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Abstract

African swine fever (ASF) is a severe haemorrhagic infectious disease affecting suids, thus representing a great economic concern. Considering the importance of the early diagnosis, rapid point of care testing (POCT) for ASF is highly demanded. In this work, we developed two strategies for the rapid onsite diagnosis of ASF, based on Lateral Flow Immunoassay (LFIA) and Recombinase Polymerase Amplification (RPA) techniques. The LFIA was a sandwich-type immunoassay exploiting a monoclonal antibody directed towards the p30 protein of the virus (Mab). The Mab was anchored onto the LFIA membrane to capture the ASFV and was also labelled with gold nanoparticles for staining the antibody-p30 complex. However, the use of the same antibody for capturing and as detector ligand showed a significant competitive effect for antigen binding, so required an experimental design to minimize reciprocal interference and maximize the response. The RPA assay, employing primers to the capsid protein p72 gene and an exonuclease III probe, was performed at 39°C. The limit of detection of the method was assessed using a plasmid encoding the target gene and resulted in 5 copy/µL. The new LFIA and RPA were applied for ASFV detection in the animal tissues usually analysed by conventional assays (i.e., real-time PCR), such as kidney, spleen, and lymph nodes. A simple and universal virus extraction protocol was applied for sample preparation, followed by DNA extraction and purification for the RPA. The LFIA only required the addition of 3% H2O2 to limit matrix interference and prevent false positive results. The two rapid methods (25 min and 15 min were needed to complete the analysis for RPA and LFIA, respectively) showed high diagnostic specificity (100%) and sensitivity (93% and 87% for LFIA and RPA, respectively) for samples with high viral load (Ct<27). False negative results were observed for samples with low viral load (Ct>28) and/or also containing specific antibodies to ASFV, which decreased antigen availability and were indicative of a chronic, poorly transmissible infection. The simple and rapid sample preparation and the diagnostic performance of the LFIA suggested its large practical applicability for POC diagnosis of ASF.

Introduction

African swine fever (ASF) is a severe haemorrhagic infectious disease affecting pigs and wild suids[1]. The disease is caused by African swine fever virus (ASFV), a large and complex double-stranded DNA virus of the genus *Asfivirus* within the *Asfarviridae* virus family[2]. ASFV widespread transmission is

due to the several modes of its transmission, including arthropod vector, soft ticks of the Ornithodoros genus in the sylvatic cycle, mainly in Africa, direct or indirect contact with contaminated, as well as inanimate fomites (e.g., clothes, transport vehicles, carcasses, etc...). The symptoms lead to a haemorrhagic fever, due to cytokine and arachidonic acid-caused impairment of the haemostasis, destruction of megakaryocytes, impaired thrombocytopoiesis, and severe lymphopenia, and is characterised by high mortality[2]. After the first record outbreak in Kenya in 1907, many others took place worldwide in the last century[3], especially in South Africa[4,5]. In Sardinia (Italy), for instance, where the first appearance was in 1978, the ASF become endemic and this had heavy economic consequences for the export of local porcine food products[6]. Ironically, since the rising demand for pork and concomitant increase in transboundary movements of pigs and pork products is likely to increase the risk of transmission and spread of ASF[5], researchers and pig industry must face both the origin and the symptoms of the problem at the same time. In 2015, Sardinia started an eradication strategy (EP-ASF-15-18),[7], which proven to be effective so that at the end of 2022 the Implementing Regulation (EU) 2022/2486 provided for the zoning of the Sardinia region, reclassifying a large part of its extension as free from ASF.. Recently, a new outbreak in Europe has appeared[8] and again we must face the emergency with an improved state of the art.

Currently, the ASF diagnosis is made by means of virus or antibodies detection tests[9] . As reported in the “ASF diagnosis workflow in case of ASF suspicion” section of the guidelines from the European Union Reference Laboratory (EURL) for ASF, Animal Health Research Centre (CISA-INIA), the PCR is by far the most sensitive method for the detection of the agent and the method of choice for first-line laboratory diagnosis at the EURL and national reference laboratories (NRL)[10]. EURL standard operating procedures (SOP) for ASF diagnosis include UNE-EN ISO/IEC 17025:2017 accredited genome detection methods (conventional, real-time and Taqman probe), antibody detection methods (ELISA, immunoblotting and immunoperoxidase), isolation, titration and identification by the haemadsorbing (HAD) test in swine primary cell culture (in swine peripheral blood monocytes and in porcine alveolar macrophages), growing and titration on established cell lines[10,11]. Nevertheless, laboratory testing requires expensive equipment, qualified personnel, relatively long time for sampling, delivering, and processing of the samples.

