

RESEARCH NOTE

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# Validation of a direct multiplex real-time reverse transcription PCR assay for rapid detection of African swine fever virus using swine field samples in Vietnam

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## Abstract

**Objective** This study validates a direct multiplex real-time reverse transcription polymerase chain reaction (rRT-PCR) assay which was previously established for enabling rapid and simultaneous detection of African swine fever (ASF) virus (ASFV) and classical swine fever virus. The assay eliminates the need for viral nucleic acid purification using a buffer system for crude extraction and an impurity-tolerant enzyme. However, the assay had not yet been validated using field samples of ASFV-infected pigs. Therefore, to address this gap, we tested 101 samples collected from pigs in Vietnam during 2018 and 2021 for validation.

**Results** The rRT-PCR assay demonstrated a diagnostic sensitivity of 98.8% and a specificity of 100%. Remarkably, crude samples yielded results comparable to those of purified samples, indicating the feasibility of using crude samples without compromising accuracy in ASFV detection. Our findings emphasize the effectiveness of the rRT-PCR assay for the prompt and accurate diagnosis of both swine fever viruses, which is essential for effective disease prevention and control in swine populations.

**Keywords** African swine fever, Multiplex real-time RT-PCR, Crude samples, Validation

## Introduction

African swine fever (ASF) poses a significant threat to swine industries worldwide. This lethal viral disease, caused by the ASF virus (ASFV), affects domestic pigs (*Sus scrofa domestica*) and wild boars [1]. ASFV belongs to the genus *Asfivirus* within the family *Asfarviridae*, harboring a linear double-stranded DNA genome. The virus has been widely distributed in Europe, Eurasia, Asia–Pacific, and Caribbean countries [2]. In Asia, since the first confirmation of the ASF outbreak in China in August 2018 [3], the disease spread to Vietnam (February 2019; [4]) and other neighboring countries.

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Another febrile swine disease, classical swine fever (CSF), remains a significant concern for the global swine industry due to its substantial economic impact and potential for devastating outbreaks. CSF virus (CSFV), the causative virus of this disease, has a positive single-stranded RNA genome [5]. Because ASF and CSF exhibit similar clinical manifestations in affected pigs, a tentative diagnosis of suspicious cases based on clinical observation or postmortem examination must be confirmed by laboratory investigation. We previously developed a direct multiplex real-time reverse transcription polymerase chain reaction (rRT-PCR) assay that provides simplified but accurate detection of ASFV and CSFV in one reaction without the need for nucleic acid purification [6]. The effectiveness of this assay in detecting ASFV and CSFV was intensively validated using serum samples, whole blood samples, and tissue samples collected from pigs, boar–pig hybrid, and wild boars under experimental conditions, and it was demonstrated that at least three ASFV genotypes (I, II, and X) can be detected. Furthermore, for CSFV detection, we performed validation assays using field samples collected from infected and uninfected pigs and wild boars. Nevertheless, because of the absence of field cases of ASF in Japan, no validation assay using field samples of ASF-positive animals has yet been conducted. Therefore, in this study, our aim was to validate the rRT-PCR assay for detecting ASFV using naturally infected pig field samples collected in the recent ASF epidemic in Vietnam.

## Materials and methods

### Clinical samples

A total of 101 samples comprising whole blood samples, serum samples, and tissue homogenate samples were collected from pigs by veterinary officials on affected farms in the northern part of Vietnam during 2018–2021. The viral nucleic acid of the samples were purified using the QIAamp DNA Mini Kit (QIAGEN, Venlo, The Netherlands) and the real-time PCR assay for ASFV based on the WOAHP recommendation [7]. In total, 81 samples identified as positive for ASFV and 20 samples identified as negative for ASFV both by the real-time PCR assay were sent to the National Institute of Veterinary Research (NIVR), Vietnam, and used in this study.

### Preparation of crude samples for the multiplex rRT-PCR assay

Test samples used for the multiplex rRT-PCR assay were prepared as follows: whole blood, serum, and supernatants of tissue homogenates obtained from pigs were mixed with an equal volume (5  $\mu$ L) of Lysis Buffer S (code no. 9812, Takara Bio Inc., Shiga, Japan), incubated at room temperature ( $\sim$ 25  $^{\circ}$ C) for 5 min, and placed on ice until use. A portion of the clinical samples, 17 tissue

homogenate samples and 2 whole blood samples, was also subjected to crude sample preparation using Solution N (code no. 9815, Takara Bio Inc.), designated for the rapid preparation of crude samples not only in serum and tissue homogenates but also whole blood and hemolyzed samples. Solution N was first diluted with saline to a one-tenth concentration and then mixed with an equal volume (20  $\mu$ L) of tissue homogenate samples or whole blood samples. The mixture was heat-incubated at 98  $^{\circ}$ C for 3 min, brought down to temperatures  $<$ 40  $^{\circ}$ C, and then centrifuged at  $\geq$ 1200  $\times$ g for 2 min. The resulting supernatant was collected and placed on ice until use.

