFV iiPCR for detecting above clinical samples was evaluated by comparing test results of a real-time PCR assay. Nucleic acid extracts of clinical samples were tested in parallel. Among the 56 tested clinical samples, 30 samples were tested positive by ASFV iiPCR and real-time PCR (Table 3). The iiPCR S/N ratio of ASFV positive samples ranged from 3.96 to 9.85 and the Ct values of real-time PCR were from 22.75 to 34.65. The above results revealed ASFV iiPCR has comparable sensitivity with an OIE recommended real-time PCR on testing clinical samples.

To test if clinical blood samples could be directly used as the PCR template in ASFV iiPCR without nucleic acid extraction, 10 plasma

Table 2
Specificity analysis of ASFV iiPCR.

Virus[a]	Strain	ASF iiPCR		ASFV Real-time PCR (Ct)
		Results	S/N	
ASFV	Plasmid (2000 copies)	+	4.17	29.96
CSFV	C strain	–	0.97	N/A
PRRSV	CH-1R	–	0.99	N/A
PRV	Bartha-K61	–	0.98	N/A
PCV2	HH3	–	1.02	N/A
PEDV	HN1301	–	0.99	N/A
TGEV	HN1203	–	1.01	N/A
PRoV	HN03	–	0.96	N/A
PPV	HN-2011	–	1.01	N/A

[a] ASFV, African swine fever virus; CSFV, classical swine fever virus; PRRSV, porcine reproductive and respiratory syndrome virus; PRV, pseudorabies virus; PCV2, porcine circovirus 2; PEDV, porcine epidemic diarrhea virus; TGEV, transmissible gastroenteritis virus; PRoV, porcine rotavirus; PPV, porcine parvovirus.

Table 3
Comparison of ASFV iiPCR with real-time PCR in clinical samples.

		Real-time PCR		Total
		Positive	Negative	
iiPCR	Positive	30	0	30
	Negative	0	26	26
	Total	30	26	56

Table 1
Comparison of detection limits between ASFV iiPCR and real-time PCR.

Dilutions	Copy number/reaction	ii PCR result (S/N)					Real-time PCR result (Ct)				
10^{-6}	2000	+(4.75)	+(4.11)	+(4.32)	+(4.08)	+(4.29)	+(30.79)	+(29.93)	+(31.22)	+(30.07)	+(31.21)
10^{-7}	200	+(3.23)	+(3.18)	+(3.69)	+(4.11)	+(3.75)	+(34.12)	+(33.87)	+(34.76)	+(33.52)	+(34.69)
10^{-8}	20	+(2.02)	+(2.34)	+(1.89)	+(2.15)	+(2.11)	+(37.45)	+(37.23)	+(37.95)	+(37.89)	±(38.23)
10^{-9}	2	–(1.01)	–(1.00)	–(1.02)	–(0.99)	–(0.98)	–(N/A)	–(N/A)	–(N/A)	–(N/A)	–(N/A)

Notes: "+" indicates positive results, "–" indicates negative results, "±" indicates suspected result. The cutoff Ct value for the real-time PCR was set as 38 according to the manual instruction.

samples (seven ASFV negative and three ASFV positive samples) were prepared by a briefly spun in a mini centrifuge. Consistent with previous results, three ASFV positive plasma samples had S/N values of 9.23, 9.34 and 8.85, respectively. The seven ASFV negative plasma samples had S/N values from 0.97 to 1.04. Next, the above three ASFV positive plasma samples were 10-fold serially diluted with ASFV negative plasma as the PCR templates. In parallel, the nucleic acids were extracted from 100 μL of plasma samples and eluted with same volume of TE buffer. The extracted nucleic acids were then serially 10-fold diluted with TB buffer. As shown in Table 4, the detection limits for both unextracted or extracted samples were 10^{-4} dilutions, which indicated the unextracted plasma samples could be directly used as template in ASFV iiPCR without compromising its sensitivity.

The early and accurate diagnosis of ASFV was prerequisite for ASFV control to implement stringent measures and stamping-out policy before the onsite of clinical symptoms which appear as early as 4 days post-infection (Zhao et al., 2019). Currently, the OIE-recommended real--time PCR is widely used to diagnose ASFV in China. However, the sophisticated thermocycler, complicated procedures and laboratory-dependent operations limited their applications in point-of-care testing. Moreover, it could take several days to collect and transport samples to ASFV reference laboratory for real-time PCR test. The delayed testing may endanger nearby pig herds or farms exposed to ASFV infection. Therefore, a rapid, highly sensitive and specific, and field-deployable assay is necessary for ASFV control.

Relying on the fluorescent probe hydrolysis driven by natural liquid convection in a capillary tube, iiPCR was recently developed and applied to several swine viral pathogens including CSFV, Foot and mouth disease virus, and Senecavirus A (Lung et al., 2016; Ambagala et al., 2017; Zhang et al., 2019). Compare to the real-time PCR, iiPCR has several advantages. Firstly, ASFV iiPCR is simple and rapid to diagnose with the comparable sensitivity and specificity as real-time PCR does. Excluding the time needed to extract DNA, it only takes 25 minutes to read the results. Second, the compact iiPCR device is field-deployable for onsite disease surveillance and outbreak monitoring. It could be used in remote, resource-deprived areas to get rapid results at a relatively low cost. Third, the automated interpretation by iiPCR machine makes it easier to read the results for untrained users who work in veterinary clinics, pig farms, slaughtering houses, and diagnostic laboratories.

Tran et al. had established an iiPCR assay for rapid and on-site detection of ASFV (Tran et al., 2021). In their study, the established ASFV iiPCR has a good sensitivity as real-time PCR by testing two ASFV positive samples (one blood and one tissue homogenate) with 10-fold dilution. To valid the feasibility of ASFV iiPCR in the field, more clinical samples should be included in their study. Therefore, 56 clinical samples were used in our study and the results proved that ASFV iiPCR could be applied in the field with high sensitivity and accuracy as real-time PCR (Table 3).

Besides conventional and real-time PCR, the loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) were developed for onsite ASFV diagnosis (James et al., 2010; Miao et al., 2019). These molecular methods need nucleic acid extraction from clinical samples, which is inconvenient to perform in the field. To overcome this bottleneck, plasma samples were directly used as templates in the established ASFV iiPCR in this study. The results showed that ASFV iiPCR had same detection limits by the positive results of 10^{-4} dilution for both unextracted and extracted samples (Table 4). Since the plasma and sera are the commonly used in the field for ASFV diagnosis, ASFV iiPCR could work as a practical onsite tool for virus detection using only 5 μL volume of samples.

Table 4
Sensitivity comparison of ASFV iiPCR between unextracted and extracted plasma.

Dilution/ Sample ID	Unextracted positive plasma sample (S/N)			Extracted positive DNA sample (S/N)		
	1	2	3	1	2	3
10^{-1}	+(8.77)	+(8.61)	+(8.13)	+(9.01)	+(8.84)	+(8.75)
10^{-2}	+(6.33)	+(6.25)	+(5.69)	+(6.78)	+(6.31)	+(6.14)
10^{-3}	+(3.21)	+(3.42)	+(2.87)	+(3.97)	+(3.72)	+(3.03)
10^{-4}	+(1.53)	+(2.20)	+(1.64)	+(2.14)	+(1.98)	+(1.77)
10^{-5}	–(1.00)	–(0.99)	–(0.99)	–(1.01)	–(0.98)	–(1.02)

Footnotes

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