Considering ASFV is highly contagious and the socio-economic impact of the ASF outbreaks, rapid point of care testing (POCT) is recognised as a powerful tool for the control of the spread of the disease[12,13]. Along the years, rapid tests to diagnosis ASF have been also developed, in particular molecular[14] and antibody tests[15].

Some portable analytical devices have been developed for ASF molecular diagnosis, including CAS12a-mediated biosensors[9], pen-side thermocycler for real-time polymerase chain reaction[16,17], recombinase polymerase amplification (RPA)[18–21], and loop mediated isothermal amplification (LAMP)[9,22–24]. The detection of ASFV by LAMP showed concordance with results from real-time PCR but required four or more complex primers. The RPA developed by TwistDx (Cambridge, United Kingdom) is an isothermal DNA amplification technology that can be performed in the field due to its low resource requirements, allowing rapid and specific DNA amplification, diversified and simple readout. It employs the recombinase and its cofactor to bind with oligonucleotide primers and can be further improved by adding a sequence-specific fluorescent probe in search for homologous DNA.

Among the POCT techniques, the lateral flow immunoassay (LFIA) is by far the most popular for the diagnosis of infectious diseases, because of rapidity, simplicity, and the fact it is cheap and equipment-free. A typical LFIA includes several partially overlapping pads to guide the flow of the liquid sample in a defined direction. The sample is applied to the sample pad, flows through a

conjugate pad (where it redissolves the labelled reagent stored in dried form), and a nitrocellulose membrane (where the capture reagents are immobilised onto reactive zones, called test and control lines) towards the absorbent pad, which collects the liquid and promotes the flow. No external forces or energy are required to operate the device. The standard operating procedures (SOPs) by the EURL consider ASF diagnostic tools intended either as those directly targeting the virus and those able to reveal the serological response to ASFV. LFIA devices have been almost exclusively developed for serological testing of ASF, employing various signal reporters, such as quantum dots[25] or Eu-doped fluorescent microspheres for fluorescence[26] and Au nanoparticles[27] for visual detection, respectively. Antigenic rapid tests for ASF diagnosis have been developed also by some research groups[28–30]; though SPOs only accept molecular methods for the direct virus detection. Previously reported antigen tests are characterised by low sensitivity, as they showed positive response for high viral load (Ct<25) [30][31].

A major limitation of existing POCT for ASF diagnosis, both based on LFIA and RPA, is the applicability confined to whole blood or serum[32] , which collection becomes impracticable on carcasses and long dead animals. In fact, wild animals, typically, are found several days after the death due to ASFV and, tissues still available from the carcasses (bone marrows, spleen, kidney, and lymph nodes) are usually considered for applying reference diagnostic methods and are preferred matrices also for on field testing. Fortunately, these tissues also show high viral load[12,13]; however, their employment as samples required the development of an appropriate extraction method, which ideally should be suited for onsite application and, at the same time, compatible with requirement of sensitivity and specificity of the assays. As an example, the RPA developed by Miao et al [18] showed excellent sensitivity and a perfect agreement with RT-PCR on clinical samples; however, tissue homogenates were treated with formaldehyde, which hamper the on-field and user-friendly applicability of the method.

In this work, we describe the development of two rapid tests for the detection of ASF virus. A general and versatile sample preparation, which could be easily accomplished on field, was also developed for extracting the virus from tissues. As expected for so complex matrices, co-extracted components strongly affected results from both methods and required the investigation of appropriate strategies for mitigating their interference.

One method developed here is a molecular test based on RPA for the detection of a highly conserved region of the B646L gene, encoding the virus major capsid protein p72 and the other is an antigenic LFIA for the ASFV detection in the main target tissue. B646L gene is a commonly used target region for molecular detection and epidemiology studies by OIE Reference Laboratories and many commercial tests as well as research studies [33–35]since it was demonstrated to be highly conserved even in strains isolated in different parts of the world, including natural mutants recently identified in China[36,37]. RPA uses the *Escherichia coli* RecA, recombinase and single-strand DNA binding protein (SSB) for DNA denaturation instead of heat denaturation. Subsequently, the recombinase protein uvsX from T4-like bacteriophages binds to single stranded oligonucleotide primers in the presence of ATP and a crowding agent, forming a recombinase-primer complex. The complex then scans double stranded DNA seeking a homologous sequence and facilitate strand invasion by the primer at the cognate site and the formation of a D-loop structure. The complimentary strand is stabilized by SSB proteins, and the recombinase is disassembled from the nucleoprotein filament. A strand displacing DNA polymerase binds to the 3’ end of the primer to elongate it in the presence of dNTPs. Cyclic repetition of this process results in the exponential amplification in less than 30 minutes in a constant temperature within the range of 37 °C to 42 °C[38] (**Figure 1**). The detectable