### Preparation of purified nucleic acids for the multiplex rRT-PCR and conventional PCR assays

A total of 19 samples (17 tissue homogenate samples and 2 whole blood samples) used for the crude sample preparation mentioned above was subjected to purification of viral nucleic acids using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Basel, Switzerland). For the purification, 100  $\mu$ L of the sample was used, and the purified nucleic acid fractions were eluted in 50  $\mu$ L of elution buffer according to the manufacturer's instructions and stored at  $-$ 20  $^{\circ}$ C until use.

### Multiplex rRT-PCR assay

The reaction mixture was prepared using CSFV/ASFV Direct RT-qPCR Mix & Primer/Probe with ROX Reference Dye (code no. RC212A, Takara Bio Inc.) according to the manufacturer's instructions. To the 23  $\mu$ L of rRT-PCR reaction mixture, we added 2  $\mu$ L of the crude sample or purified nucleic acid sample, each containing theoretically identical amounts of nucleic acids equivalent to 1  $\mu$ L and 4  $\mu$ L of the original sample, respectively. The multiplex rRT-PCR assays were performed using an Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA) as previously reported [6].

### Statistical analysis

Data obtained by the multiplex rRT-PCR assay testing crude samples and purified nucleic acids prepared from the 19 samples mentioned above were analyzed using a paired t-test with two-tailed analysis to determine the statistical significance of differences. The  $C_t$  values obtained by testing purified nucleic acids were adjusted by adding 2 for comparison based on theoretically identical amounts of the original samples. The reason for the addition of 2 to the  $C_t$  values was that purified nucleic acids contained four times as much original sample as crude samples, and the efficiency of amplification of this assay was 105.293% for ASFV [6]. Therefore, when purified nucleic acid and a crude sample were prepared from the same original sample, the  $C_t$  value of purified nucleic

acid was expected to be approximately 2 lower than that of a crude sample.

### Conventional PCR assay

For ASFV detection from the 19 samples subjected to the purification of viral nucleic acids using the conventional PCR assay, 2  $\mu\text{L}$  of purified viral nucleic acids was subjected to PCR using a KOD One PCR Master Mix (TOYOBO, Osaka, Japan) and a set of PPA-1 (forward) and PPA-2 (reverse) primers in a total volume of 25  $\mu\text{L}$  of reaction mixture [7]. PCR was performed using a SureCycler 8800 (Agilent Technologies, Santa Clara, CA, USA) under the following conditions: 98 °C for 10 s, 55 °C for 5 s, and 68 °C for 1 s, 35 cycles. The amplified products were separated by electrophoresis on a 1% agarose gel. The products were then stained with ethidium bromide and visualized under UV light transillumination.

## Results

### Diagnostic sensitivity and specificity of the rRT-PCR assay

Of the 81 samples (53 whole blood samples, 10 serum samples, and 18 tissue homogenate samples) that were identified as ASFV-positive in the real-time PCR assay described in the WOAHA manual [7], 80 tested positive in our rRT-PCR assay, with only one tissue sample tested negative. A total of 20 serum samples identified as ASFV-negative by the real-time PCR assay [7] tested negative by our rRT-PCR assay. Thus, the diagnostic sensitivity and specificity of our rRT-PCR assay were 98.8% and 100%, respectively (Table 1). The  $C_t$  values for all the samples are presented in Supplemental Table S1. The tissue sample with ID 1394, which tested ASFV-positive in the real-time PCR assay [7] but negative in our rRT-PCR assay, was subsequently subjected to the purification of viral nucleic acids. The purified nucleic acids of that sample had a  $C_t$  value of 39.3 according to our rRT-PCR assay.

