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Rapid Extraction and Detection of African Swine Fever Virus DNA Based on Isothermal Recombinase Polymerase Amplification Assay

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List of abbreviations

%	percentage
°C	degrees Celsius
ASF	African swine fever
ASFV	African swine fever virus
ASSURED	affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, deliverable to end users
CISA-INIA	Animal Health Research Centre - National Institute for Agricultural and Food Research and Technology
CSF	Classical swine fever
dsDNA	double-stranded deoxyribonucleic acid
e.g.	exempli gratia
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
FAT	fluorescent antibody test
FLI	Friedrich-Loeffler-Institut
HA	haemadsorption test
HAD50/ml	50% haemadsorbing doses per ml
i.e.	id est
IBT	immunoblotting test
IFAT	indirect fluorescent antibody test
IL-1	interleukin-1
IPT	indirect immunoperoxidase test
Kbp	kilo base pairs
LFD	lateral flow device
nm	nanometers
OIE	World Organisation for Animal Health
p.i.	post infection
PCR	Polymerase Chain Reaction

PONT	point-of-need test
PRRS	Porcine reproductive and respiratory syndrome
RPA	Recombinase Polymerase Amplification
Se	sensitivity
Sp	specifity
TAD	transboundary animal disease
TNFα	tumor necrosis factor alpha
µl	microliter

1. Introduction

African swine fever (ASF) is a devastating and highly infectious viral disease affecting domestic and wild pigs (*Sus scrofa*) which has, as of today, impacted swine production systems throughout the world. Due to its massive implications with national economies, veterinary services as well as animal welfare policies, this transboundary animal disease (TAD) is of great significance and because of that it is listed as notifiable to the World Organisation for Animal Health (OIE).

Since 2020, ASF has been circulating in Germany, which means that the country has lost its "disease-free status". The best strategy to contain an outbreak is to rapidly detect infected pigs in farms or the carcasses of infected wild boars. Gold standard for ASF genome detection is Polymerase Chain Reaction (PCR) since it is highly sensitive and specific. Nonetheless, it is time-consuming and requires an equipped laboratory and trained personnel. An equally sensitive, but faster, simpler, and more user-friendly test approach offers significant advantages. Point-of-need tests (PONT) are a viable solution, bringing these characteristics for diagnostic assays to the field. Time between sample taking and agent identification can be significantly reduced while maintaining high performance. To assure this, validation of new diagnostic assays for infectious diseases are based on guidelines by the OIE. Different steps are needed, including preliminary considerations for assay development, analytical characteristics, diagnostic characteristics, reproducibility and implementation (HEALTH 2018).

The aim of this study was to further close the last-mentioned gap regarding immediate and effective on-site ASFV detection methods. For this purpose, a new rapid diagnostic assay based on recombinase polymerase amplification (RPA) was developed to detect the circulating ASFV. This single-tube isothermal method is executed at a constant temperature (37-42 °C) and only takes up to 15 minutes to amplify the target nucleic acid sequence detectable in real-time using fluorescence (PCR takes between 2-6 hours at least). To accelerate the process of sample to result readout, the key aspect of DNA extraction was addressed, and the ASFV-RPA assay was tested with two different templates: firstly, DNA extracted with a silica spin-column based kit and secondly, samples directly treated with a rapid heat and lysis protocol. The accuracy of the isothermal ASFV-RPA assays was compared to an established real-time PCR. Additionally, the field validation of the RPA-ASFV-assay was conducted in a pilot field study in Uganda with local samples of a suspected ASF outbreak and performed in a mobile suitcase lab.

2. Literature overview

2.1 African Swine Fever

2.1.1 Aetiology

2.1.1.1 Classification and taxonomy

The causative agent of African Swine Fever is African Swine Fever Virus (ASFV). Formerly, it was included into the family *Iridoviridae*. Today, however, it is designated as the sole member of *Asfarviridae* family, as well as the single species of its genus *Asfivirus*. “Asfar”-viridae is an acronym derived from *African swine fever and related viruses*. It is the only known DNA Arbovirus (arthropod-borne virus) (DIXON et al. 2013). Thus, it is a unique agent, only seeming to be related closer to unclassified giant viruses, including Kaumoebavirus, Faustoviruses and Pacmanvirus, sharing about 30 genes with them (ANDREANI et al. 2017).

2.1.1.2. Viral structure and genome

The virion of ASFV has a complex structure, consisting of genomic dsDNA, nucleoprotein core shell, inner lipid membrane, icosahedral protein capsid (= intracellular virion) and an outer lipid-containing envelope (= extracellular virion) (figure 1). The diameter of an extracellular virus is 175-215 nm (SALAS and ANDRÉS 2013).

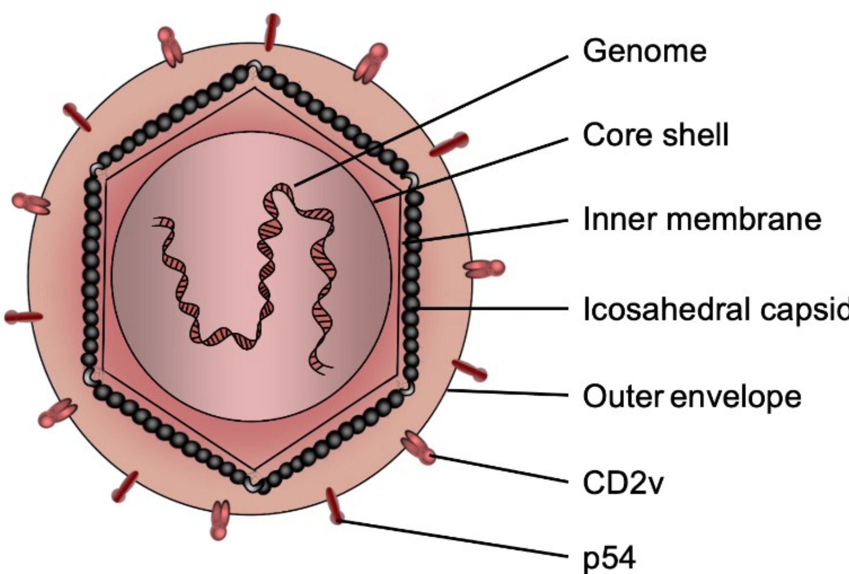

Figure 1. Illustration of the extracellular ASF virion, displaying its nucleoid and protein core shell, inner membrane, icosahedral capsid, and outer lipid envelope including its two known glycoproteins p54 and CD2v. Figure prepared according to WANG et al. (2021a).

The genome of ASFV consists of a covalently close, linear, double-stranded DNA. It encodes between 151-167 open reading frames and, depending on the isolate, the DNA varies between 170 and 190 Kbp. The viral genome encodes at least 68 different structural proteins and non-structural proteins intended for replication, immune modulation and repair. Nonetheless, the function of around half of the genes is not fully understood (ALEJO et al. 2018).

2.1.1.3 Genetic typing and antigenic variability

The main component of the viral capsid is the p72 protein encoded by the B646L gene. This gene is commonly used for genotyping. Hitherto, 24 different genotypes are described in Africa (BOSHOFF et al. 2007). However, only genotypes I and II are circulating globally (WADE et al. 2019). Thus, p72 typing is used to identify closely related strains and its possible origins (BASTOS et al. 2003). Sequencing the central variable region within the B602-L gene as well as the E183-L-gene encoding the p54 protein can further help to map closely related strains (NIX et al. 2006, GALLARDO et al. 2009, GALLARDO et al. 2011).

Nonetheless, emerging new strain variants can be easily identified using whole-genome sequencing technology (ZANI et al. 2018).

Importantly, genotypes do not determine the strains pathogenicity nor virulence. Better suited for this purpose is the classification of ASFV isolates into serogroups based on the CD2v protein encoded by the EP402R gene. This viral haemagglutinin protein is responsible for the haemadsortion property of the viral particle (MALOGOLOVKIN et al. 2015). Hence, this gene is assumed to be important for pathogenesis as well as replication in the vector (ROWLANDS et al. 2009). The antibody-mediated inhibition of haemadsorption leads to a virus infection neutralization *in vitro* and partially protects against ASFV challenge *in vivo* (RUIZ-GONZALVO et al. 1996, BURMAKINA et al. 2016). In addition, it is likely that more serogroups exist based on the haemadsorption properties of the C-type lectin-like protein (BURMAKINA et al. 2016).

Altogether, ASFV is considered as very stable and presents a low overall mutation rate (DIXON et al. 2020). Nonetheless, the virus shows genetic and antigenic variability. This is not only due to the serotyping based on the specific haemadsorption properties but also because of the five different multigene families where gene deletion and duplication lead to a different genome length (DE LA VEGA et al. 1990).

2.1.2 Epidemiology

2.1.2.1 Disease distribution

The disease ASF is endemic in East and Southern Africa in a sylvatic cycle between common warthogs (*Phacochoerus africanus*) and soft ticks (*Ornithodoros moubata)*, where it allegedly originated (MULUMBA-MFUMU et al. 2019). The disease was first reported in domestic pigs in Kenya 1921 (MONTGOMERY 1921). Due to the importation of European domestic pigs into Africa shortly before the first outbreak, the balance of the ancient natural cycle between hosts and the causative agent was disrupted (SCOTT 1965, PINI and HURTER 1975). By the end of the 1950s, ASF was found in most countries in Eastern, Southern and Central Africa (WILKINSON and PENSAERT 1989, PLOWRIGHT et al. 1994). The virus then spread to Western Africa (NATIONS 2000). The first international case in Europe was reported in 1957 in Portugal, probably due to importation of infected pork products fed as swill (MANSO RIBEIRO et al. 1963). Over the course of 50 years (1957 – 2007), the virus appeared in central American countries, Brazil (ANDRADE 1981, WILKINSON and PENSAERT 1989) and in several European countries (CWYNAR et al. 2019). All non-African regions, except Sardinia, managed to eradicate ASF by depopulation until the turn of the century (ARIAS and SÁNCHEZ-VIZCAÍNO 2002). In sub-Saharan African countries ASF is still endemic (GAVIER-WIDÉN et al. 2020).

With growing globalisation, increasing demand of pork and the still very diverse biosecurity measures and outbreak response systems, the disease reappeared 2007 in the Republic of Georgia (ROWLANDS et al. 2008), steadily spreading to other Transcaucasian countries, Baltic States and Russia (GOGIN et al. 2013). Upon the arrival of the virus to China in 2018 (WANG et al. 2018), the disease had made the jump to the Asian continent, where it spread rapidly, even reaching remote regions such as Papua New Guinea (MIGHELL and WARD 2021). So far, only the continent of North America did not report an ASF case (figure 2).

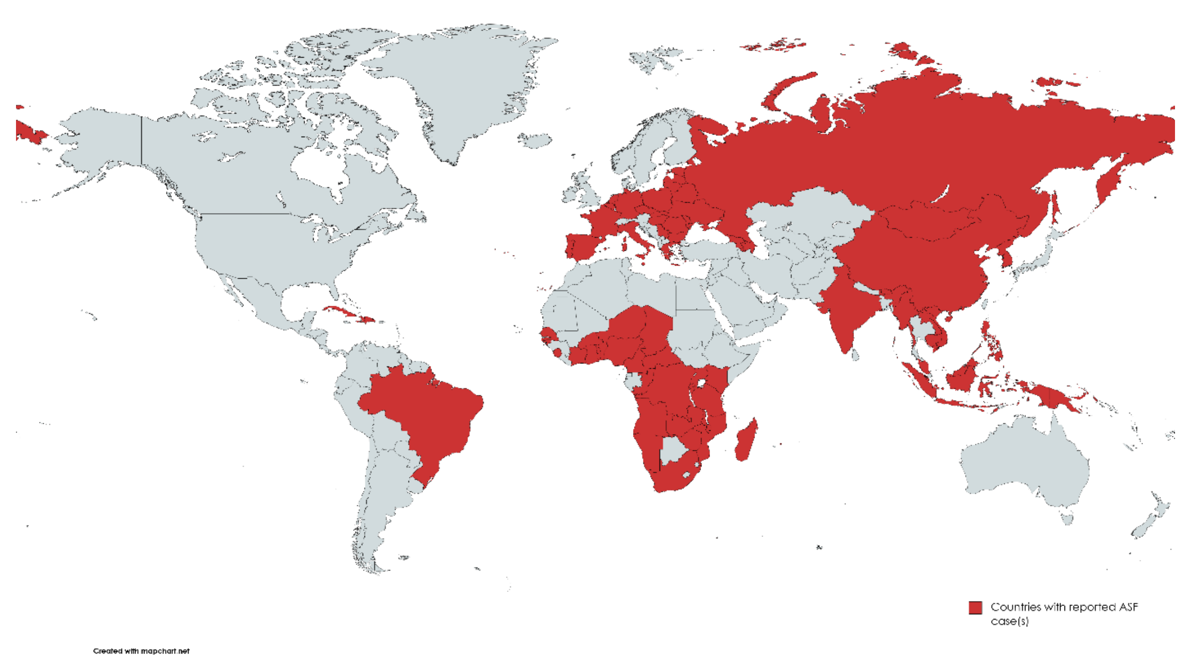

Figure 2. Countries where ASF outbreaks in wild boar or domestic pigs have been confirmed since the agent's identification. Information collected from OIE-WAHIS (ANON. 2021b) and CISA-INIA (ANON. 2021a). Accessed on: 01/08/2021.