amplification signal has been detected by real-time monitoring, so avoiding the need of off-line detection methods, such as through gel-electrophoresis or lateral flow dipstick, and reducing the assay time and operations, compared to previously reported approaches [29, 32].
The principle of the newly developed LFIA is a non-competitive, sandwich-type immunoassay, based on a monoclonal antibody (mAb) targeting the ASFV p30 antigen. Though most antigenic LFIA for ASFV detection target the p72 antigen [21,24,25], the p30 antigen has been reported as a highly antigenic protein[39], enabling the achievement of higher sensitivity for the detection of the ASFV by immunological methods[30,40]and was then chosen as the target antigen in this work. In addition, only one anti-p30 mAb was used for the capture and for the detection of the viral antigen (**Figure 2**). As the capture, the antibody was drawn to form the test line, while for the detection the mAb was adsorbed onto the surface of gold nanoparticles to produce a bright red coloured probe. The virus, contained in positive samples, reacted with the probe, and accumulated at the test line, resulting in an intense red colour line appearing. No signal was visible at the test line for negative samples, which does not contain the virus, as no antigen was available for bridging the immobilized and labelled mAbs. To confirm the validity of the assay, a bacterial ligand targeting immunoglobulins G was drawn at the control line, to bind the probe independently from the presence of the virus. In a previous work we observed that, despite the general approach for sandwich-type immunoassay is to use large excess of bioreagents to promote the formation of high number of immunocomplexes, when just one mAb is used, the competition among labelled and immobilized antibodies for binding to the same epitope of the antigen compromises the results[41,42]. Therefore, we applied a full factorial design of experiments to define the optimal amount of the detection antibody, which depends on the amount of the antibody molecules linked to the AuNPs and the amount of the probe (mAb-AuNP) applied to the single device.
The two devices were validated by analysing 234 PCR ASFV-assessed samples, comprising 115 ASFV-positive samples and 119 negative samples, as classified according to a reference laboratory-based molecular method. Samples were analysed in parallel by the two rapid methods, also to compare the performance of the two approaches for on-field analysis.

Materials and Methods

Materials

B646L gene sequences belonging from 38 ASFV strains were aligned using Geneious software (Geneious ver. 11.1.2). Highly conserved regions, without nucleotide mismatches among the different strains, were chosen for P72 primer design following Twist Amp exo assay design manual guidelines (www.twistdx.co.uk). The primers and the probe were synthesized by Eurogentec (Seraing, Belgium).
Plasmid bearing the whole synthetic gene encoding for p72 viral protein was obtained in a precise quantification by Eurofins (https://www.eurofins.it/). Viral DNA extracted from cell culture infected with the strain BA71V was gently provided by the National Reference Center for Pestiviruses and Asfiviruses of Istituto Zooprofilattico Sperimentale Umbria e Marche (CEREP). Infected culture supernatant, treated with 1% NP40 (IGEPAL CA-630), was also provided by CEREP and used in preliminary experiments to set up the LFD prototype. Gold (III) chloride trihydrate (ACS reagent), sucrose, tri-sodium citrate, sodium caseinate and bovine serum albumin (BSA) were obtained from

Sigma–Aldrich (St. Louis, MO, USA). The anti-p30 mAb and the IVC were provided by IZSLER. Tween20 and other chemicals were purchased from VWR International (Milan, Italy). Nitro-cellulose membranes with cellulose adsorbent pad and blood separator sample pads were purchased by MDI membrane technologies (Ambala, India) and glass fiber conjugate pads were obtained from Merck Millipore (Billerica, MA, USA). The monoclonal antibody used in this study is included in the biological bank of CEREP and derives from the supernatant of hybridoma cultures secreting antibodies to the p30 protein of the ASF virus. The antibody was purified with the Melon gel Monoclonal IgG purification kit (Thermofischer Scientific) according to the manufacturer's instructions. The purified IgG were quantified by the Bradford method.

