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Development of a novel real-time PCR assay targeting p54 gene for rapid detection of African swine fever virus (ASFV) strains circulating in Vietnam

Thi Bich Ngoc Trinh[1] | Thang Truong[2] | Van Tam Nguyen[1] | Xuan Dang Vu[1] | Le Anh Dao[1] | Thi Lan Nguyen[1] | Aruna Ambagala[3] | Shawn Babiuk[3] | Jinsik Oh[4] | Daesub Song[5] | Van Phan Le[1]

[1] College of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam

[2] National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Manitoba, Canada

[3] National Centre for Foreign Animal Disease, Canadian Food Inspection Agency, Winnipeg, Manitoba, Canada

[4] Median Diagnostics, Chuncheon-si, South Korea

[5] College of Pharmacy, Korea University, Sejong, South Korea

Correspondence
Van Phan Le, College of Veterinary Medicine, Vietnam National University of Agriculture, Hanoi, Vietnam.
Email: letranphan@vnua.edu.vn

Thi Bich Ngoc Trinh and Thang Truong contributed equally to this article.

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Abstract

African swine fever (ASF) continues to cause outbreaks throughout regions of Africa, Europe and Asia. The disease can cause severe morbidity and mortality resulting in serious economic losses. Since there is no vaccine available to control ASF, early detection is critical to contain and control the disease. The aim of this study was to develop a novel real-time PCR assay based on highly conserved ASFV gene E183L (p54). The limit of detection of the assay, VNUA-p54 real-time PCR, was 2.63 copies/reaction and 2 Log_{10} HAD_{50}/ml. The VNUA-p54 real-time PCR was able to detect fifteen different ASFV reference strains representing p72 genotypes I, II and V. The assay was specific and did not amplify other swine viruses including CSFV, FMDV, PRRSV and PEDV. The diagnostic sensitivity of the real-time PCR assay was evaluated using 200 field clinical specimens collected from swine farms located in different provinces in Vietnam. The VNUA-p54 real-time PCR assay is an additional tool for ASF diagnostics and can be used in combination with other p72 based ASFV real-time PCR assays as a rapid confirmatory assay.

KEYWORDS
African swine fever, real-time PCR, VNUA-p54

1 | INTRODUCTION

African swine fever (ASF) was first described in 1921 in Kenya (Montgomery, 1921). It is a highly lethal swine disease caused by a large double-stranded DNA virus belonging to the *Asfarviridae* family (Dixon et al., 2005). African swine fever virus (ASFV) infects exclusively suids including domestic swine, wild boars and warthogs. The disease can cause high mortality rates up to 100% resulting in large economic losses to the swine industry in affected countries due to loss of production and trade restrictions (Costard et al., 2013). ASF transmission can occur between a healthy and an infected pig through direct contact (Costard et al., 2013) or by a bite of an ASFV infected Ornithodoros soft tick (Plowright, 1977) which is responsible for maintenance of the sylvatic cycle in Africa.

Since its first discovery in Kenya, ASF has spread outside Africa twice. The first ASF outbreak in Europe was reported in Portugal in

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1957 which later spread to Caribbean and South America between the 1970s and 1980s. This outbreak was completely eradicated from Europe and Americas at a great cost with the exception of Sardinia (Sánchez-Vizcaíno et al., 2015). In 2007, ASF entered Europe for the second time through Georgia (Costard et al., 2013; Rowlands et al., 2008) from where it spread to the Caucasus region and Russia (Gogin et al., 2013; Khomenko et al., 2013). Then it continued its spread into Eastern Europe and entered China in 2018 where the virus became endemic (Zhao et al., 2019). Subsequently it spread to Mongolia (Heilmann et al., 2020), Vietnam (Le et al., 2019), Cambodia, Republic of Korea (Kim et al., 2020), Laos, Philippines, Myanmar and Timor-Lester, Papua New Guinea and most recently to India (OIE). Since there is no vaccine for ASF, control of this disease relies on rapid detection and elimination of the infected animals (Oura et al., 2013). Real-time PCR is the preferred first-line diagnostic for ASF. It is highly sensitive and specific, rapid and highly scalable (Fernández-Pinero et al., 2013; King et al., 2003). Several real-time PCR assays have been developed and validated for ASF detection and most of them target ASF p72 gene (Fernández-Pinero et al., 2013; Tignon et al., 2011; Wang, Jia et al., 2020; Wang, Xu et al., 2020; Zsak et al., 2005). Here we describe development of a novel real-time PCR assay (VNUA-p54) targeting a highly conserved region of the ASFV E183L gene that encodes an essential structural protein p54 (Brookes et al., 1998; Rodriguez et al., 1996; Mai et al., 2021).