2.1.2.2 Host range and epidemiological cycles

ASFV has a narrow host range. It includes vertebrae hosts from the *suidae* family as well as invertebrate hosts from the soft tick genus *Ornithodoros* as arthropod vectors (JORI and BASTOS 2009). No zoonotic potential of the agent has been reported so far. This may be due to the lack of related viruses in human hosts that could be used as recombination partners and the low recombination and mutation rate as a result of an accurate DNA proof-reading polymerase (DIXON et al. 2020).

The transmission cycle of ASF depends on various factors, including geographical distribution of hosts, climate, pig production system and humane interaction. Therefore, transmission pattern is different in Asia, Europe and Africa as illustrated in figure 3.

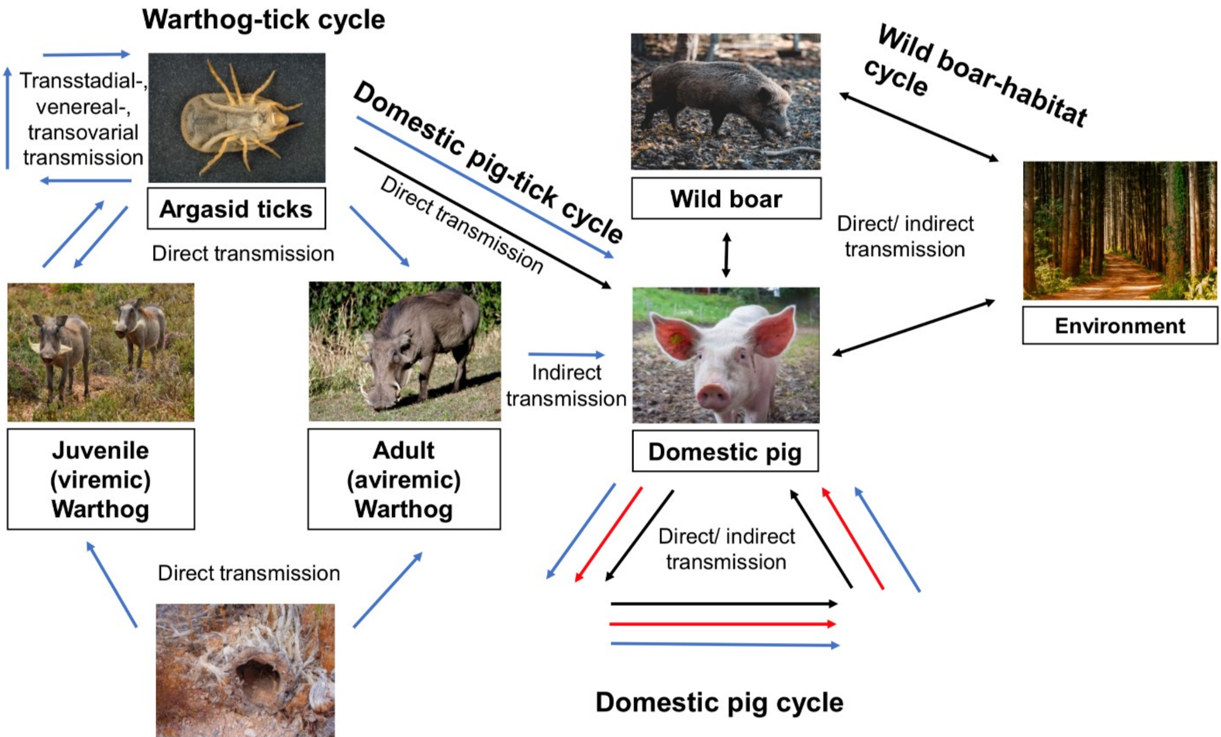

Figure 3. Different ASF transmission cycles and correlation between its agents. Blue arrows represent transmission routes in Africa, black arrows in Europe and red arrows in Asia.

2.1.2.2.1 Warthog-tick cycle

This sylvatic cycle is common in Eastern and Southern Africa because of endemicity of genotypes I–XXII and XXIV (BASTOS et al. 2003, BOSHOFF et al. 2007, ACHENBACH et al. 2017). There is no clear evidence describing the presence of this cycle in Western and Central Africa. The cycle is maintained between the virus's natural hosts, i.e. soft ticks from the *Ornithodoros moubata* complex transmitting to warthogs. Other swine species including bushpigs *(Potamochoerus spp.*) and giant forest hogs (*Hylochoerus meinertzhageni*) are known to be susceptible for ASFV (MONTGOMERY 1921), but no exact estimate of prevalence within these hosts are recorded (JORI et al. 2007). Moreover, bushpigs are nocturnal, secretive animals, less populated, show lower infection rates (WILKINSON et al. 1988) and, in case of *Hylochoerus* species, live in densely forested areas. Thus, it appears highly unlikely that these species are implicated in the transmission of ASFV.

Warthogs remain asymptomatic carriers and cannot transmit the disease to other vertebrae hosts. Therefore, this cycle relies on the ticks (THOMSON et al. 1980, PLOWRIGHT et al. 1981). Tick bites infect warthogs within the first 6-8 weeks of life, while they are still in the burrow (the natural habitat of Argasid ticks). Consequently, a viraemia is developed in young warthogs after

2-3 weeks which can lead to the infection of other ticks. Viral levels of up to 10^3 50 % haemadsorbing doses per ml (HAD_{50}/ml) are found in the bloodstream after one week of infection and decrease over time. After a short period warthogs recover and show no clinical signs (THOMSON et al. 1980). ASFV can be found in lymphatic tissues of warthogs of any age, with high viral levels of over 10^6 HAD_{50}/g (THOMSON et al. 1980). Warthogs they remain infected throughout their lifetime (WILKINSON et al. 1988). Another key aspect favourable to the long-term persistence of the disease is the long infectivity of ticks in various seasons due to diverse transmission routes: transstadial, venereal and transovarial transmission within tick populations allow the virus to be independent of viraemic hosts (GAUDREAULT et al. 2020).

2.1.2.2.2 Domestic pig-tick cycle

Occasional spill-over to domestic pigs have been described in sub-Saharan Africa (COSTARD et al. 2013), especially when they share common grounds with warthogs or ticks establish burrows on farms. Another viable option is bringing back infected warthog carcasses to a populated area after hunting. Thus, infected pigs and/or ticks reach the premises of pigs pens. In Eastern and Southern Africa this cycle is linked with genotypes VIII and XXIV and historically in the Iberian peninsula with genotype I (PENRITH 2020). On Portuguese ground, ASFV has been linked to *Ornithodoros erraticus* as the arthropod vector, which can be present in pig farms (BOINAS et al. 2011). Having found ticks as reservoir and being transmitted mostly between the domestic pigs, ASFV can persist without the need of African wild suids (WILKINSON 1984).

2.1.2.2.3 Domestic pig cycle

Within this cycle, once ASFV is introduced from wild suids to naïve domestic pigs, the virus circulates among domestic pig herds without the active contribution of wild suids nor ticks (BELTRAN-ALCRUDO et al. 2017). Genotypes I–X, XII, XIV–XXIV have been identified in domestic pigs within the area where the classic sylvatic cycle is also present (HAKIZIMANA et al. 2021). On the other hand, genotypes I or II circulate among domestic pigs causing ASF outbreaks on a global scale (ROWLANDS et al. 2008, GE et al. 2018). Transmission modes in this cycle are diverse. Direct transmission can be carried out through contact between healthy and infected animals via oro-nasal ingestion of contaminated secretions, especially blood and excretions. Nevertheless,

indirect transmission due to human intervention plays the key role for the rapid and wide disease spread: infected pork products that are fed as swill as well as contaminated fomites (vehicles, premises, clothes) that are transported to other naïve pig populations can transmit the virus (CHENAIS et al. 2019). Ticks can be involved, but do not play a major role in ASF spread within domestic pig herds in Europe (FRANT et al. 2017). In addition, mechanical transmission through other arthropod vectors poses a possibility but is still not considered to play a crucial role (BOKLUND et al. 2018, BALMOȘ et al. 2021). Another knowledge gap is the carrier-domestic pig as a potential source of infection in non-endemic areas. Although this transmission route was demonstrated in a small study by EBLÉ et al. (2019), there is still no clear evidence regarding its viability in long-term real settings (STÅHL et al. 2019).

2.1.2.2.4 Wild boar-environment cycle

The Eurasian wild pigs, i.e. feral pigs *(Sus scrofa ferus)* and wild boars (*Sus scrofa domesticus*), play a pivotal role in this European cycle, where genotype II is circulating since 2007 (MALOGOLOVKIN et al. 2012). This cycle was first described when ASF reached the Spanish peninsula in the 1960s, which is a natural habit for *Sus scrofa*. After 2007, this pattern was further reported in Central and Eastern Europe, where wild boars are widely present and freely move through borders. Here, it was reported that Eurasian wild boars, which are densely present especially in Poland and the Baltic States, were able to maintain ASFV without the need of domestic pigs reintroducing the virus (CHENAIS et al. 2019). Quantitatively, wild boars excretes the same amount of virus as domestic pigs and, thus, the epidemiological dynamic is similar between wild boar and domestic pig herds (ARIAS and SÁNCHEZ-VIZCAÍNO 2002). Carcasses and the surrounding area can retain ASFV for long periods of time and facilitate further viral spread. Transmission can occur either directly through scavenging wild pigs or indirectly *via* contaminated soil (CHENAIS et al. 2019).

2.1.2.3 Tenacity, transmission, and infectivity

ASFV shows great resistance to physical and chemical factors. Since it is a highly stable virus, it remains viable for long periods in blood, faces and tissues as well as in the environment and contaminated raw pork products. Especially cold, humidity and high protein content are factors

that enhance the viability of the virus. European Food Safety Authority (EFSA) published a “scientific review on African Swine Fever” in 2009 listing the viability in different matrices (SANCHEZ-VIZCAINO et al. 2009). The virus can survive for up to 3 years in frozen pork products and for 15 weeks in chilled meat. Uncooked pork products can remain infectious for 3 to 6 months. These contaminated pork products seem to play a pivotal role in the viral spread at an international level (KOLBASOV et al. 2018). In blood, ASFV can survive up to 18 months at room temperature and up to 6 years at +4 °C. Up to 10^9 HAD_{50} /ml could be detected in blood and up to 10^5 HAD_{50}/ml in faeces, saliva and urine (GUINAT et al. 2014). Moreover, the virus can survive in putrefied blood for 15 weeks and in faeces for 11 days (SANCHEZ-VIZCAINO et al. 2009).

Blood is thus the matrix that carries the highest viral load among body fluids and is considered to be the raw material with highest infectivity (BLOME et al. 2013). Although other body fluids contain less virus and their viability depend mainly on the environmental temperature, they can pose a risk particularly when climate conditions and biosecurity measures are advantageous (AUTHORITY 2014).

Additionally, it was discovered that ASFV persisted for 150 days at 4 °C in skeletal muscle and for 6 months in bone marrow at -4 °C (KOWALENKO et al. 1965). These reports might explain why carcasses of wild suids play a role in ASF spreading.

There is no reliable evidence for vertical transmission of ASFV during the gestation period. In addition, no sexual transmission in pigs has been documented, notwithstanding the fact that viral particles are found in genital fluids (DE CARVALHO FERREIRA et al. 2012). Thus, this route of infection is still considered as a possibility, which encouraged the OIE to publish a recommendation to reduce the transmission risk especially regarding artificial insemination (HEALTH 2019c).

Another direct route of transmission that has been shown to be effective to carry infectious particles is through aerosols in short distances (DE CARVALHO FERREIRA et al. 2013, OLESEN et al. 2017).

Indirect transmission through contaminated inanimate fomites, such as clothes and vehicles, is possible (SÁNCHEZ-VIZCAÍNO et al. 2013, MAZUR-PANASIUK et al. 2019). Furthermore, *in-vitro* studies showed that pig ration can carry viral particles (NIEDERWERDER et al. 2019, STOIAN et al. 2019). Nonetheless, there are no reports on ASFV being detected in animal feed so far

(SHURSON et al. 2021). Further research is needed to fully understand fomites as a potential source of infection.

The stability of ASFV in soil depends on pH, ambient temperature and soil structure (CARLSON et al. 2020). The virus is highly stable at a pH range of 4-11 (HEALTH 2019a). Not favourable for virus survival are rather acidic soils. For example, adding citric acid and calcium hydroxide resulted in complete inactivation in all soil types (CARLSON et al. 2020). Additionally, peracetic acid also succesfully decreases ASFV infectivity in various soil types (TANNEBERGER et al. 2021). Since ASFV is enveloped, lipid solvents, detergents and commercial disinfectants based on phenolic compounds and iodine are effective for virus inactivation (DE LORENZI et al. 2020). Each country has its own licenced ASF disinfection products. For this purpose, the OIE has listed all recommended chemicals for ASFV (HEALTH 2019a). Regarding temperature, the virus is easily inactivated with heat: recommended are 56 °C for 70 minutes or 60 °C for 20 minutes (HEALTH 2019a).

Finally, morbidity within the herd might vary greatly depending on the swine species, type of pig production system, management, and biosecurity measures present. Although highly lethal in naïve pig populations, ASF is not as infectious as other transboundary animal diseases, such as foot-and-mouth disease (BELTRAN-ALCRUDO et al. 2017) or classical swine fever (CSF) (SCHULZ et al. 2017).