Samples

Different set of archive positive samples were used. They included 22 EDTA-blood samples and 93 tissue samples (66 spleen, 22 kidneys, 2 lymph nodes, 1 gut, 1 bladder, 1 lung). The latter were collected during the 2014-2018 eradication campaign in Sardinia, where ASF is endemic since late seventies. The set comprises 53 samples from active surveillance in domestic pigs (mainly collected during outbreak or clinically suspicious animals), 30 from active surveillance from wild boar and free-ranging pigs and 10 from passive surveillance. Additional information on complementary tests were available, including direct (Haemadsorption test, fluorescent antibody test, antigen ELISA) and indirect (antibody ELISA, immunoblot) methods. The latter were introduced in wild boar active surveillance (hunted animals) during the final step of eradication program in Sardinia for surveillance purpose. Among the negative samples, 20 fresh EDTA-blood samples were collected from negative swine and 99 frozen wild boar tissues (spleen, kidney, and lymph node) were collected during the 2019 hunting season in Piedmont. All samples, but fresh blood, were stored at -20°C or -80°C. All samples were previously tested by conventional real time PCR at the time of sampling and classified as positive or negative accordingly. At the time of the study, samples were re-tested by real time PCR and the Ct values from the repeated RT-PCR were considered for comparison with the rapid tests. Samples were homogenised again, the DNA extracted and re-tested by real time PCR at the time of LFIA and RPA analysis. Briefly, tissue samples were homogenized in Phosphate Buffer Saline 10% w/v and centrifuged at 1000xg for 10', supernatant was use for DNA extraction. DNA extraction was performed from 200 µl of tissue homogenates using MagMax Core Kit (Thermo Fisher Scientific) in the automated nucleic acid purification system MagMax 96 (Thermo Fisher Scientific), according to the manufacturer's instructions. PCR assay was performed as already described[43,44] using 7500 Fast Real-Time PCR System (Applied Biosystems), the TaqMan Fast Advanced Master Mix (Applied Biosystems), 0.8µM of sense and anti-sense primers, 0.2µM of TaqMan probe in a total volume of 25µl containing 5µl of extracted DNA. The incubation profile was established as follows: 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 60 s, after an initial denaturation step at 95 °C for 10 min.

Tissue homogenates

The tissues were prepared according to a previously reported protocol[41] and the homogenates were used for RPA and LFIA. In details, 0.2g of sample was grinded for 2 minutes and extracted with 1 mL of the tissue running buffer (Tris-glycine 0.12M supplemented with 0.25% w/v casein, 1% w/v BSA, 1% v/v Tween20, and 0.02% v/w NaN_3 pH 8.2).

Detection of ASFV in target tissues by RPA

91 positive and 50 negative tissue samples were homogenized as described above and analyzed by the RPA. The Axxin T8-ISO (Axxin, Fairfield, Australia) for molecular diagnostic isothermal assay was employed in the study. DNA extraction and purification were carried out as described for the preparation of samples for RT-PCR. ASFV-RPA assay was performed in a 50µL volume using the TwistAmp exo kit (TwistDx, Cambridge, United Kingdom). The reaction mixture included 29.5µL rehydration buffer, 5µL extracted DNA template, 4.8µl primers/probe mix (2.1µL 10µM forward primer, 2.1µL a0µM reverse primer, 0.6µL 10µM probe), 10.7µL of 65mM magnesium acetate. All reagents, except for the viral template and magnesium acetate, were prepared in a master mix and dispensed into the 0.2 mL tubes containing the dried enzyme pellet. Five microliters of viral DNA were then added to each tube. Subsequently, magnesium acetate was pipetted into each tube lid, the lids were carefully closed, and briefly vortexed. The tubes were immediately placed in a T-8 fluorometer device (Axxin, Fairfield, Australia) to start the reaction at 39 ◦C for 25 min with a mixing step after 4 min of amplification. The ROX fluorescence signal was recorded in real-time every 20 seconds. The output signal was reported in millivolt (mV). To identify the detection limit of the real-time RPA assay, serial dilutions of the plasmid bearing the protein capsid p72 gene were prepared to achieve DNA concentrations in terms of copy number per µl, starting from 10^5 to 2,5 copy number per microliter. A volume of 5µL of each DNA dilution was used as template. Threshold time was plotted against the corresponding amount of DNA detected. The decision algorithm was set considering the main isothermal amplification parameters (initial average, gradient and amplitude parameters).