### Comparison of the sensitivity of the rRT-PCR assay using crude samples and purified nucleic acids

Crude samples and purified nucleic acids prepared from the 19 samples (17 tissue homogenate samples and 2 whole blood samples) were subjected to the rRT-PCR assay to compare its sensitivity using templates. The  $C_t$  values obtained by testing three types of templates

prepared using Lysis Buffer S (Takara Bio Inc.), Solution N (Takara Bio Inc.), and the High Pure Viral Nucleic Acid Kit (Roche Diagnostics), which contained theoretically identical amounts of nucleic acids, were compared using paired  $t$ -tests. The  $p$  values for the two-tailed  $t$ -tests of  $C_t$  values between Lysis Buffer S-treated crude samples and purified nucleic acids, as well as between Solution N-treated crude samples and purified nucleic acids, were 0.160 and 0.066, respectively. These results revealed no significant difference in  $C_t$  values for ASFV between the same clinical samples prepared differently (Fig. 1). Original  $C_t$  values for all the templates are shown in Supplemental Table S2. To further clarify the presence or absence of ASFV gene in the 19 samples, purified nucleic acids were subjected to the conventional PCR for ASFV detection as described in the WOAHA manual [7], and all samples were identified as ASFV-positive, except one sample (ID 1394) (Supplemental Table S2).

## Discussion

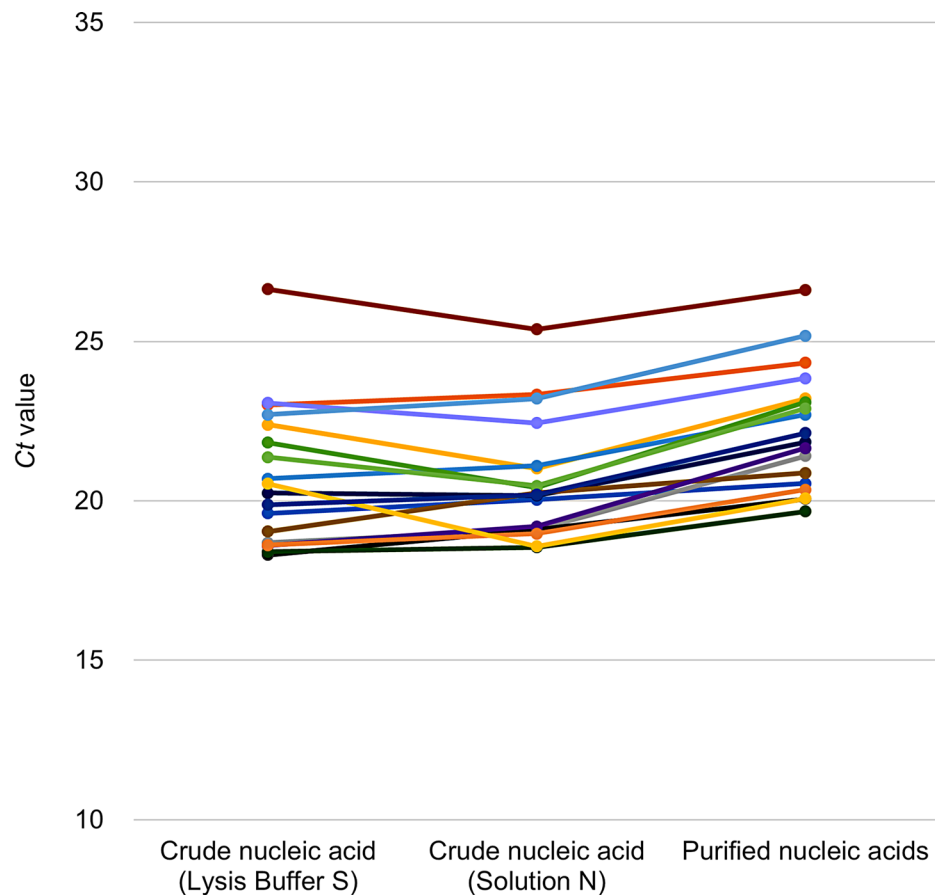
Our study demonstrates that the utilization of field-collected crude samples does not compromise sensitivities for ASFV detection. The results of 100 of 101 samples tested using our new rRT-PCR assay were consistent with those of real-time PCR assay for ASFV detection based on the WOAHA manual (Table 1) [7]. The one sample with a discordant result yielded no  $C_t$  value for ASFV when crude sample was used as a template, but a  $C_t$  value of 39.3 was observed when purified nucleic acids were used in the rRT-PCR assay. These data suggest that a minute amount of ASFV-derived DNA present in test samples results in differences in test results depending on whether the template is crude or purified, which is similar to our previous observations for CSFV detection in wild boar-derived samples [6]. Nevertheless, both diagnostic sensitivity (98.8%) and specificity (100%) of the assay using crude field samples provide simple, rapid, and reliable diagnosis to facilitate prompt and effective implementation of preventive measures against ASF.