2.1.3 Pathophysiology

2.1.3.1 Pathogenesis

Key aspects of ASFV pathogenesis include a severe lymphoid depletion, leading to a state of immunodeficiency, and vascoendothelial damages inducing the characteristic hemorrhagic lesions of the disease (SALGUERO 2020).

The main infection occurs *via* oro-nasal route, where the virus reaches the tonsils and the respiratory tract. The first replication takes places in the lymphoid tissues of the nasopharynx. Other routes have been described, including skin injuries, injections and tick bites (PENRITH and VOSLOO 2009). ASFV replicates primarily in the cytoplasm of monocytes and macrophages of the lymph nodes close to the entry point. The incubation period of the virus ranges between 3-19 days (HEALTH 2019c). Afterwards, viral spreading in the host occurs through the bloodstream

(associated with erythrocyte membranes) or the lymphatic system around 2-8 days post-infection. This viraemia usually persists for longer periods of time due to the lack of effective neutralizing antibodies. ASFV can then spread to different organ systems, including lymph nodes, kidney, lungs, spleen, liver, and bone marrow. At this stage of infection, secondary replication of ASFV takes place in affected organs and can also affect other cells, including megakaryocytes, neutrophils, fibroblasts, hepatocytes, dendritic cells and endothelial cells (TRUYEN et al. 2015).

At the cellular level, ASFV causes damage in different ways. Infected cells show high phagocytic activation and secretory behaviour. Thus, macrophage activation and a secretion of cytokines (e.g. IL-1 und TNFα), complement factors and arachidonic acid derivatives are stimulated. Haemostasis is then compromised and further leads to endothelial cell damage. The resulting consequence is microthrombosis. The proinflammatory cytokines furthermore provoke lymphocyte apoptosis (lymphopenia), leading to a generalized immunodeficiency of the host. Additionally, the effect of megakaryocytes and the coagulopathy lead to a thrombocytopenia during ASFV infection. Characteristic lesions with haemorrhagic tendency ensue. Summarizing, the pathogenic effect of ASFV on primary target cells lead to acute phase reactions, activation of endothelial cells, apoptosis and inflammation (BLOME et al. 2013).

Although these processes are widely regarded as proven by the scientific community, there are still knowledge gaps regarding ASFV pathogenesis: viral receptors, cellular and humoral interactions and viral immune evasion mechanisms are not yet fully understood (WOZNIAKOWSKI et al. 2016, BLOME et al. 2020).

2.3.1.2 Clinical signs and pathological findings

The disease can affect all ages and both sexes in swine herds.
Clinical picture of ASF can be divided into four forms, and are mainly associated with strain virulence (table 1). Other factors affecting the clinical course of ASF are route of infection, individual host background, infection dose as well as endemic status of the affected area.

Table 1. Summary of the ASF forms and its characteristics. Source of information: BELTRAN-ALCRUDO et al. (2017).

Form	**Virulence**	**Mortality**	**Clinical signs**	**Post-mortem findings**	**Comments**
Peracute	High	90-100%	High fever; appetite-, and vitality loss; sudden death	Usually no clear lesions are found	Sudden death may occur without developing clinical signs or pathological lesions
Acute	High	60-100%	Fever; inactivity; huddle behaviour; increased respiratory rate; cyanotic areas; (necrotic-) haemorrhages on ears, abdomen, and hint legs; ocular and nasal discharge; hyperaemic areas on chest, abdomen and extremities; constipation; diarrhoea (mucoid or bloody); vomiting; late abortion; death may occur after 6-15 days p.i.	Enlarged-, oedematous-, and haemorrhagic lymph nodes; hemorrhagic or hyperaemic splenomegaly; petechiae on the kidneys capsule; generalized oedemas; skin haemorrhages	Most common form; Similar signs and pathological lesions are observed in feral pigs and wild boar, although they are not so obvious due thick darker skin and fur
Subacute	Moderate	20-80%	Similar to the acute form but milder	Commonly enlarged and haemorrhagic spleen; interstitial pneumonia; congested and oedematous lung	Mainly in endemic regions; animals either die 7-20 days p.i. or recover after 30 days p.i.
Chronic	Low/ attenuated	10-30%	Mild fever; respiratory symptoms; joint swelling	Possible hyperaemic-necrotic skin areas; pneumonia with caseous-mineralized necrosis; fibrinous pericarditis; oedematous-haemorrhagic mediastinal lymph nodes	Described in regions with longer disease history, i.e. Italy, Spain, Angola
Asymptomatic	Low/ attenuated host/ adapted host	0%	-	-	Described especially in endemic areas (ABWORO et al. 2017, CHANG'A et al. 2019, CHAMBARO et al. 2020, PATRICK et al. 2020).

2.3.1.3 Differential diagnosis

A definite clinical picture is not found in case of infection with ASFV. An affected animal can show none, one or more sets of clinical signs, especially in the early stages of the disease. Therefore, only laboratory diagnosis is conclusive.

Generally, any febrile disease in swine associated with haemorrhages and high mortality should lead to ASF suspicion. Clinical signs and pathological findings are often non-specific. Thus, ASF needs to be differentiated from other diseases (BELTRAN-ALCRUDO et al. 2017, HEALTH 2017, HEALTH 2019a) (table 2).

Table 2. Summary of differential diagnosis of ASF and its distinctive traits.

Disease	Aetiology	Main distinguishing clinical signs	Main distinguishing post-mortem findings
Classical swine fever (CSF)	Pestivirus *(Flaviviridae)*	Conjunctivitis; ataxia and central nervous symptoms in young pigs; yellow-grey diarrhoea; prolonged clinical course	Encephalitis; "button" mucosal ulcers of the gastrointestinal tract, larynx, and epiglottis
Porcine reproductive and respiratory syndrome (PRRS)	PRRS-Virus *(Arteriviridae)*	Intensified respiratory distress	No enlarged spleen; thyme atrophy
Aujeszky's disease	Suid herpesvirus 1 *(Herpesviridae)*	Great variation, including hypothermia, trembling, ataxia, rhinitis and sneezing	Necrotic enteritis; encephalomyelitis; pathognomonic white spots on liver of young piglets
Porcine dermatitis and nephropathy syndrome	Porcine circovirus 2 *(Circoviridae)*	Symptoms mostly in adult pigs	Increased fluids in body cavities and synovia; subcutaneous oedemas; enlarged pale kidneys
Poisoning	Various compounds, e.g. coumarin-based poisons, fungal toxins, pesticides	Rapid death, few clinical signs and lesions and afebrile hosts	Various
Erysipelas	*Erysipelothrix rhusiopathiae*	Characteristic diamond-shaped skin lesions in adult pigs	Arthritis; endocarditis; pleural-, peritoneal haemorrhage
Bacterial septicaemias	e.g. *Salmonella enterica, Staphylococcus spp., Streptococcus spp., Haemophilus parasuis, Actinobacillus pleuropneumoniae*	Sings vary in severity and especially juvenile animals are affected	Endocarditis; enteritis; occasional encephalitis; miliary necrotic foci in the liver; absence of vascular lesions in the spleen and lymph nodes

2.2 Available diagnostic tools for ASFV

The presence of ASF can only be confirmed through laboratory diagnosis, either detecting the agent or its immune footprint. Which approaches are suited best depend on the laboratory capacity of the affected area and the epidemiological situation. The OIE published validated detailed guidelines for recommended ASF diagnostics, where aim, performance and fitness of purpose is described for each technique depending on the epidemiological context (HEALTH 2019b). Here, sensitivity, workload and significance of test results are depicted as main characteristics. Figure 4 provides a summarising overview of recommended ASF diagnostic tools.

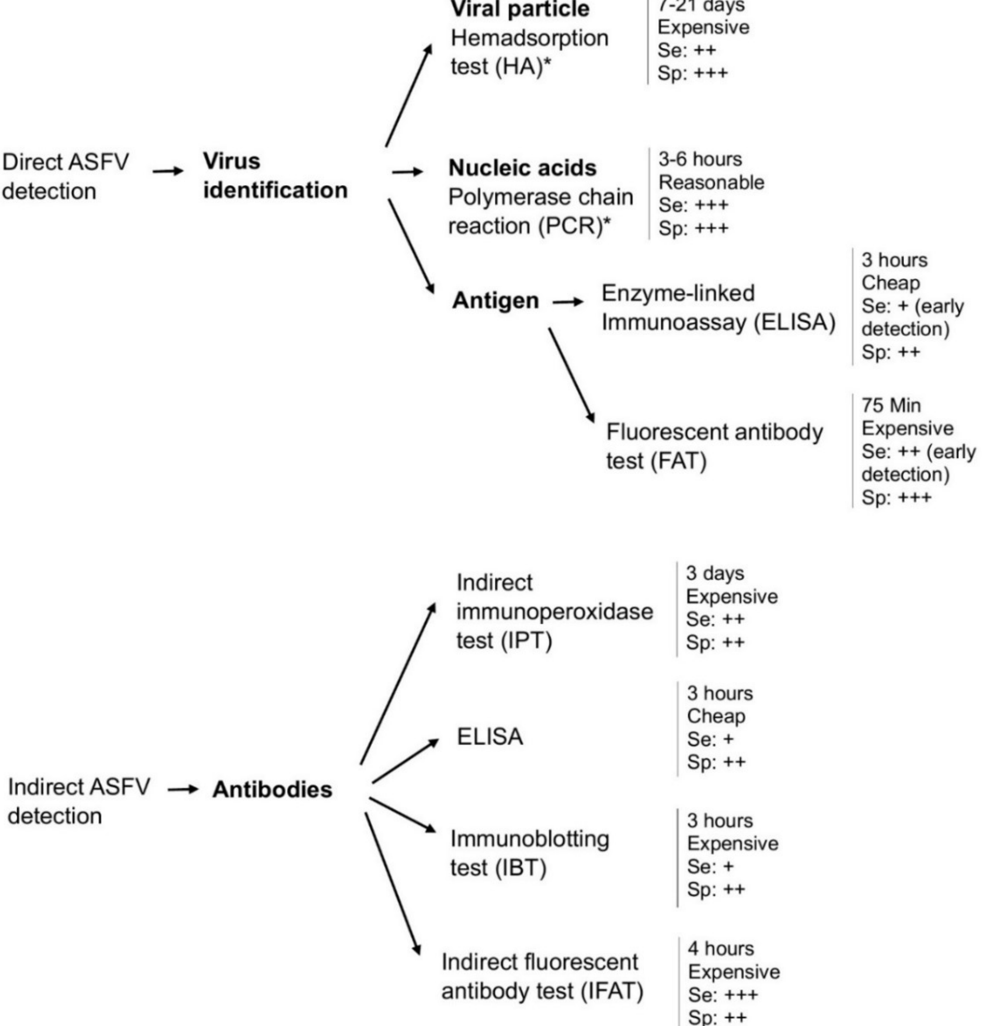

Figure 4. Diagram showing recommended diagnostic techniques for ASF, including time needed, cost implication, sensitivity (Se) and specificity (Sp). Data summarized from a report on ASF (BELTRAN-ALCRUDO et al. 2017).

2.2.1 Diagnosis based on immune response

Since there is no vaccine available, antibodies against ASFV always indicate an infection. Antibodies are usually developed after 7-10 days post-infection and persist over months or even years. However, there are no fully neutralising antibodies (ARIAS and SÁNCHEZ-VIZCAÍNO 2002). Immune response against ASF depends furthermore on the strain virulence, high virulent isolates usually cause death without antibody development. Thus, they are not suited for early agent identification. In contrast, infection with certain low virulent strains may only be traced using the serological approach. Serological tests are rather simple and low-cost, which makes them a good screening candidate for blood or serum samples. The bottleneck remains the sensitivity of these tests, which is generally lower than nucleic acid-based tests (BELTRAN-ALCRUDO et al. 2017)

To confirm clinical cases, an enzyme-linked immunosorbent assay (ELISA) for antibody detection in serum or plasma is commonly used (GALLARDO et al. 2019a). Indirect ELISAs have also been validated (ESCRIBANO et al. 1989, PASTOR et al. 1990) and several ELISA commercial kits are available. Nonetheless, inaccurate sample handling and preparation can result in false positive results and a clean workflow is of utmost importance. Therefore, secondary confirmatory tests are always recommended for positive or doubtful results (BELTRAN-ALCRUDO et al. 2017) such as indirect immunoperoxidase test (GALLARDO et al. 2015), indirect fluorescent antibody test (SANCHEZ-VIZCAINO 1987) and immunoblotting test (PASTOR et al. 1989).

2.2.2 Diagnosis based on direct agent identification

Different methods are validated for direct ASFV detection. Samples that can be tested include anticoagulated blood (EDTA), serum or tissues (especially lymph nodes, kidney, tonsils, bone marrow, spleen, lung).

Most used for viral genome detection is Polymerase Chain Reaction (PCR) due to its superior sensitivity, specificity, and high sample throughput. Additionally, PCR can identify ASFV even in degraded or putrefied tissues. However, it does not discriminate between infectious or non-infectious viral particles. A positive PCR results (with live or inactivated virus) is enough to determine the agent’s circulation in disease-free countries. For this reason, an assays sensitivity is of utmost importance. Validated conventional PCR (BASTOS et al. 2003, AGUERO et al. 2004,

BASTO et al. 2006) and real-time PCR (KING et al. 2003, TIGNON et al. 2011, FERNANDEZ-PINERO et al. 2013) are widely in use.