LFIA development and Strip preparation

The LFIA device was composed of a nitrocellulose membrane, layered with sample, conjugate and adsorbed pads, The anti-p30 antibody (1.0 mg/mL) and SpG (0.5 mg/mL diluted in 0.02M phosphate buffer pH7.4) were drawn onto the nitrocellulose membrane at 1 µL/cm by means of an XYZ3050 platform (Biodot, Irvine, CA, USA) to form the test (TL) and control (CL) lines, respectively. The conjugate pad was pre-adsorbed with the “storage” buffer (ESI) and dried at 60 °C for 1 h. Subsequently, it was dipped into the probe solution diluted in the storage buffer until complete saturation. Then, it was dried at room temperature for 2 h. The membranes were dried at 37 °C for 60 min under vacuum, layered with sample, conjugate, and adsorbent pads, inserted into plastic cassettes (Kinbio, Shanghai, China). To define the probe amount and characteristics, a full factorial experimental design (FF DoE) was used. In details, the optimal amount of the anti-p30 mAb to be adsorbed to a mL of AuNP (OD1), the probe amount (measured as the OD of anti-p30_AuNP conjugate to be applied to the conjugate pad) and the size of AuNPs, were investigated. Previously, the stabilizing amount of antibody needed to prevent AuNP aggregation (titre, T) was defined by the salt-induced aggregation test (**Figure S1**) and was measured to be 8 ug per mL of AuNP with optical density equal to 1. The FF DoE explored 3 levels of AuNP size, 3 levels of optical density, and 5 levels of mAb-to-AuNP ratios; therefore, nine anti-p30_AuNP conjugates were prepared (details are reported in the ESI) and characterized by the visible spectra in the wavelength range 400–700 nm. Spectra were acquired by using an Agilent Cary 60 (Palo Alto, CA, USA) spectrophotometer ((SBW 0.5 nm, rate 900 nm/min) and were reported in **Figure S2**. Each anti-p30-AuNp conjugate was diluted to different OD values and applied to the LFIA strip as above. Two experiments were conducted for each probe by applying solutions of negative and positive controls (see below) in duplicate. In summary, a total of 180 experiments were conducted (**Table 2**). The mean colour intensity of the test line was measured and used as the parameter for the decision. The intensity of the test line was quantified by acquiring strip images with a scanner (OpticSlim 550 scanner, Plustek Technology GmbH,

Norderstedt, Germany), and the area of the coloured lines was quantified by means of QuantiScan 3.0 software (Biosoft, Cambridge, UK). The positive control used to develop and optimize the assay was an inactivated viral culture (IVC) diluted 1+1 with the running buffer. The inactivation was carried on a viral culture with 5 % of IGEPAL CA-630. As the negative control the running buffer was used. The formulation of the running buffer was Tris-glycine 0.12M supplemented with 0.25% w/v casein, 1% w/v BSA, 1% v/v Tween20, and 0.02% v/w NaN_3 pH 8.2.

Detection of ASFV in EDTA-blood and target tissues by LFIA

Blood samples were diluted 1:10 in blood dilution buffer (Tris-glycine 0.12M supplemented with 0.5% w/v casein, 1% w/v BSA, 1% v/v Tween20, and 0.02% v/w NaN_3 pH 8.2) and 3 drops (ca 100µl) added to sample well. The background was sufficiently reduced to allow for interpreting the result by visual observation. Homogenates from lymph node and spleen tissues showed a significant false positive rate, when analysed by the LFIA. Therefore, different modifications of the running buffer were investigated, to the aim of reducing the interference. In particular, the following additives were evaluated: in proteins (2% w/v casein, 2% w/v BSA), surfactants (5% v/v Tween20,0.5% v/v TritonX-100), viral inactivators (5% v/v IGEPAL CA-630), and oxidisers (3% v/v H_2O_2). Finally, all positive and negative tissues were extracted as described above. Then, 3 drops (ca 100uL) of a 30% v/v of H_2O_2 were added and the mixture was immediately analysed by the LF device. Approximately 100 µL of the mixture was applied to the sample well of the LF device and the result was visually evaluated after 15 and 30 minutes from sample applications. Samples were classified according to the appearance of a colour at the test line, as coherently judged by at least two operators.

Statistical analysis

Concordance between the two rapid methods was evaluated in a sub-set of 50 negative samples and 89 positive samples by Cohen's Kappa. Concordance, Specificity and Sensitivity were calculated for each method using the conventional RT-PCR as the reference method.

LFIA device stability

Cassettes prepared as described above were stored in the dark in plastic bags containing silica. To study their shelf-life, LFIA devices were stored at room temperature for three months (real-time stability) and at 37°C (accelerated ageing)[45], after which the positive and negative controls were analysed in duplicate.