An advantage of our assay is that the nucleic acid preparation does not involve purification or washing using columns or magnetic beads. We previously reported a simple procedure for preparing PCR templates from sera or tissue homogenates using Lysis Buffer S [6]. In

**Table 1** Diagnostic sensitivity and specificity of the multiplex rRT-PCR assay for ASFV detection

Multiplex rRT-PCR assay using crude samples as templates	Real-time PCR assay using purified nucleic acid as templates [7]						Total
	Positive			Negative			
	WB	TH	SE	WB	TH	SE	
Positive	53	17	10	0	0	0	80
Negative	0	1	0	0	0	20	21
Total	81			20			

WB, whole blood; TH, tissue homogenate; SE, serum Sensitivity: 98.8% (80/81). Specificity: 100% (20/20). All the samples tested negative for CSFV by the rRT-PCR assay



**Fig. 1** Comparison of the sensitivity of the rRT-PCR assay for ASFV detection using different nucleic acids as templates. Lysis Buffer S (Takara Bio Inc.) and Solution N (Takara Bio Inc.) were used for the preparation of crude samples, and the High Pure Viral Nucleic Acid Kit (Roche Diagnostics) was used for the preparation of purified nucleic acids. The crude samples and purified nucleic acids were prepared from whole blood samples and tissue homogenate samples obtained from pigs in Vietnam and were used to compare Ct values between templates prepared from the same samples using the three different procedures. The Ct values obtained by testing purified nucleic acids are adjusted by plus 2 for comparison based on 1  $\mu$ L of the original sample. Original Ct values for all the templates are shown in Supplemental Table S2

the present study, we demonstrated a novel method for preparing similar templates using Solution N instead. Although this method requires additional heat treatment at 98 °C for 3 min, followed by low-speed centrifugation ( $\geq 1200 \times g$ ) for 2 min, it can be applied to various field samples (blood, hemolytic serum, putrefied tissue) at different levels of quality. These methods eliminate the need for specialized equipment and reagents traditionally used in nucleic acid purification, thereby reducing the risk of cross-contamination during sample processing. These simplified sample preparation methods are superior to conventional methods because they require only a small amount (generally 2–20  $\mu$ L per assay) of sample as a test material, thus simplifying the procedure for sample collection. Currently, several real-time PCR assays for ASFV detection have been reported [8]. However, to our knowledge, no simpler procedure has been described for preparing templates for real-time PCR assay to detect ASFV. Although loop-mediated isothermal amplification

(LAMP) and recombinase polymerase amplification assays for ASFV detection are also developed as simplified genetic test methods [9–12], they do not allow the quantitative analysis of target materials.

In conclusion, our study emphasizes that this novel rRT-PCR assay, in combination with simplified sample preparation methods, is useful for diagnosing ASF for various purposes in both ASF-endemic and -free areas.

### Limitations

Our study validated the rRT-PCR assay using 101 samples collected from pigs in ASF-endemic areas in Vietnam. While this sample size provided valuable insights, further study using more samples from different geographic locations or environmental conditions would be necessary to ensure the assay's robustness and broad applicability.

### Abbreviations

ASF African swine fever  
ASFV African swine fever virus

CSF	Classical swine fever
CSFV	Classical swine fever virus
LAMP	Loop-mediated isothermal amplification
NIVR	National Institute of Veterinary Research
rRT-PCR	Real-time reverse transcription polymerase chain reaction
WOAH	World Organisation for Animal Health

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13104-024-06898-2>.

Supplementary Material 1

Supplementary Material 2

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## Author contributions

T.K. (Takehiro Kokuho), K.M., and T.N. conceived and designed the experiments. H.S., T.N., T.K. (Tomoya Kitamura), M.W., and K.M. performed experiments and analyzed the data. H.S. and T.N. contributed to the writing of paper drafts. H.V.D., H.T.T.T. and A.D.T. provided clinical samples and assisted in performing the experiments. All authors have read and approved the manuscript.

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## Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

### Ethics approval and consent to participate

The study was conducted in compliance with the institutional rules for the care and use of laboratory animals and a protocol approved by the Ministry of Agriculture and Rural Development (MARD), Vietnam (TCVN 8402:2010).

### Consent for publication

Not Applicable.

### Competing interests

The authors declare no competing interests.

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