Haemadsorption test (HA) can be used for confirmation of viral presence and is considered as gold standard (CARRASCOSA et al. 2011, DE LEÓN et al. 2013). This is based on the aggregation of pig erythrocytes to the surface of ASFV infected monocyte-macrophage-lineage cells (haemadsorption), producing typical rosette forms (ENJUANES et al. 1976). Nonetheless, not all ASFV isolates show a HA phenotype (THOMSON et al. 1979, GONZAGUE et al. 2001, GALLARDO et al. 2019b). Other matrices including bone marrow cells, primary leukocyte or alveolar macrophages cultures can be used for virus isolation through cell infection, regardless of the HA phenotype. Indirect fluorescent antibody test (IFAT) is used for infected cell identification.

ASFV antigens can be used for agent identification. Nonetheless, antigen detection has a relatively low sensitivity and should always be confirmed with a gold standard method (PCR or HA). Commercial kits exist based on sandwich ELISA (HEALTH 2019b). Another viable antigen detection method is direct FAT, where a positive result from the first week post-infection together with a clear clinical picture can lead to a presumptive ASF diagnosis. Moreover, it can be used to detect antigens from non-haemadsorbing ASF strains, since antigens can be detected in leukocytes which show no HA (HEALTH 2019b).

2.3 Gaps in ASFV diagnostics

Surveillance and ASFV detection are essential for disease prevention. Not only border controls need to be improved to minimize the risk of disease introduction to new regions, but also passive and active surveillance are to be enhanced (ÁLVAREZ et al. 2019). Knowledge gaps regarding ASF diagnostics can be divided into three main pillars.

The first pillar represents the knowledge gap of in-depth understanding of the virus's characteristics. These include the viral tenacity in the environment, antibody development and virus growth kinetics (especially in survivors and persistently infected swine). All of these characteristics lead to different epidemiological scenarios and thus are important to understand the infection dynamics. Additionally, whole ASFV genomic analyses and genetic markers are

needed to adapt diagnostic test development to new strains and variations arising (BLOME et al. 2020).

The second pillar comprises the further optimization of validated diagnostic tools. Existing techniques used, e.g. virus isolation, must be improved and cell lines should be further developed for sufficient and effective virus replication. Moreover, there is a generalized need for advanced and harmonized protocols. For example, standard operating procedures for diagnostics testing already exist in Europe and are made available by the European Reference Laboratory for African swine fever, the Animal Health Research Center (CISA-INIA) (BLOME et al. 2020).

The third and last group deals with surveillance research gaps, as postulated by all ASF stakeholders. There is an urgent need for improved carcass detection methods, optimized sampling protocols (e.g. animal ration, carcasses, wild boar) as well as sensitive and rapid field diagnostic tests (ÁLVAREZ et al. 2019).

3. Publication

3.1 Statement of contribution

My own contribution to the publication of my thesis comprised the conceptualization of the manuscript, interpretation and summary of the findings under supervision of Ahmed Abd El Wahed. All involving literature research, tables and figures as well as the statistical analysis where carried out by me. I wrote and submitted the manuscript with the support of and in agreement with the co-authors.

3.1.1 Publication

Rapid Extraction and Detection of African Swine Fever Virus DNA Based on Isothermal Recombinase Polymerase Amplification Assay

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Article

Rapid Extraction and Detection of African Swine Fever Virus DNA Based on Isothermal Recombinase Polymerase Amplification Assay

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Abstract: African swine fever virus (ASFV) is the causative agent of a deadly disease in pigs and is spread rapidly across borders. Samples collected from suspected cases must be sent to the reference laboratory for diagnosis using polymerase chain reaction (PCR). In this study, we aimed to develop a simple DNA isolation step and real-time recombinase polymerase amplification (RPA) assay for rapid detection of ASFV. RPA assay based on the p72 encoding B646L gene of ASFV was established. The assays limit of detection and cross-reactivity were investigated. Diagnostic performance was examined using 73 blood and serum samples. Two extraction approaches were tested: silica-column-based extraction method and simple non-purification DNA isolation (lysis buffer and heating, 70 °C for 20 min). All results were compared with well-established real-time PCR. In a field deployment during a disease outbreak event in Uganda, 20 whole blood samples were tested. The assay's analytical sensitivity was 3.5 DNA copies of molecular standard per µL as determined by probit analysis on eight independent assay runs. The ASFV RPA assay only detected ASFV genotypes. Compared to real-time PCR, RPA diagnostic sensitivity and specificity were 100%. Using the heating/lysis buffer extraction procedure, ASFV-RPA revealed better tolerance to inhibitors than real-time PCR (97% and 38% positivity rate, respectively). In Uganda, infected animals were identified before the appearance of fever. The ASFV-RPA assay is shown to be as sensitive and specific as real-time PCR. Moreover, the combination of the simple extraction protocol allows its use at the point of need to improve control measures.

Keywords: African swine fever virus; recombinase polymerase amplification; DNA extraction; molecular detection

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1. Introduction

African swine fever causes a highly lethal, contagious disease in pigs, threatening the global swine industry and national economies. Accordingly, the virus is placed on the list of notifiable diseases of the World Organization for Animal Health (OIE).

The virus is a member of the *Asfarviridae* family [1] with an envelope and a large double-stranded DNA genome of 170–193 kbp [2]. In total, 24 genotypes and 8 serotypes were discovered mainly in Africa, [3–8], where ASFV was first described in Kenya in 1921 [9]. The virus is circulating in a sylvatic cycle among African wild suids (mainly Warthogs, *Phacochoerus africanus*) and *Ornithodoros* soft ticks in sub-Saharan Africa. This cycle is not accompanied by overt disease [10]. Globally, genotypes I and II are the major causes of outbreaks with direct transmission between wild and domestic pigs. Since its first

Introduction into Portugal in 1957 [11], the virus had been circulating through southern European countries until the late 1990s. Europe has faced the remerging of ASFV in Georgia in 2007 [12]. Recently, many cases have been identified in wild pigs in Germany and Poland [13–16]. A key aspect that facilitates its widespread transmission is the various transmission modes: arthropod vector (sylvatic cycle) mainly in Africa, direct or indirect contact with contaminated secretions (of either wild boars, Sus scrofa, or domestic pigs), as well as inanimate fomites (e.g., clothes, transport vehicles, carcasses, contaminated pork) [17].

Clinical signs associated with ASF are highly variable, ranging from peracute (lethality 90–100%) to asymptomatic, depending on various factors, e.g., the virulence of the virus, viral infectious dose, and host genetic background [18]. The most common form is the acute infection that induces high fever, lethargy, respiratory and digestive dysfunctions (often with hemorrhagic tendency), abortion, and sudden deaths. Since it shows great similarities with other infectious diseases regarding clinical and pathological pictures (e.g., classical swine fever (CSF)) [19], differential laboratory diagnosis is essential. The host range of ASFV is restricted to swine and no records of other livestock or human infection have been reported. Since neither effective treatment nor vaccination are available, the most essential control measures are identification of infected animals in wild or domestic pigs and immediate culling and movement restriction.

African swine fever virus can be isolated on macrophage cultures or on bone marrow cells, which requires a highly equipped laboratory. There are recommended direct (antigen or whole virus) and indirect (antibody) detection methods for ASFV. Indirect techniques comprise serological assays based on antibody enzyme-linked immunosorbent assays (ELISA), indirect immunoperoxidase test, and immunoblotting. Direct methods include hemadsorption test, virus isolation on macrophages, antigen detection by fluorescent antibody test, or antigen ELISA. The gold standard, however, is molecular genome detection based on polymerase chain reaction (PCR), either conventional or real-time. Many PCR assays were established over the past 20 years and recommended by the OIE [20–22], but PCR testing is limited to regional or reference laboratories, because of the complexity of the PCR and for biosafety reasons. A simpler and more standardized approach has been shown to be useful in less equipped laboratories [23]. Moreover, an on-site detection system will save time and decrease the duration between sample collection and results, which lead to the immediate implementation of control measures. Recently, promising isothermal amplification methods were developed and used to detect other animal pathogens [24,25]. Nevertheless, a key aspect that makes the implementation of molecular point-of-care tests still challenging is the lack of simple and effective on-site nucleic acid extraction. Among rapid molecular assays is recombinase-based isothermal amplification: Recombinase polymerase amplification (RPA) and recombinase-aided amplification (RAA). The chemical process relies on three core enzymes and proteins: a recombinase (uvsX of T4 phage for RPA or the recombinant enzyme from E. coli for RAA), single-stranded DNA-binding protein (SSB), and the polymerase. These chemicals replace the denaturation, annealing, and extension steps of the PCR, but at a constant temperature of 37–42 °C for a maximum of 15 min. Furthermore, the detection of real-time amplification is based on a synthetic molecular probe [26], which emits fluorescence upon binding to the amplicon.

In this study, a rapid DNA extraction and RPA assay targeting the B646L gene (encoding the capsid protein p72) of ASFV was developed. The limit of detection, cross reactivity, and clinical performance were also determined. All results were compared with a reference silica gel-column-based extraction method and real-time PCR.

2. Materials and Methods

2.1. Clinical Samples and Ethical Statement

In total, 52 whole blood samples from experimentally infected domestic pigs were used in the study. The animal experiment was externally approved by the competent authority (Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF)

Mecklenburg-Vorpommern) under reference number 7221.3-2-011/19. In addition, 21 serum samples from routine diagnosis submitted to the faculty of Veterinary Medicine, Leipzig University, Germany, were screened.

2.2. Molecular DNA Standard and RPA Oligonucleotides

The B604L gene was used as the target for the developed RPA assay. A 417-nucleotide-long molecular standard (GenBank accession number: MK554698.1; nt: 1491-1908) was synthesized by Thermo Fisher Scientific GENEART (Regensburg, Germany). The RPA oligonucleotides were selected based on multiple alignment of 24 sequences representing the ASFV genotypes (Accession Numbers: AF302816, AM999764, AF270706, FJ528594, DQ250120, AF302818, AY494553, AF270711, AF302818, AF270705, AY351564, AF449463, AY351522, AY351543, AY351542, AY351555, AY494552, AY494551, DQ250119, DQ250122, DQ250127, DQ250109, DQ250125, DQ250117, KT795360, KY353989) using Geneious 2020.2.3 (https://www.geneious.com, accessed on 2 October 2020). Six primers and one exo-probe were designed and screened in this study (Table 1). The primers/probe combination yielding the highest signal in RPA (threshold time (TT) in minutes and fluorescence intensity in millivolt (mV)) was selected for further assay validation. TIB MOLBIOL GmbH (Berlin, Germany) synthesized all oligonucleotides.

Table 1. RPA oligonucleotides sequences.

ID	Sequence (5′ to 3′)
Probe	ATCGATAAATTTCCATCAAAGTTCTGCAGC-BHQ1-THF-FAM-TACATACCCTTCCAC
FP1	TGGTATCAATCTTATCGATAAATTTCCATCAA
FP2	CCTATTATTAAAAACATTTCCGTAACTGCTCA
FP3	ATATTAGCCCCGTTACGTATCCGATCACATTA
RP1	AATTCTCTTGCTCTGGATACGTTAATATGACC
RP2	ACTGGGTTGGTATTCCTCCCGTGGCTTCAAAG
RP3	CAAAGGTAATCATCATCGCACCCGGATCATCG

2.3. RPA Conditions

A real-time RPA assay was performed in a 50 µL volume using the TwistAmp Exo kit (TwistDx, Cambridge, UK). The reaction mix comprised 29.5 µL rehydration Buffer, 8.2 µL nuclease-free water, 2.5 µL magnesium acetate (280 mM), 2.1 µL of each primer (10 µM), 0.6 µL probe (10 µM), and 5 µL template (or 1 µL for samples treated with the rapid extraction protocol), which was added into the lid of the reaction tube containing the freeze-dried pellet. Water was used instead of the DNA template for the negative control. For RNA viruses tested for cross reactivity, 8.2 µL (500 U) of RevertAid reverse transcriptase (Thermo Scientific, Regensburg, Germany) was used instead of nuclease-free water. The tube was closed, centrifuged, mixed, centrifuged, and placed immediately into the T8-ISO Instrument (Axxin, Fairfield, Australia). The incubation temperature was 42 °C for 15 min. A mixing and centrifuging step was conducted at 320 s after the test start. The FAM fluorescence signal was recorded in real time. The TT was determined using the T8 Desktop Application (version 2.8.0.0, Axxin) based on the first derivative values.

2.4. Analytical Sensitivity and Specificity

To determine the real-time ASFV-RPA assay's analytical sensitivity, eight replicates of serial dilutions of the molecular standard (10^2-10^0 DNA Copies per µL) were tested. The limit of detection was calculated using RStudio version 1.3.1093 [27] performing a probit regression analysis and visualized using the ggplot2 package (v3.3.3) [28]. Cross reactivity of the real-time RPA assay was determined using nucleic acids of viruses listed in Table 2.

Table 2. List of viruses whose nucleic acids were included in the cross-specificity testing.