Results and Discussion

Development of the RPA

Primers and probe for the RPA were designed to amplify a 220 bp segment of the major capsid protein p72. The B646L gene of ASFV-BA71V strain (ASU18466) was compared with twenty other ASFV reference strains (GenBank accession No. AY261360, AY261361, AY261362, AY261364, FN557520, LR536725, LR899193, MN270969, MN270970, MN270971, MN270972, MN270973, MN270974, MN630494, MT180393, MW396979, NC_044946, NC_044947, NC_044949, NC_044955) using Muscle v5[46] to screen out conserved fragments. Two highly conserved regions were identified, and a pilot study was conducted on different primers/probe panels in order to choose the

best performing time/temperature parameters and primer set (data not shown). Selected primers and probe are shown in **Table 1**. The Axxin T8-ISO software enables the use of three possible algorithm pass criteria to determine results: i) the initial average, which is used to test that the assay starts correctly as it verifies that the assay started with a low value and only subsequently rises higher ; ii) the gradient, which is based on the slope of the assay curve; and iii) the amplitude, which verifies whether the assay curve rises above a threshold value. The users should define the algorithm (or a combination of algorithms) and the parameters to determine pass criteria. According to manufacturer's instruction, the initial average criterium was set to 150 mV and the amplitude criterium to determine the positive / negative results was established at 1000mV. By using a serially diluted plasmid bearing the target gene, we observed that 5 copy number/µL of the plasmid displayed a fluorescence curve exceeding the 1000mV threshold, while further diluting the plasmid to 2.5 copy number/ µL the signal dropped to the background level (**Figure S3**). Therefore, we considered 5 copy number/ µL as the limit of detection of the method and confirmed the suggested threshold.

The RPA was evaluated on 50 negative samples, and the maximum amplitude registered was below 100mV, with one exception reaching 279mV, well below the established threshold (**Figure S4**)

Among positive samples, 91 were analyzed by RPA and 58 showed amplitude larger than 1000mV and were determined as positives. As far as threshold cycle in RT-PCR is concerned, with few exceptions, samples with a Ct value <25 were mainly positive in RPA assay (Se 96%), while for Ct value between 25 and 35, the number of RPA positive samples decreased. One option of the Axxin c allows an algorithm to be tested on an existing set of data acquired on the T8 instrument and to define a new threshold. This avoids having to re-run a real world test every time the algorithm is tweaked. According to results obtained for negative samples, the amplitude criterium could be modified and possibly the threshold decreased below 1000mV to reach a greater sensitivity. However, this option was not explored yet.

In this context, RPA has successfully been used for different kinds of target organisms with diverse sample types[47] as well as in the presence of known PCR inhibitors, such as haemoglobin, ethanol, and [48,49]

DoE for the development of the ASFV-LFIA device

The availability of one single mAb requires us to perform a full factorial experimental design to optimise the analytical signal[41,42] (colour). The levels of the anti-p30 mAb to be adsorbed to AuNP were defined as 2, 4, 8, 12 and 16 µg/mL$_{OD1}$. The levels of the amount of the probe to be applied to the conjugate pad considered in the study were OD 2, 3, and 4, and the sizes of AuNPs were 27, 32, and 36nm. Two experiments were conducted for each probe by applying solution of negative and positive controls (see below). In summary, a total of 45 experiments (1 experiment = 2 replicates Infected culture supernatant + 2 replicates for the tissue running buffer alone) were conducted (**Table 2**). The mean intensity of the test line was measured and used as the parameter for the decision. The positive control used to develop and optimize the assay was an infected culture supernatant that was diluted 2-fold with the tissue running buffer. The inactivation was carried with 5 % of NP40 as the negative control the tissue running buffer was used. The formulation of the tissue running buffer was Tris-glycine 0.12M supplemented with 0.25% w/v casein, 1% w/v BSA, 1% v/v Tween20, and 0.02% v/w NaN_3 pH 8.2. As explained above, we explored 3 levels of optical density, 3 levels of size of the AuNP used as the signal reporter and 5 levels of mAb-to-AuNP ratio (T factor).

The levels are reported in the **Table 2** and the intensities of the signals are reported in the **SI, Table S1**.

The results of the FF-DoE confirmed the presence of a competition between the detection and the capture antibodies for the same epitopes of the virus. The competition explained the decrease of the signal with the increase of the antibody quantity, both in terms of amount of antibody adsorbed per Au nanoparticle, and in terms of quantity of probes. The size of the gold nanoparticles influenced the shapes of the intensity curves, with a more gradual decrease as a function of the mAb-to-AuNP ratio, for the 36nm- compared to the 32nm-, and even more to the 27nm AuNPs suggesting a combined effect of the mAb-to-AuNP ratio and AuNP dimension (**Table S1**, **Figure S5**). A possible explanation could be that the lower size of the nanoparticles exasperated the saturation effect, while the 36nm AuNPs may not saturate the antigen surface due to steric hindrance. As an alternative, or in combination with the effect described above, the higher number of small mAb-AuNP could increase the probability of interaction with the antigen compared to the lower number of large nanoparticles. Interestingly, the conjugates with 0.25xT anti-p30 mAb have a peculiar behaviour showing the highest signal intensities in each combination, lower proneness to saturation, and lower colour decrease with the optical density. Among the combinations including 0.25xT the one obtained with the 32nm AuNPs appears to suffer less the signal decrease as a function of the optical density. Therefore, we considered the 0.25x T, with 32-nm-diameter AuNPs and an optical density of 2 as the optimal combination and used this probe for the development of the LFIA device.