2 | MATERIALS AND METHODS

2.1 | Clinical samples

Two hundred samples including whole blood ($n = 127$), serum ($n = 17$), spleen ($n = 40$) and kidney ($n = 16$) were collected from pigs displaying clinical signs of ASF from farms located in the different provinces in Vietnam during 2019–2020 outbreaks (Table S4). A sample volume of 200 μl of whole blood, serum or 10% tissue homogenate were used for nucleic acid extraction using DNeasy Blood & Tissue Kit (QIAGEN, Germany) according to the manufacturer's instructions.

2.2 | Determination of analytical sensitivity and specificity

The limit of detection is defined as the highest dilution factor where 95% of the positive samples can be detected. To test the limit of detection of the VNUA-p54 real-time PCR assay, 10-fold dilutions of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 HAD_{50}/ml of VNUA/HY/ASF-1/Vietnam/2019 ASFV strain (Le et al., 2019) were generated and the real-time PCR was performed on a LightCycler® 96 Instrument (Roche, Switzerland). Additionally, the detection limit of the real-time PCR was also conducted using the known amount of DNA templates of 0.1, 1, 2, 5 and 10 copies for each reaction. Each DNA concentration was run 12 times by VNUA-p54 real-time PCR assay as in the previous recommendation (Uhlig et al., 2015). To determine the diagnostic specificity of the real-time PCR assays, fifteen different ASFV reference strains representing p72 genotypes I, II and V (Table S1) and other swine viruses including classical swine fever (Strain Vietnam/ND20/2014, GenBank accession no. MH979232), porcine reproductive and respiratory syndrome (Strain HUA/HP1963; GenBank accession no. KF699844), porcine epidemic diarrhea (Strain HUA-14PED96, GenBank accession no. KT941120) and foot and mouth disease (Strain O/VN/PT555/2018, GenBank accession no. MN379784) were used in this study.

2.3 | Development and optimisation of the VNUA-54 real-time PCR assay

In order to design an ASFV p54-based real-time PCR, the E183L (p54) gene sequences representing all 24 ASF p72 genotypes of ASFV were aligned by the Geneious software, and a highly conserved 100 bp region between nucleotide positions 287 and 386 was selected, and primers (forward: 5′-CAAGTGTAGGCAAGCCAGTC-3′ and reverse: 5′-GCCATGACTAGTCTGTCCGT-3′) and a TaqMan® probe (5′-FAM ACGGGCAGACCGGCAACAAA-3′TAM) were designed. The primer and probe concentrations and cycling conditions were extensively optimised and the optimised reaction mixture contained 5 μl of 4X TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems™); 10 μM of forward and reverse primers, and 10 μM of probe; 5 μl of extracted DNA and DNase & RNase-free water in a 20 μl reaction. The optimal thermal profile for the VNUA-p54 real-time PCR assay is 50°C for 5 min; 95°C for 20 s; followed by 40 cycles of amplification (3 s at 95°C and 30 s at 58°C). All real-time PCR reactions were performed on a LightCycle™ 96 (Rocher, Switzerland) and data was analysed by the manufacturer's software. For comparison, an ASF real-time PCR developed by Tignon et al. (2011) was used as described.

3 | RESULTS

3.1 | Analytical sensitivity of the VNUA-p54 real-time PCR assay

The limit of detection of the VNUA-p54 assay was analysed and compared with the validated p72-based Tignon real-time PCR assay (Tignon et al., 2011) using ASFV DNA ranging from 0.1 to 10 genome copies. Both assays were able to detect 10 and 5 ASF genome copies, while the VNUA-p54 assay detected 10/12 times and the Tignon assay 9/12 times 1 and 2 genome copies. Both assays failed to detect 0.1 ASFV genome copies (Tables S2 and S3). Based on these findings LOD was calculated for both assays using https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory. The LOD was 2.63 copies for the VNUA-p54 real-time PCR assay and 3.29 copies for the Tignon real-time PCR assay (Table 1).