Virus Name	Virus Type	Number of Samples
African swine fever virus	DNA, enveloped, double-stranded	10
Classical swine fever virus	RNA, enveloped, single-stranded	11
Porcine parvovirus (NADL-2)	DNA, non-enveloped, single-stranded	1
Foot and mouth disease virus	RNA, non-enveloped, single-stranded	10
Modified vaccinia Ankara	DNA, enveloped, double-stranded	1
Porcine circovirus-2	DNA, non-enveloped, single-stranded	1

2.5. Nucleic Acid Extraction Procedures

DNAs from samples were extracted by two different methods. First, a standardized silica-based DNA extraction kit (DNAeasy Blood & Tissue Kit, QIAGEN GmbH, Hilden, Germany) was used for the purification of total DNA, as instructed by the manufacturer. A total of 5 µL was used as a template in the RPA reaction. Second, the same clinical samples were incubated with 200 µL QIAGEN ATL lysis buffer at 70 °C for 20 min. Then, 1 µL of the processed sample was diluted in 9 µL nuclease-free water, and 1 µL of the mix was used as a template.

2.6. Real-Time PCR Conditions

The molecular standard as well as all clinical samples were tested with an established real-time PCR targeting the same gene region of the ASFV-RPA assay [20]. The real-time PCR was performed on the Stratagene M × 3000 P QPCR from Agilent Technologies (Santa Clara, California, United States). The reaction of QuantiNova Probe PCR kit (QIAGEN GmbH, Hilden, German) consisting of 12.5 µL of the QuantiNova Probe PCR Master Mix, 5 µL H_2O, 1 µL of each primer (10 µM), 0.5 µL of probe (10 µM), and 5 µL template, reaching a total volume of 25 µL. The following temperature profile was used: 95 °C for 2 min for initial denaturation; 40 cycles of amplification including 10 s at 94 °C and 30 s at 60 °C for denaturation and annealing, respectively.

2.7. Pilot Field Deployment

On request of a small farm in Kibaale district in Uganda, 20 whole blood samples of suspected ASF domestic pigs were screened at Makerere University (Kampala, Uganda). DNA was extracted using a Quick-gDNATM MiniPrep kit from ZYMO Research (Irvine, CA, United States) according to the manufacturer's instructions. For the heating/lysis buffer method, samples were incubated with 200 µL genomic lysis buffer from the Miniprep kit at 70 °C for 20 min. RPA was performed as mentioned above.

3. Results

3.1. Selection of RPA Primers and Probe

All possible primer combinations were tested using a concentration of 10^5 of the ASF molecular standard. Best results were achieved using FP1 and RP3 with a TT of 2.66 min and a fluorescence signal of 5000 mV (Supplementary Figures S1–S3). This primer combination was used for further assay validation.

3.2. Analytical Sensitivity and Specificity

To compare the performance of the RPA to the real-time PCR using molecular standard, a serial dilution of $5 * 10^6$–$5 * 10^0$ DNA molecules/reaction was prepared and tested. Both assays were able to amplify and detect down to one DNA molecule/µL (Figure 1).

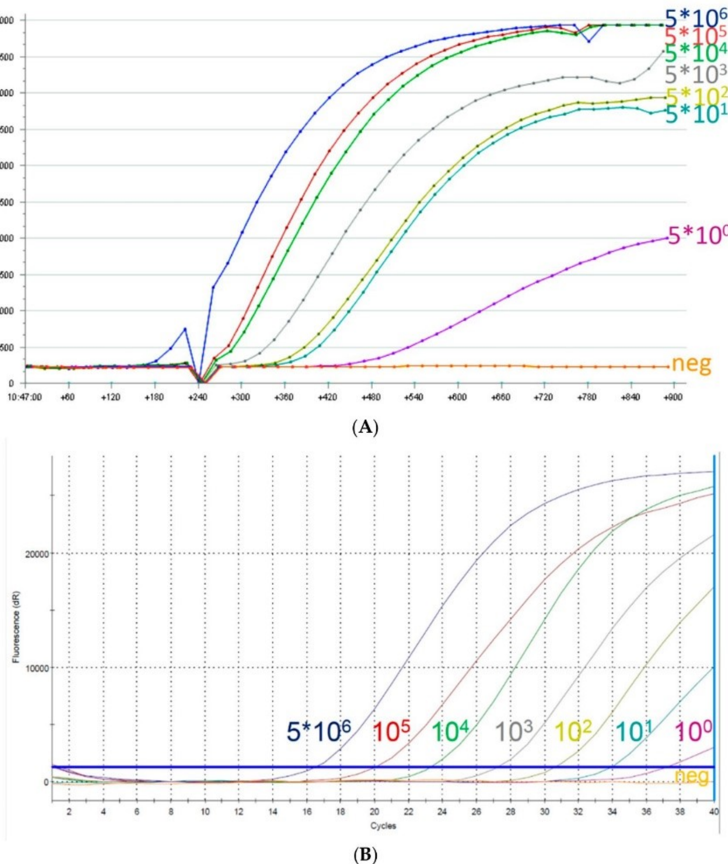


Figure 1. Amplification curves of RPA (**A**) and real-time PCR (**B**). Both assays detected down to one DNA molecule per µL.

To determine the RPA assay limit of detection, eight RPA runs of 100, 10 and 1 molecular standard DNA molecules/µL were performed. The 100 and 10 copies/µL were detected in all the 8 runs (8/8 runs), while the 1 copy/µL was identified in 3/8 runs. With this dataset, a probit regression analysis was performed and yielded a limit of detection of 3.5 copies per µL (95% CI) (Figure 2A). The reaction speed was under 7 min (Figure 2B). The ASFV-RPA assay detected all tested ASFV nucleic acids as positive (Table S1) and did not cross-react with nucleic acids of other viruses (Table 2).

3.3. Clinical Samples

DNAs from silica gel extraction protocol and simple heat/lysis step were screened simultaneously in both real-time PCR and RPA assay. By using the pure DNA from the silica-gel-based method, both real-time PCR and RPA have correctly detected 37 as positive and 36 as negatives (Table 3, Table S1 Supplementary file). No correlation between the Ct of the real-time PCR and the TT of the RPA was detected (Figure 3). When applying the simple heat/lysis step, 36 samples were assigned as positive, 1 as a false negative, and 36 as negatives. In contrast, in real-time PCR, only 14 out of the 37 positive samples were detected (Table 3, Supplementary Table S1).

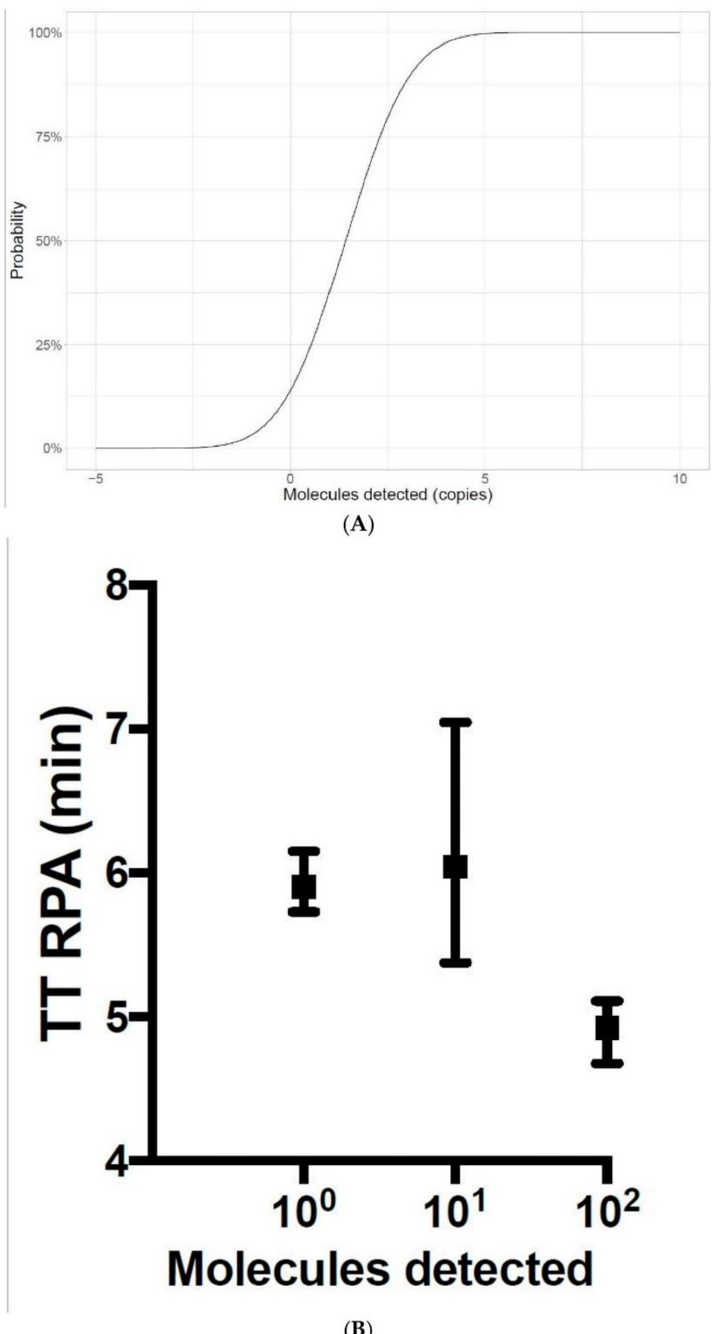


Figure 2. Limit of detection (**A**) and reproducibility (**B**) of the ASF RPA assay. Dataset of 8 RPA runs of the molecular standard 100 to 1 DNA copy/µL was used. Limit of detection is 3.5 copies/µL (A). The speed of the assay was between 5 and 7 min (B).

Table 3. Sensitivity and specificity of ASFV-RPA and real-time PCR using two extraction procedures. Sensitivity was significantly lost in PCR using rapid heat/lysis extraction with blood samples.

Extraction Method	Sensitivity (n = 37)		Specificity (n = 36)	
	RPA	Real-Time PCR	RPA	Real-Time PCR
Qiagen DNeasy Blood & Tissue kit	100%	100%	100%	100%
Heated sample in lysis buffer	97%	38%	100%	100%

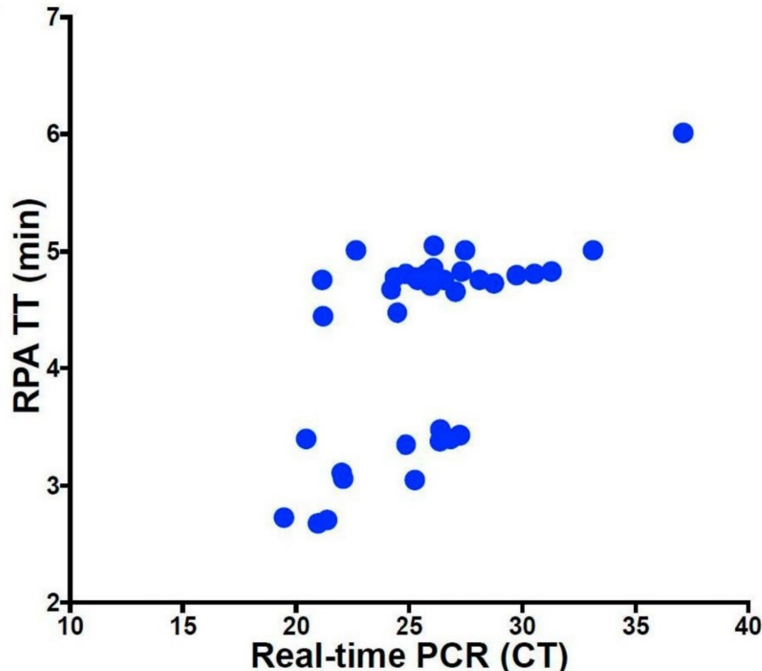
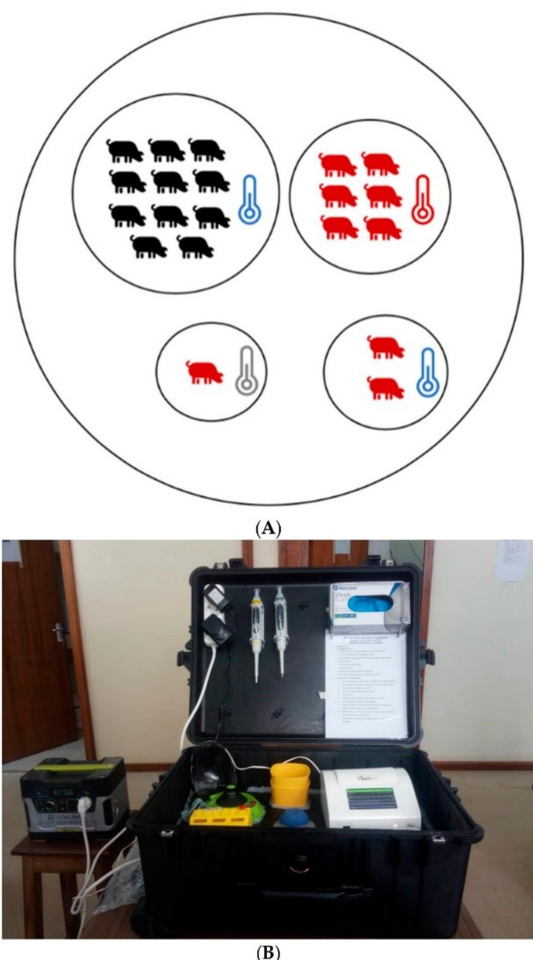


Figure 3. Comparison between the TT value of the RPA and Ct value of the real-time PCR. No correlation was found (R^2 = 0.34) as the RPA is much faster than the real-time PCR.