Detection of ASFV in blood and tissue samples by LFIA

The LFIA was applied to detect ASFV in 42 EDTA-blood samples (22 ASFV positive, 20 negative). As expected, the frozen positive blood samples were highly haemolytic upon storage, giving rise to a high background on the LFD. Few samples were treated with HemogloBind reagent (BSG, NJ USA) to reduce background, however the increased dilution of samples due to the protocol suggested by the manufacturer resulted in a decrease of the signal and a general reduction of sensitivity. So, this option was not further investigated. We then simply diluted the samples 1:10 with the blood dilution buffer and this allowed for data collection without interference from haemolysis; however, at the expenses of the assay sensitivity (Se), which resulted to be limited. Among 22 positive blood samples, 13 were found positive (Se 59%). Sensitivity was higher for samples with a RT-PCR Ct value <30 (100%), slight lower for samples with Ct ranging from 30 and 35 (67%) and negligible for Ct value >35 (42%). All negative bloods were negative by LFIA.

The first analysed tissues belonging to freshly hunted wild boars (7 lymph nodes and 7 spleens) and assessed as ASFV-negative by RT-PCR, showed false positive results (**Figure S6a**). Lymph nodes and spleen are part of the immune system and contain structures able to bind to immunoglobulins, such as monocytes and complement factors, in a particularly relevant quantity. We hypothesised that these structures may act as bridges between immunoglobulins adsorbed onto AuNP and those immobilized onto the membrane to form the Test line, so resulting in false positive result. Interestingly, the effect was not observed in long-term frozen samples (**Figure S6b**). The presence of false positivity can be attributed to several non-specific interactions, such as electrostatic interactions, protein-protein interactions, and so on, or can be due to specific components (anti-antibodies or macrophages) able to bind the Fc of the capture or/and detection antibodies. Common strategies to overcome this problem, which is often observed in immunoassay, is the addition of surfactants and/or proteins to the sample diluent. Accordingly, we produced four modified sample diluents, which formulation was obtained doubling the concentration of: the surfactant, BSA, casein, all additives. No one of these solutions was effective (**Figure S7**).

As the same samples were tested several times, we noticed that sometimes of freezing reduced the false positivity even with the conventional diluent (**Figure S8**). These experiments strengthened the hypothesis that the false positivity was due to the reactivity of some cellular or macromolecular structure (i.e., macrophages or Ca complement protein)[50] able to bind to the antibodies. Therefore, another set of sample diluents was prepared with the aim of promoting cell lysis or disrupting the tetrameric structure of Ca protein. A hypotonic buffer to promote osmotic shock, and formulations supplemented with concentrated surfactants (5% Tween20, 0.5% of Triton X-100, and 0.5% of IGEPAL CA-630) were prepared to cause macrophage and dendritic cell lysis. We also attempted to disrupt Ca protein structure by using a Ca-chelating agent (5mM of EDTA) and by treating with 3% of H_2O_2 (as a di-sulphide bridge and glycosyl oxidising agent)[51]. The buffers with 5xTween20, and TritonX-100 were completely ineffective, while a reduction of the false positive signals was obtained with the hypotonic buffer, and buffers supplemented with IGEPAL CA-630, EDTA, and H_2O_2 (**Figure S9**). However, the only diluent that completely extinguished the false positive signals and which did not compromise the sensitivity, as ascertained by the signal measured for the positive control, was the diluent with 3% of H_2O_2. The reaction of H_2O_2 was clearly visible as oxygen gas produced upon addition to the sample extracts and suggested the interfering substances were susceptible to oxidative disruption, while the components of the LFIA were not.

We applied the treatment with hydrogen peroxide to all previously tested samples and confirmed that non false positive results were observed. Despite spleen and lymph nodes tissue are unconventional matrices for LFIA analysis, and that their composition and content is very peculiar, the interference of complement proteins or other protein structures able to bind to immunoglobulins and mimic the sandwich formation in the absence of the specific antigen can affect in a general way the performance of LFIA in various biological matrices. The effectiveness of the treatment with H_2O_2 suggests it as a possible approach for neutralizing them. Considering the high reactivity of hydrogen peroxide we opted for prudentially adding it just before applying the extract to the LFIA device. Therefore, the preparation of the sample was established as follows: homogenization of the tissue (ca. 150-250 mg) with a mL of the buffer, sedimentation of the particulate material, addition of 0.1 mL of a 30% solution of hydrogen peroxide, transfer of about 0.1 mL of the extract to the sample well of the LFIA device for the analysis. As a confirmation of these explanation, the 21 negative kidneys did not show any false positive results even without the addition of hydrogen peroxide. In fact, the presence of the interfering substances in kidney is negligible.