The sensitivity of the VNUA-p54 assay compared with Tignon real-time PCR assay was further evaluated using a 10-fold dilution series of the ASFV strain VNUA/HY/ASF-1/Vietnam/2019. The results showed

TABLE 1 LOD_{95} value of real-time PCR in detecting ASFV p54 gene with VNUA-p54 primers and p72 gene with Tignon primers

	VNUA-p54		Tignon	
Copies/reaction	Positive samples	LOD_{95}	Positive samples	LOD_{95}
0.1	0/12	2.63	1/12	3.29
1	10/12		9/12	
2	10/12		9/12	
5	12/12		12/12	
10	12/12		12/12	

that both assays were able to detect ASFV at viral titres of 10^7, 10^6, 10^5, 10^4 and 10^3 HAD_{50}/ml. The Ct values obtained for VNUA-p54 assay were 20.78, 24.23, 27.69, 30.92 and 34.39 and Tignon assay were 22.03, 25.29, 28.58, 32.2 and 35.16, respectively. In addition, at viral titre 10^2 HAD_{50}/ml, VNUA-p54 assay could result in Ct value of 37.66 while Tignon assay was negative. Both assays failed to detect ASFV at viral titre of 10 HAD_{50}/ml. Basing on the Ct values generated from each known virus titres, a linear relationship was observed between VNUA-p54 and Tignon real-time PCR assays (Figure 1). Pearson's correlation test was used to determine the correlation of HAD_{50} values between VNUA-p54 and Tignon assay and the result showed that the Pearson's correlation coefficient was equal to 0.89 ($p < 0.05$) (Figure 2).

3.2 | Analytical specificity of the VNUA-p54 real-time PCR assay

To determine the analytical specificity of the VNUA-p54 real-time PCR assay, a total of 15 different ASFV reference strains of p72 genotypes I, II and V were used and the result showed that the VNUA-p54 real-time PCR assay was able to detect all of them (Table S1). The specificity of the VNUA-p54 real-time PCR was also tested with other swine virus strains of CSFV, FMDV, PRRSV and PEDV and the assay did not have non-specific reaction with any of those pathogens (Data not shown).

3.3 | Field sample evaluation of the VNUA-p54 real-time PCR assay

A total of 200 field clinical samples including whole blood ($n = 127$), serum ($n = 17$), spleen ($n = 40$) and kidney ($n = 16$) collected from pigs displaying ASF- clinical signs were tested by both VNUA-p54 and Tignon real-time PCR assays. The results showed that both assays were able to detect ASFV in all sample types. The mean Log_{10} HAD_{50} value of VNUA-p54 assay was 7.34 and Tignon assay was 7.00. Diagnosis results according to the type of samples showed that the mean Ct values obtained from spleen, kidney, serum and whole blood samples were 7.54, 7.31, 5.94 and 7.42 for VNUA-p54 assay and 7.18, 6.98, 5.87 and 7.06 for Tignon assay, respectively (Table 2).

4 | DISCUSSION

It has been almost a hundred years since ASF was first described in Kenya and today ASF is a greater threat to pig populations throughout the world. Despite numerous efforts by research groups across the globe, there is no vaccine for ASF and the disease is difficult to eradicate once established and has increased the geographic prevalence. Control of this disease relies on detection and containment and therefore rapid and sensitive diagnostics for ASF are really critical and important. Currently, there are numerous molecular and serological methods available to identify ASFV-infected animals (Abad et al., 1998; Fernández-Pinero et al., 2013; King et al., 2003; Tignon et al., 2011). Serological assays are used to determine if an animal has been exposed to ASFV. Molecular tests can detect the presence of ASFV in pigs even before to the clinical signs appear. A number of molecular assays including laboratory based, portable conventional and real-time loop-mediated isothermal amplification (LAMP) assays have been developed and validated for ASFV genome detection. Real-time PCR is the preferred assay used in many diagnostic laboratories since it is quantitative and faster compared to conventional PCR (Daigle et al., 2020; King et al., 2003; Tignon et al., 2011; Wang, Jia et al., 2020; Wang, Xu et al., 2020). Most of these assays are based on B646L (p72) gene.