3.4. Field Deployment in Low-Resource Settings

Blood samples from domestic pigs from an outbreak in Uganda were tested with the ASFV-RPA assay combined with either heating/lysis buffer method or routine silica-gel-based nucleic acid purification method. Eleven samples were assigned as negative and nine as positive using both extraction methods (Figure 4). TT values between the two extraction methods did not differ considerably. Using the silica-based extracted DNA, TT values were between 3.1 and 5.45 min; using the rapidly extracted DNAs between 5.11 and 6.05 min.



(**A**)

(**B**)

Figure 4. Field deployment of mobile suitcase lab in Uganda: (**A**) ASFV-RPA assay results of 20 samples from suspected ASF domestic pigs in Uganda. Eleven afebrile pigs tested negative, while six febrile pigs, one pig without temperature reading and two afebrile pigs tested positive. Red is animal tested positive. Black is animal tested negative. Blue thermometer indicates normal body temperature, red is pig with fever, and grey is pig with no body temperature measured. (**B**) Mobile suitcase lab.

4. Discussion

ASFV detection relies on well-equipped reference laboratories performing established diagnostic methods. In the present study, we developed a sensitive and specific real-time RPA assay for the rapid detection of ASFV. The B646L gene, encoding for the major capsid protein p72, was chosen as a target since it is a highly conserved region [29,30]. The ASFV-RPA assay was as sensitive as the OIE-recommended real-time PCR being able to detect down to one DNA copy/µL. Moreover, no cross reaction was observed with other viruses with a similar clinical picture. The bottleneck of molecular point-of-need testing remains sample inactivation and extraction. Therefore, the ASFV-RPA assay was also

combined with a simple heating and lysis buffer procedure for blood samples, showing a 97% positivity rate.

The gold standard detection method is real-time PCR, which takes up to several hours to deliver results. Two modified PCR assays have been developed to speed up the testing and simplifying the extraction method [31,32]. The total run time was two hours for nine samples implementing a cartridge-based kit, which is easily deployable but required various hands-on steps per sample [32]. When using a magnetic-bead-based extraction protocol, an automated expensive device was required [31]. Direct use of blood in PCR inhibited the reaction as observed in our study (sensitivity 38%) and by others [31,33]. Around 5 to 10 shifts in the Ct values were observed in our study when comparing highly purified DNA and non-processed blood samples. In contrast, RPA is better suited for crude blood samples without further purification steps (sensitivity 97%). No differences were recorded in the TT RPA values between the DNAs of the two extraction approaches. The tolerance of the RPA assay to inhibitors such as milk, hemoglobin, ethanol, and heparin was reported [34,35].

The assay speed is crucial, especially at the point-of-need testing. When comparing the performance of RPA and real-time PCR using linear regression analysis, no correlation was found between TT and Ct values (Figure 3). The reason is the explosive nature of the RPA reaction at a single constant temperature leading to a non-linear amplification outcome [36], while the thermal cycling profiles needed for the PCR reaction lead to more regular exponential amplification curves [37]. Both the speed and robustness of the ASFV-RPA make it an ideal candidate for point-of-need testing. Other advantages are the stability of reagents at ambient temperature (around 40 °C for up to 3 months) and operation in a portable mobile suitcase laboratory [34,38–40]. The field study in Uganda showed the successful deployment of the ASFV-RPA assay in low-resource settings. In addition, afebrile animals carrying the virus were detected before the onset of clinical signs. Thus, a deployment for early ASFV screening is viable and can help early control of the disease. Moreover, our study is the first to test clinical ASF samples both from Europe and eastern Africa using a point-of-need setup.

Many isothermal amplification assays have been developed over the past two decades for identifying ASFV. Loop-mediated isothermal amplification (LAMP) [41,42] and cross-priming amplification (CPA) [43,44] detected ASFV with sensitivity of 90 and 70%, respectively [45]. Both required 3-6 sets of primers to amplify the target region, in addition, the run time was around 30–60 min at temperatures >50 °C. The results visualization was based on SYBR Green. In ASFV-RPA assay, five DNA copy was amplified using two primers in less than 10 min and with higher specificity applying an exo-probe-based system.

RPA assays for the detection of ASFV based on separate steps of amplification and visualization using lateral flow were developed [33,46]. The clinical sensitivities of these assays were ranging between 70 and 100% with a turnaround time of 30 min. This approach is subjective to high cross-contamination risk since the post-amplification pipetting step is needed to transfer the amplicon to the lateral flow cartridge. RPA assay relying on CRISPR as a reporter is highly sensitive but has a runtime similar to the real-time PCR and the reagents must be stored at −20 °C [46]. Other ASFV-RPA amplification monitoring based on SYBRE Green dye is not field applicable because of the need to open the post-amplification tube to add the dye [47]. The developed real-time exo-probe based ASFV-RPA assay in this study is highly sensitive, produces faster results (<10 min), and utilizes an all-in-one tube reaction mix. The only drawback is the need for a fluorometer, which adds to the start-up costs. Two other real-time ASFV-RPA and RAA assays have been established but were limited to samples originated from China [48,49] and did not amplify properly our isolate from Germany (Supplementary Figure S4). Therefore, we recommend testing local isolates before implementing diagnostics for ASFV to avoid false negatives.

In conclusion, the developed probe-based real-time RPA assay is shown to be a highly sensitive and specific detection method for ASFV. Furthermore, the simple and effective heating/lysis buffer extraction procedure eases the on-site applicability of the assay. When

combining ASFV-RPA with a portable lab setup, e.g., mobile suitcase lab, it can be deployed in the field as point of need testing method. This would allow faster detection of ASF cases since it can significantly reduce the time between sample collection and result.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/v13091731/s1. Figure S1: Possible primer combinations tested with 5×10^5 ASFV molecular standard; Figure S2: The three best primer combinations tested with 5×10^5 ASFV molecular standard, Figure S3: The two best primer combinations were tested with $5 \times 10^{3\text{-}1}$ ASFV molecular standard, Table S1: ASFV positive samples. ASFV Genotype and sample matrix were listed, Figure S4: RPA assay using $10^{3\text{-}1}$ of the ASFV molecular standard based on Wang et al. 2020.

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4. Discussion

Sensitive and fast African swine fever (ASF) diagnosis is the first step towards effective disease control. On-site testing options are being continuously developed but need further improvement, harmonization, and implementation.

In our study we developed a highly sensitive and deployable assay for direct ASFV diagnosis based on recombinase polymerase amplification (ASFV-RPA). The limit of detection of ASFV-RPA was 3.5 DNA copies per µl. All screened ASFV genotypes (including European and African isolates) were detected while no other viral nucleic acids were identified. Using the standardized DNA extraction method in ASFV-RPA, and compared to real-time PCR, diagnostic sensitivity and specificity were 100%. To ease the applicability on site a rapid heat/ lysis buffer extraction protocol was tested and showed promising results, achieving 97% of positivity rate compared to a 38% of the real-time PCR. When deploying the assay in a field study in Uganda, ASFV-RPA detected 11 samples as positive, including 2 known afebrile animals (thus detecting the circulating virus even before onset of clinical symptoms).

Generally, PCR is regarded as gold standard for animal and human pathogens and widely accepted by all communities due to its high sensitivity and specificity. Point-of-need tests (PONT), however, are still considered as rather new, although they have been on the market for the past two decades and are continuously being improved. An added difficulty for PONT in animal science represents the lack of concrete regulations for its development and implementation. They can be an asset for animal disease surveillance and outbreak investigations in the field, reducing the time between sample taking and result delivery coupled to surveillance systems. PONT can deliver essential first-line surveillance data, which is a key aspect for an outbreak control and strategies to run effectively and ease decision making. Nonetheless, broad acceptance and faster validation regulations are still lacking (HANSEN and ABD EL WAHED 2020). Furthermore, field applicability of PONT differs within high income countries and low- and middle-income countries. While in developed countries the impact might not vary as much from gold standard testing procedures due to the existing infrastructure, countries with lower resources generally present a heavy burden of disease and often lack the necessary framework (URDEA et al. 2006). Moreover, other important factors posing a problem include lack of compensation in case of an animal disease outbreak, insufficient legal structures and their

enforcement (QIU et al. 2021). In these settings, PONT could improve testing outcomes and animal-health related data. Official validation criteria for such on-site tests for animal diseases are still lacking. In human science, tests that are performed at the patients bed-side are known as "point-of-care", and the World Health Organization denominated the "ASSURED criteria" for their development: affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free and deliverable to end users (WU and ZAMAN 2012). There is a need for formulation and application of these criteria to animal PONT, especially for transboundary diseases like ASF, which have severe consequences and rely on fast diagnosis.

In high income countries such as Germany, PCR is the recommended tool to determine ASF outbreaks. The German infrastructure is well established and financial resources allow this detection method to be regularly used. Depending on the number of samples, a primary result can be obtained usually in around 6 hours (BORGWARDT 2014). In case of a positive or doubtful result, the sample needs to be examined by the national reference laboratory (Friedrich-Loeffler-Institut) (FRIEDRICH-LOEFFLER-INSTITUT 2019). In this setting, ASFV-RPA, as a PONT, could offer an asset for on-site ASFV detection: blood testing of suspected outbreaks in wild boar or in pig production farms could ease the pressure on rapid PCR sample transport and laboratory testings. However, they could induce the implementation of first important control measures. A negative result could further help better and faster assess the epidemiological situation directly on-site, minimizing risks and pressure on the involved stakeholders.

In low- and middle-income countries, where infrastructure and resources are scarce and not always available, PCR results can take many days to be delivered. Correct sample transport (avoiding PCR inhibitors, contamination and deterioration) and cold-chain maintenance can also pose a problem and cause false negative results (SCHRADER et al. 2012, BELTRAN-ALCRUDO et al. 2017). These bottleneck aspects in ASF detection could be avoided with a PONT test like ASFV-RPA. With high sensitivity and specificity, being robust and deployable and thus reducing the need of sample transport, ASFV-RPA could offer field-data in real time.

However, not only the actual detection of the agent can be difficult, but also the consequences that come with a defined ASF outbreak. A compensation and incentive policy in case of an ASF outbreak remains still a key aspect to overcome in low-resource settings, as well as keeping social and cultural issues within the pig farmer's heterogenicity in mind (BARNES et al. 2015) . In

addition, international and multi-centered collaboration can be significant for successful on-site ASF test implementation. The needs “in the field” must be acknowledged by all researchers, developers and actors involved (DERDA et al. 2015), maximizing efforts and knowledge exchange. Within this line, interdisciplinary research can enhance and fasten PONT development (WANG et al. 2016). Thus, close cooperation among stakeholders, appropriate trainings as well as making supplies available are necessary to evaluate disease-control agendas effectively and make them more sustainable. For ASF test implementation, this might include the preliminary testing of all known ASFV genotypes, an appropriate legal and logistical context, staff training, assessment of the test’s practicality in the field as well as the valuable feedback of those implementing it on site.

Many on-site testing methods have been developed for ASF. Nevertheless, the field applicability of every technique varies.
Antigen and antibody detection in form of lateral flow devices (LFD) have been developed for ASF, being easily applicable in the field as it is a simple and cheap visualization method (SASTRE et al. 2016a, SASTRE et al. 2016b). Furthermore, no expensive equipment is needed. Nonetheless, sensitivity of pen-side LFD still needs to be improved to be comparable to laboratory-based testing. Moreover, antigen detection based on ELISA or FAT is only suitable for early disease detection (HEALTH 2019b). However, ASF-antibodies are not always suited to correctly assess the ASF epidemiological situation. Direct viral detection in the field can be more accurate to determine whether the agent is circulating or not, especially in disease-free countries where early detection is pivotal (HEALTH 2019c).

In the last few years, efforts are being made to adapt gold-standard PCR at point of need for direct ASFV detection, as it is the most sensitive and specific method and which allows high-sample throughput with automation (HEALTH 2019b). The aim is to reduce workload and equipment while maintaining its high performance. Different formats are used for this purpose, including LFD (ZENG et al. 2020), pen-side tests (LIU et al. 2019, DAIGLE et al. 2020) and nanofluidic chips (JIA et al. 2021). Results are very promising, but contamination of the PCR reaction must be avoided, and production costs are still high since specific equipment is needed. Moreover, cold chain for reagent storage must be maintained. Reagents lyophilization is being further developed to ease the practicality on site (WANG et al. 2020). Especially the nucleic acid extraction procedure from samples still poses a challenge for accurate molecular diagnosis (ALI

et al. 2017). Faster and effective extraction approaches including cartridge-based kits and magnetic beats have been applied and show good applicability. The drawbacks include the need of additional pipetting steps and/or expensive magnetic devices (LIU et al. 2019, DAIGLE et al. 2020, CHEN et al. 2021). Another simple method, only needing basic equipment and consisting of one heating step, lysis buffer and dilution, has been shown to be effective for ASFV inactivation and extraction, both in this study and in others (CERUTI et al. 2021, ZHANG et al. 2021). The dilution step, however, still needs to be optimized since it can affect the assay's sensitivity, especially when using PCR (LIU et al. 2019). This heat/lysis procedure is easy to handle, affordable since no magnetic device is needed and suited for on-site testing. This method was used together with an isothermal amplification method and LFD, achieving promising results (ZHANG et al. 2021). Isothermal amplification has gained a lot of attention in the last few years as a genomic detection method. Common isothermal techniques, such as cross-primer amplification and loop-Mediated Isothermal Amplification, are described for ASFV with sensitivities between 70-90%, 25-60 minutes reaction time, 56-66 °C reaction temperature and more than two sets of primers (WOŹNIAKOWSKI et al. 2018). Another isothermal method, named Recombinase Polymerase Amplification (RPA) is emerging in the market over the past decade (LI et al. 2018) and was used in the present study. RPA only needs two primers, 39-42° C and around 15 minutes reaction time. For ASFV, sensitivities over 90% have been reached, including readouts based on LFA (MIAO et al. 2019, ZHAI et al. 2020, ZHANG et al. 2021), direct dye addition (ZHANG et al. 2020) and fluorescent detection (FAN et al. 2020, WANG et al. 2021b). The reagents can be stored at room temperature and the workflow is easy to handle. While sample contamination of any kind is a factor to consider when taking tests into the field, closed fluorometers reduce this risk. When using LFA and direct addition of dye to the RPA reaction, contamination can happen more easily. Nonetheless, closed-tube devices are usually more costly and need a power supply. Addressing the common PONT problems of contamination, complex extraction protocols and low sensitivity, ASFV-RPA offers a closed-tube system and a cheap and fast extraction method while maintaining its high sensitivity.

