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

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August 28, 2020

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-54 real-time PCR, was 2.63 copies/reaction and 2 Log10 HAD50/ml. The VNUA-54 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 CSF, FMD, PRRS, and PED. The diagnostic sensitivity of the real-time PCR assay was evaluated using 187 field clinical specimens collected from swine farms located in different provinces in Vietnam. The VNUA-54 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, Vietnam*

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) that 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 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 become endemic (Zhao et al., 2019). Subsequently it spread to Mongolia (Heilmann et al., 2020), Vietnam (Le et al., 2019), Cambodia, Republic of Korea (H. J. 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 (Fernandez-Pinero et al., 2013; Tignon et al., 2011; A. Wang et al., 2020; Y. Wang 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).

1. **MTERIALS AND METHODS**
2. **Clinical samples**

One hundred and eighty-seven samples including whole blood (n=122), serum (n=12), spleen (n=37), 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 (Supplementary 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.

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-54 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-54 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 (Supplementary 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 optimization 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 optimized and the optimized reaction mixture contained 5 µL of 4X TaqMan Fast Virus 1- Step Master Mix (Applied Biosystems); 10µM 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-54 real-time PCR assay is 50°C for 5 minutes; 95°C for 20 seconds; followed by 40 cycles of amplification (3 seconds at 95°C and 30 seconds at 58°C). All real-time PCR reactions were performed on a LightCycle^{TM} 96 (Rocher, Switzerland) and data was analyzed by the manufacturer's software. For comparison, an ASF real-time PCR developed by Tignon et al., was used as described (Tignon et al., 2011).

3. RESULTS

3.1. Analytical sensitivity of the VNUA-P54 real-time PCR assay

The limit of detection of the VNUA-P54 assay was analyzed 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 (Supplementary Table S2 and 3). 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 ten-fold dilution series of the ASFV strain VNUA/HY/ASF-1/Vietnam/2019. The results showed that both assays were able to detect ASFV at viral titers 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 titer 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 titer of 10 HAD_{50}/ml. Basing on the Ct values generated from each known virus titers, a linear relationship was observed between VNUA-P54 and Tignon real-time PCR assays (Figure 1).

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. The specificity of the VNUA-P54 real-time PCR was also tested with other swine virus strains of CSF, FMD, PRRS, and PED and the assay did not detect any of those pathogens (Data not shown).

3.3. Field sample evaluation of the VNUA-P54 real-time PCR assay

A total of 187 field clinical samples including whole blood (n=122), serum (n=12), spleen (n=37) 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 Ct value of VNUA-P54 assay was 19.70 and Tignon assay was 20.83. Diagnosis results according to the type of samples showed that the mean Ct values obtained from spleen, kidney, serum, and whole blood samples were 19.01, 19.78, 24.42, and 19.44 for VNUA-P54 assay and 20.25, 20.91, 24.65, and 20.63 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.; King et al., 2003; Tignon et al., 2011; A. Wang et al., 2020; Y. Wang et al., 2020). Most of these assays are based on B646L (p72) gene. 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.

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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.

ETHICAL APPROVAL

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

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.

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Table legends

Table 1. LOD$_{95}$ value of real-time PCR in detecting ASFV p54 gene with VNUA-P54 primers and P72 gene with Tignon primers.

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

Table S1 . ASFV reference strains representing for p72 genotype I, II, and V used in the study.

Table S2. Ct values of real-time PCR in detecting p54 gene of ASFV with VNUA P54 primers and probe set.

Table S3. Ct values of real-time PCR in detecting p72 gene of ASFV with Tignon primers and probe set.

Table S4. Detail diagnostic results of real-time PCR for detection of ASFV in field samples collected from different provinces in Vietnam.

Figure legends

Fig. 1. Comparison of the detection limit between the VNUA-P54 and Tignon assays using VNUA/HY/ASF-1 /Vietnam/2019 ASFV strain.

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