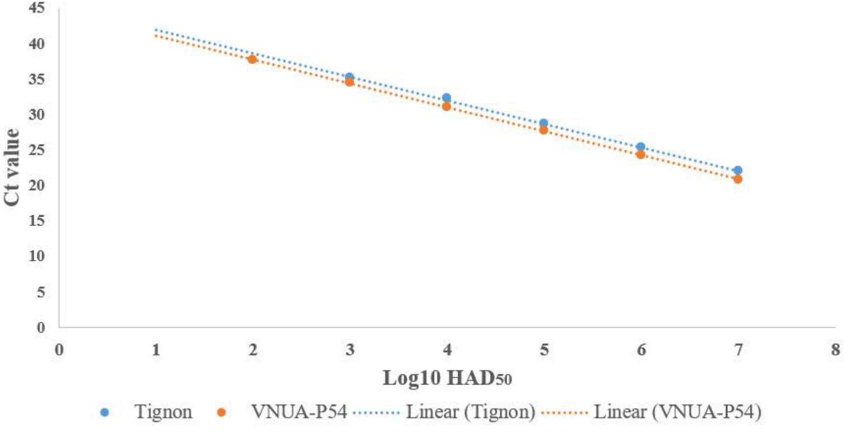

FIGURE 1 Comparison of the detection limit between the VNUA-p54 and Tignon assays using VNUA/HY/ASF-1/Vietnam/2019 ASFV strain

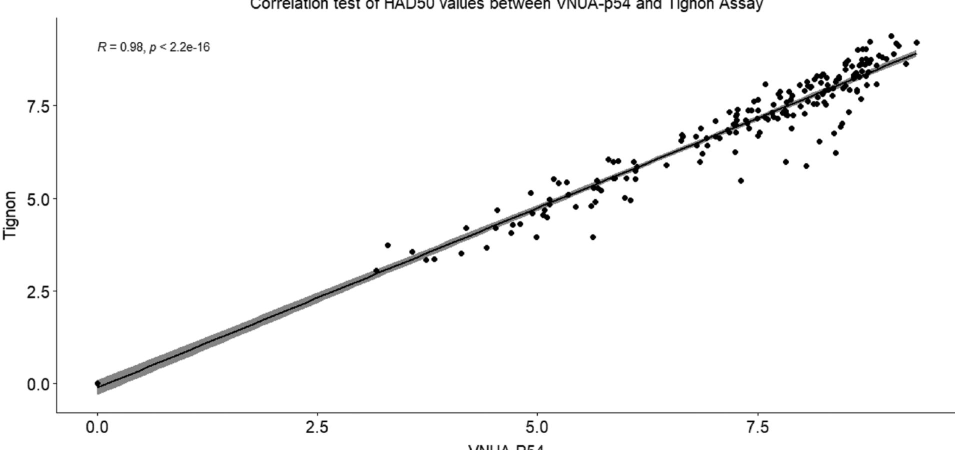

FIGURE 2 Correlation test of HAD_{50} values between VNUA-p54 and Tignon assay. Figure was created by R version 4.0.5. Pearson's correlation coefficient and *p*-value are showed in the upper-left corner

TABLE 2 Diagnostic results of real-time PCR for detection of ASFV in field samples collected from different provinces in Vietnam

		VNUA-p54 (Log_{10} HAD_{50} values)			Tignon (Log_{10} HAD_{50} values)		
Type of sample	Total samples	Positive samples	Range	Mean	Positive samples	Range	Mean
Spleen	40	37	5.67–9.18	7.54	37	5.20–8.65	7.18
Kidney	16	16	5.07–9.31	7.31	16	4.49–9.22	6.98
Serum	17	12	3.30–7.76	5.94	12	3.55–7.89	5.87
Whole blood	127	122	3.17–9.10	7.42	122	3.04–9.38	7.06
Total	200	187	3.17–9.31	7.34	187	3.04–9.38	7.00

In this study, we have developed a new assay that targets ASF p54 gene. The results obtained in this study also showed that the VNUA-p54 assay is highly specific and sensitive and performs comparable to the widely used Tignon assay. The VNUA-p54 assay can be used to detect ASFV in different sample types including blood, serum, spleen and kidney on its own or as an ancillary tool. The VNUA-p54 real-time PCR assay developed in this study will be an additional tool in our effort for rapid detection of ASFV in order to control the ongoing global epidemic.

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ETHICAL APPROVAL

Ethical Statement is not applicable because sample collection from animals has been gathered.

AUTHOR CONTRIBUTIONS

TBNT, TT and VPL conceived the idea and designed a novel real-time PCR assay. TBNT, VTN and XDV performed the experiments. VTN, AA, SB and TLN helped in revising of the manuscript. JO, DS and LAD participated in analysing the results. TBNT and VPL wrote the manuscript. All authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.605

ORCID

Thang Truong https://orcid.org/0000-0002-0707-7452
Xuan Dang Vu https://orcid.org/0000-0003-3330-1347
Aruna Ambagala https://orcid.org/0000-0003-1783-2105
Van Phan Le https://orcid.org/0000-0003-0583-4111

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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