In summary, every ASF PONT detection method sets its own unique settings in which it can be useful, especially regarding the applicability on site. Nonetheless, laboratory-based confirmatory tests or interpretation of animal test results are still regarded as necessary (BUSIN et al. 2016). Hitherto, a combination of indirect and direct ASFV detection methods can deliver the most

epidemiological information in the field. For direct detection, isothermal amplification methods meet the needs for accurate ASFV diagnosis on-site.

ASFV-RPA is shown to be a sensitive, specific, user-friendly, rapid, robust, and deliverable to end users PONT test, although one drawback is the need of a closed-tube fluorometer and the suitcaselab. However, since it is a maintenance-free set-up, this only adds to the initial costs and needs to be compared to other set-ups in terms of affordability and outcome. Thus, all "ASSURED" criteria are addressed or fulfilled. An important aspect in PONT development is its clinical validation in resource-limited settings. With this purpose in mind, ASF-RPA was deployed in a small pilot study in Uganda. In doing so, a multi-centered approach was carried out, including one center in a high-income country (German laboratory) and one in a low- or middle-income country (field-deployment in Uganda).

Usually, sample taking relies on passive reporting of symptomatic pigs. When testing for active surveillance in the field, early detection of the disease, including low virus titer in asymptomatic animals, can be an invaluable asset. Therefore, highly sensitive test methods in a portable format are necessary. ASFV-RPA was able to detect even afebrile pigs in a suspected outbreak in Uganda, thus indicating its potential to detect the virus even before symptoms appear.

Although PONT have shown to have a bright potential for ASFV control, the future of these technologies demands more general acceptance for animal diseases, field diagnostic data, automation, and higher throughput for large scale implementation. Moreover, the tests need to be affordable and national policy must endorse its regulated implementation. Additional steps complementing ASFV-PONT, such as sequencing steps and quantification algorithms, could be useful for up-to-date strain information and enhance surveillance data. Combined with an international cooperation approach, improved and deployable ASFV detection methods such as ASFV-RPA could pave the way for a rapid, accurate and sustainable test approach, depending on the countries needs and resources.

Overall, this study further underlines the potential of this isothermal amplification method for rapid and sensitive detection of ASFV. In combination with the rapid extraction procedure, ASFV-RPA represents a user-friendly, sensitive, and practical test method that can contribute to the early detection of ASF on site.

5. Summary

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Rapid Extraction and Detection of African Swine Fever Virus DNA Based on Isothermal Recombinase Polymerase Amplification Assay

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Keywords: African swine fever virus; Recombinase Polymerase Amplification; DNA extraction; molecular detection

Introduction: African swine fever virus (ASFV) causes a deadly viral disease in pigs. The virus has gradually spread throughout the world and was reported in Germany in September 2020. ASF outbreak can lead to huge economical loss. No vaccine is commercially available and thus, surveillance and early detection play a pivotal role to control an ASF outbreak. Polymerase Chain Reaction (PCR) is considered the gold standard for ASFV detection due to its superior sensitivity and specificity. However, it is time-consuming and requires well-equipped laboratories. Point-of-need tests can offer an alternative, delivering fast and reliable results directly in the field.

Objective: The aim of this study was to establish a field-deployable point-of-need test based on Recombinase Polymerase Amplification (RPA) to detect ASFV.

Material and Methods: Three sets of primers and one probe based on the B646L gene which encodes for the viral capsid protein p72 were designed. All possible combinations were screened. Analytical sensitivity was tested with eight replicates of serial dilutions of the molecular standard (10^2-10° DNA copies per µl). The limit of detection was calculated using probit analysis. ASFV-RPA's specificity was tested using various viral nucleic acids of pathogens infecting pigs. To allow the deployment at point of need, two different extraction approaches were tested in ASFV-RPA with all 73 pig blood samples included in this study: a rapid heat/lysis buffer extraction method and a standardized spin-column based extraction kit. Diagnostic sensitivity and specificity were calculated for both test approaches. All results were compared to an established real-time PCR

for ASFV. A small pilot study for ASFV-RPA assay deployment was done in Uganda with 20 blood samples of a suspected outbreak using the field-deployable suitcaselab.

Results: The calculated limit of detection of ASFV-RPA was 3.5 DNA copies per µl. All screened ASFV genotypes were detected while no other viral nucleic acids were identified. Using the standardized DNA extraction method in ASFV-RPA, and compared to real-time PCR, diagnostic sensitivity and specificity were 100%. The rapid heat/lysis buffer protocol showed very promising results, achieving 97% of positivity rate compared to a 38% of the real-time PCR. In Uganda, ASFV-RPA detected 11 samples as positive, including two known afebrile animals.

Conclusion: Immediate agent detection is a key aspect of ASF outbreak control. ASFV-RPA is as sensitive and specific as a gold standard PCR for ASFV identification. Combined with the heat/lysis buffer DNA isolation step, the duration of the assay is around 25 minutes from sample collection to result readout, with a promising positivity rate of 97% which indicates tolerance against inhibitors. ASFV-RPA is a portable detection method, as revealed during the pilot field study in Uganda. Only requiring basic equipment and solar batteries, the suitcase lab is a promising tool for on-site diagnostics in resource limited settings to detect ASFV.

6. Zusammenfassung

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Schnelle Extraktion und Nachweis von Afrikanische Schweinepest-Virus-DNA auf der Grundlage eines isothermischen Rekombinase-Polymerase-Amplifikationstests

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Schlüsselwörter: Afrikanisches Schweinepest-Virus; Rekombinase-Polymerase-Amplifikation; DNA-Extraktion; molekularer Nachweis

Einleitung: Das Afrikanische Schweinepest-Virus (ASPV) verursacht eine tödliche Viruserkrankung bei Schweinen. Dieses hat sich weltweit fortlaufend verbreitet und wurde im September 2020 erstmalig in Deutschland nachgewiesen. Der Ausbruch der Seuche kann schwere wirtschaftliche Verluste nach sich ziehen. Bis heute ist kein Impfstoff zugelassen, daher sind Überwachung der epidemiologischen Situation und der frühzeitige Erregernachweis unerlässlich für die Bekämpfung der Afrikanischen Schweinepest als Tierseuche. Die Polymerase-Kettenreaktion (PCR) gilt als Goldstandard für den Nachweis von ASPV und zeichnet sich durch eine hohe Sensitivität und Spezifität aus. Allerdings erfordert die PCR gut ausgestattete Testlabore und ist zeitintensiv. Point-of-Need-Tests können schnelle und zuverlässige Ergebnisse direkt vor Ort liefern und stellen somit eine Alternative zum Goldstandard PCR dar.

Ziel der Untersuchung: Ziel dieser Studie war es, einen Point-of-Need-Test zum Nachweis von ASPV zu entwickeln. Dieser beruht auf der Grundlage der Rekombinase-Polymerase-Amplifikation (RPA) und sollte vor Ort einsatzfähig sein.

Material und Methoden: Es wurden drei Primersätze und eine Sonde auf der Grundlage des B646L-Gens, welches für das virale Kapsidprotein p72 vom ASP-Virus kodiert, entwickelt. Alle möglichen Kombinationen wurden getestet. Die analytische Sensitivität wurde mit acht Wiederholungen von Verdünnungsreihen des molekularen Standards (10^2-10^0 DNA-Kopien pro µl) ermittelt. Die Nachweisgrenze wurde anhand einer Probit-Analyse dieser Durchläufe berechnet. Die Spezifität wurde mit verschiedenen viralen Nukleinsäuren von anderen das

Schwein infizierenden Erregern überprüft. Um den Test im Feld einsatzfähig zu gestalten, wurden mittels ASPV-RPA zwei verschiedene Extraktionsansätze mit allen 73 verfügbaren Schweineblutproben getestet: eine schnelle Hitze/Lysepuffer-Extraktionsmethode und ein standardisiertes Extraktionsverfahren auf Spin-Säule-Basis. Die diagnostische Sensitivität und Spezifität wurde für beide Testverfahren berechnet. Alle Ergebnisse wurden mit einer etablierten real-time PCR für ASPV verglichen. Eine kleine Pilotstudie zum Feldeinsatz des ASPV-RPA-Tests wurde in Uganda mit 20 Blutproben unter Verwendung des Kofferlabors durchgeführt.

Ergebnisse: Die berechnete Nachweisgrenze von ASPV-RPA lag bei 3,5 DNA-Kopien pro µl. Alle untersuchten ASPV-Genotypen wurden detektiert, aber keine anderen viralen Nukleinsäuren. Bei Verwendung der standardisierten DNA-Extraktionsmethode mit anschließender Durchführung der ASPV-RPA lag die diagnostische Sensitivität und Spezifität bei 100%, wie auch mittels der real-time PCR. Auch das schnelle Hitze-/Lysepuffer Protokoll zeigte vielversprechende Ergebnisse und erreichte eine Positivrate von 97% mittels ASPV-RPA im Vergleich zu 38% bei der PCR. In Uganda wurden elf ASPV-RPA-Proben als positiv erkannt, darunter zwei fieberfreie asymptomatische Tiere.

Schlussfolgerung: Der schnelle Erregernachweis stellt einen essenziellen Aspekt der ASP Seuchenbekämpfung dar. Die ASPV-RPA erwies sich als genauso empfindlich und spezifisch wie die Goldstandard-PCR zur Erregeridentifizierung. In Kombination mit dem Schritt der DNA-Extraktion durch Hitze/Lysepuffer benötigt der entwickelte Test etwa 25 Minuten von der Probenentnahme bis zum Ergebnis. Die Positivrate ist mit 97% vielversprechend, wobei die ASPV-RPA im Vergleich zur PCR eine höhere Toleranz gegenüber Inhibitoren aufwies. Wie die Pilot-Feldstudie in Uganda mit dem Kofferlabor zeigt, ist ASPV-RPA eine im Feld einsatzfähige Nachweismethode. Das Kofferlabor bedarf lediglich einer Grundausstattung und einer Solarbatterie. Somit stellt das Kofferlabor eine vielversprechende Diagnostikmethode dar, welche vor Ort in ressourcenarmen Umgebungen zum Nachweis des ASPV eingesetzt werden kann.

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8. Appendix

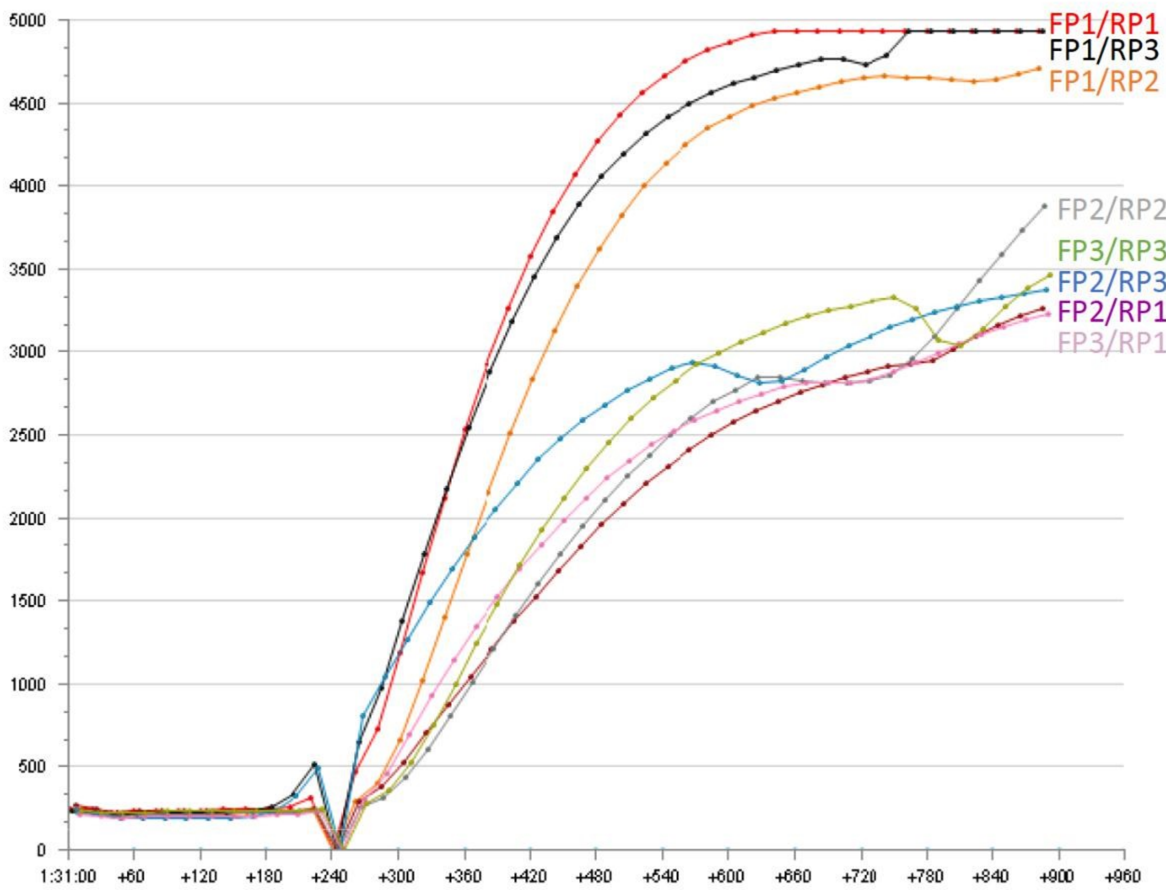

Figure S1. Possible primer combinations tested with $5*10^5$ ASFV molecular standard. FP1/RP3 had the fastest TT and maximal fluorescence signal.

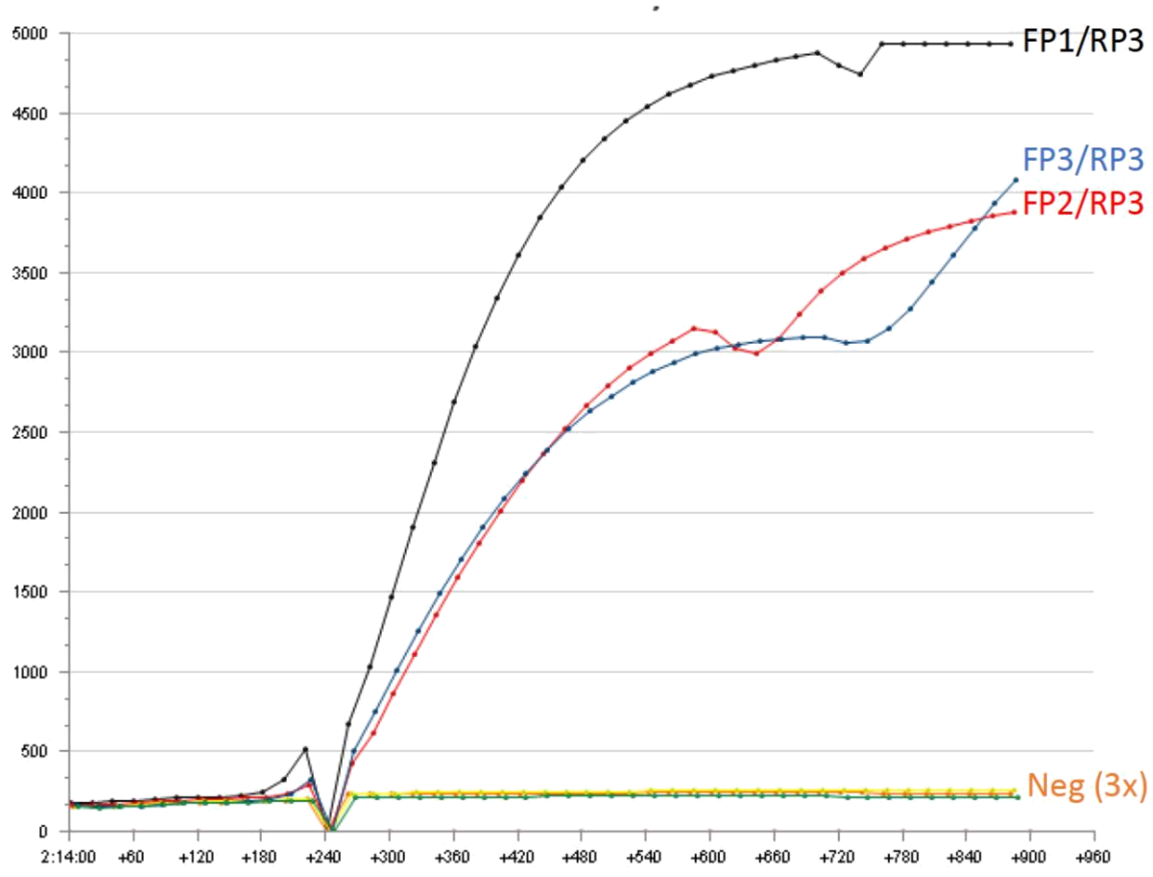

Figure S2. The three best primer combinations tested with $5*10^5$ ASFV molecular standard.

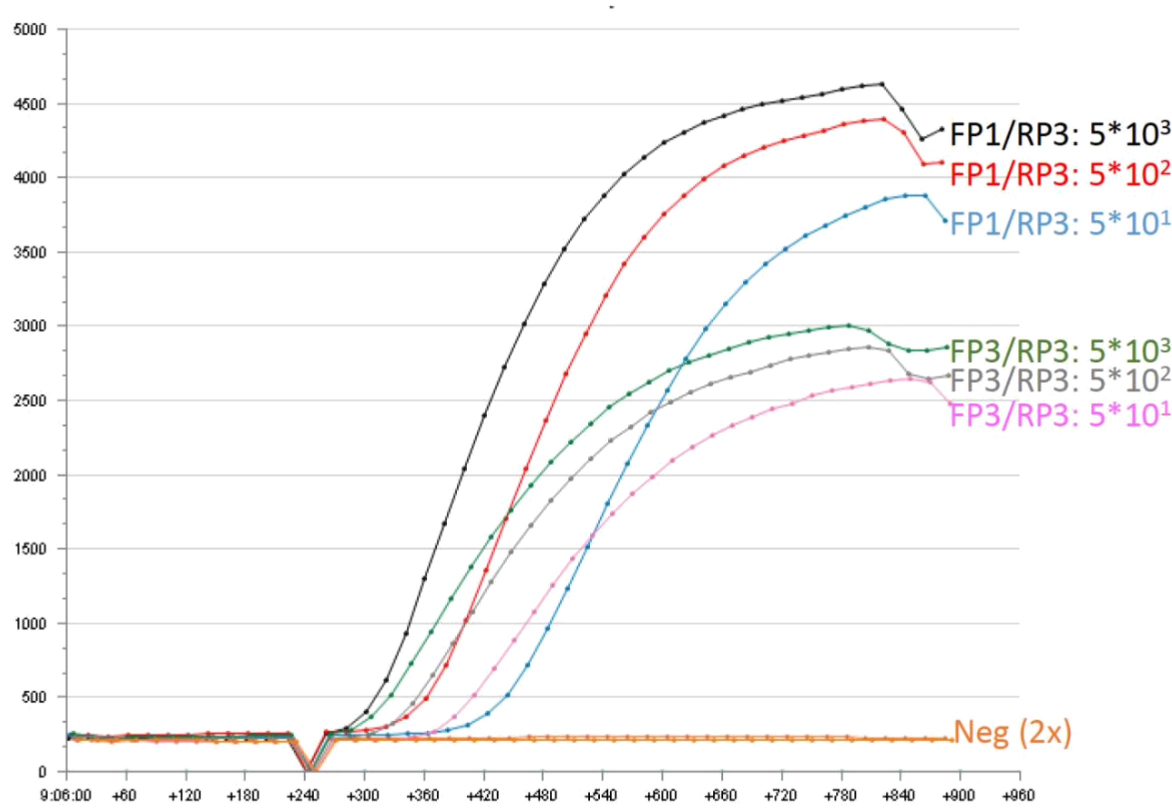

Figure S3. The two best primer combinations were tested with $5*10^{3-1}$ ASFV molecular standard. FP1/RP3 was confirmed to perform best.

Table S1. ASFV positive samples**.** ASFV Genotype and sample matrix were listed.

Sample ID	Matrix	Genotype	RPA	
			Result	TT (min)
1	Spleen	I	pos	2.35
2	Lung	I	pos	3.05
3	Macrophage culture	I	pos	3.01
4	Macrophage culture	I	pos	3.43
5	Spleen	II	pos	4.73
6	Lung	II	pos	4.76
7	Macrophage culture	II	pos	3.72
8	Blood	II	pos	4.73
9	Blood	II	pos	4.76
10	Blood	II	pos	3.35
11	Blood	II	pos	4.66
12	Blood	II	pos	3.06
13	Blood	II	pos	4.83
14	Blood	II	pos	4.76
15	Blood	II	pos	4.80
16	Blood	II	pos	4.81
17	Blood	II	pos	5.01
18	Blood	II	pos	3.05
19	Blood	II	pos	3.40
20	Blood	II	pos	3.43
21	Blood	II	pos	3.11
22	Blood	II	pos	3.48
23	Blood	II	pos	3.38
24	Blood	IV	pos	6.01
25	Blood	IV	pos	2.71
26	Blood	IV	pos	2.68
27	Blood	IV	pos	2.73
28	Blood	IV	pos	3.43
29	Spleen	IX	pos	3.12
30	Lung	IX	pos	3.48
31	Macrophage culture	IX	pos	3.73
32	Blood	XI	pos	4.76
33	Blood	XI	pos	4.78
34	Blood	XI	pos	4.81
35	Blood	XII	pos	4.45
36	Blood	XII	pos	4.48
37	Blood	XII	pos	5.01
38	Blood	XII	pos	4.71
39	Blood	XII	pos	3.40
40	Blood	XIII	pos	4.83
41	Blood	XIII	pos	4.86
42	Blood	XIII	pos	4.68
43	Blood	XIX	pos	4.76

44	Blood	XIX	pos	4.78
45	Blood	XIX	pos	4.81
46	Blood	XIX	pos	5.01
47	Blood	XIX	pos	5.05

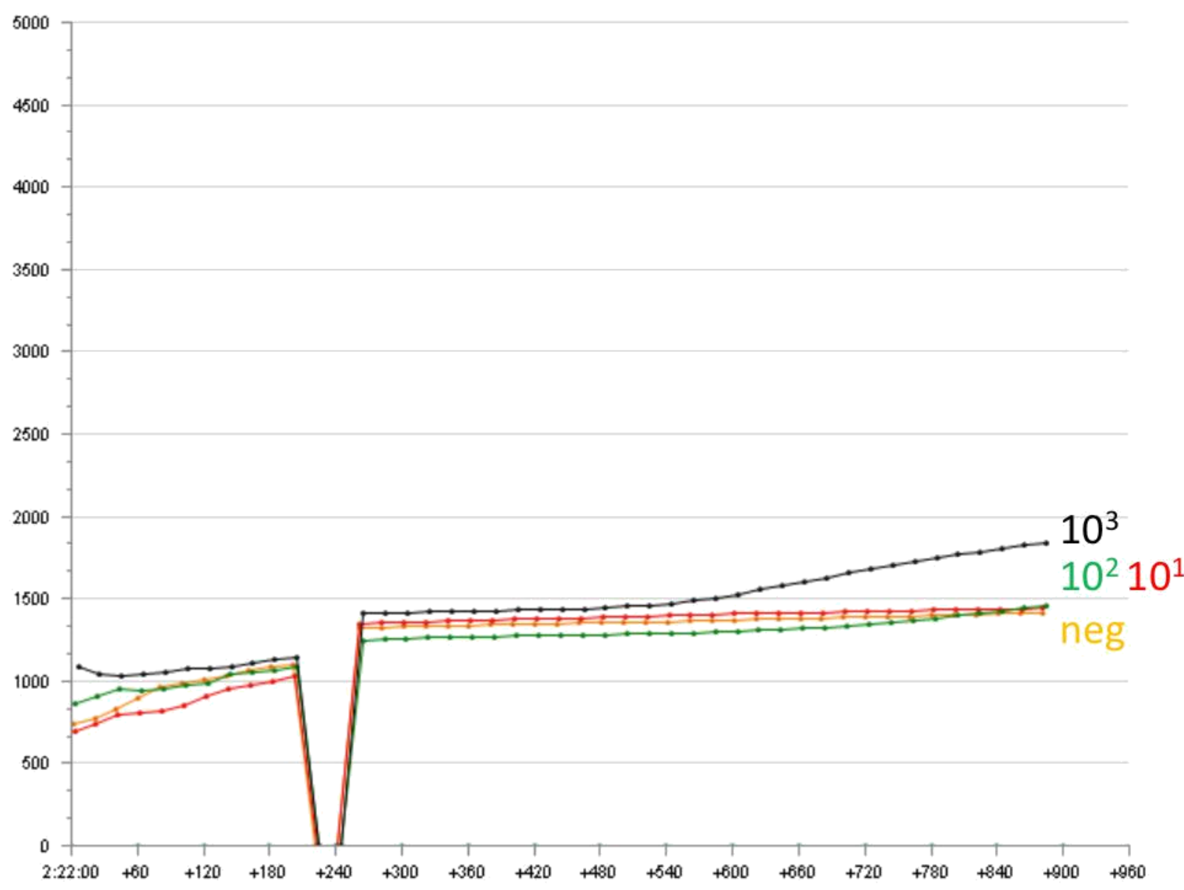

Figure S4. RPA assay using 10^{3-1} of the ASFV molecular standard based on Wang et al. 2020. Background was very high and only 10^3 showed a late weak signal.

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