Validation of the rapid tests for ASFV detection in target tissues of pigs and boars

A total of 192 tissues (93 ASFV positive and 99 negative) were analysed by the two methods. The 93 positive samples were different types of tissue (66 spleens, 22 kidneys, 2 lymph nodes, 1 gut, 1 bladder, 1 lung), as well as the 99 negatives (38 spleens, 21 kidneys, 40 lymph nodes). The collection of positive samples included 5 tissues samples belonging to boars and 5 belonging to wild pigs found dead because of ASF infection (passive surveillance), 53 tissues from domestic pigs (active surveillance on domestic animals), and 30 from wild boars and free ranging pigs (13 and 17 samples, respectively, as active surveillance on wild animals). Different specimens were collected from each animal.

The LFIA specificity was evaluated on 38 lymph node, 40 spleen and 21 kidney negative samples. All these negative samples were correctly classified by the LFIA (Specificity 100%), confirming that the pre-treatment based on the addition of 3% of hydrogen peroxide was effective to oxidise the interferences of some of these matrices (lymph nodes and spleens). RPA specificity was measured by analysing 50 negative tissue samples and turned to be 100%, as no false positive result was obtained.

The 93 ASF-positive tissues collected during the 2014-2018 eradication campaign in Sardinia were analysed by LFIA and 55 were found positive (Se 59.1%). The same samples (with two exceptions) were also analysed by the RPA and 58 were identified as positive (Se 63.7%). Results for the clinical validation of the two rapid tests are shown in **Table S2** and summarized in **Table 3.** Examples of typical results are shown in **Figure 3.**

For both analytical approaches, the sensitivity was clearly dependent on RT-PCR Ct values (**Figure 4**) either the RPA or the LFIA showed sensitivity above 90%. It should be noticed that most of the samples with high Ct values were haemolytic or dehydrated, so the antigenic material was likely very degraded while the genetic material could still be detected. On the contrary the sensitivity of the two tests overlapped for samples with Ct values between 13 and 30, which included most well-preserved tissues. Nonetheless, the RPA test demonstrated higher sensitivity than LFIA for samples with Ct>30, as expected for low viral load samples. A slight improvement of LFIA sensitivity was obtained by reading the results after 30' instead of 15' (Se 63.4%) (**Table S3, Figure S10**), however at the expenses of rapidity. We opted for considering results judged after 15 min from sample application for the evaluation of the LFIA performance. As reported in **Table 3**, among the different categories of samples, the greatest sensitivity was obtained in those from passive surveillance (RPA and LFIA: 9/10), followed by those from active surveillance of domestic pigs (38/52 and 44/53 for RPA and LFIA respectively), while samples from active surveillance of free-ranging pigs and wild boars were mostly detected as false negative by the LFIA (26/28). A little better went for the RPA assessment, which classified as false negative 18/29 samples from the last category. Again, the observation of the LFIA result after 30' instead of 15' slightly improved the performance, i.e.: the rate of true positiveness increased for the passive surveillance (10/10) and for active surveillance of domestic animals (47/53) (**Table S2**).

One-way ANOVA statistical analysis on the samples confirms the Normality of distribution of the passive, active wild, and active farm subset of populations in terms of Ct values according to Shapiro Wilk test (P=0.228, equal variance P=0.095). Performing the Holm-Sidak method for the pairwise multiple comparison (alpha overall significance level 0.05), no significantly different populations (in terms of mean and median values) was observed between passive wild and active farm subsets (P:0.526), while the active wild subsets resulted significantly different on respect to passive (P: 0.004) or active farm (P<0.001). This aspect is more evident observing the boxplot analysis (**Figure S11**). Considering the two populations as representative, we can consider the sensitivity of the two tests on respect two cut-off levels measured as the limit of the boxplot at the C.I. of 95%, Ct=27 (**Table S4**). RPA showed 87% of sensitivity for Ct≤27, while the LFIA showed 93% on the same population. Different behaviour on the active wild population could then be correlated with this statistical difference on respect to the other two populations. Most interestingly, 44 samples among the 81 belonging to the active surveillance sets resulted to contain antibodies towards ASFV, as ascertained by serological assays. Among the 38 samples that resulted as false negatives by the LFIA, 30 showed detectable levels of anti-AFSV antibodies positives, 3 were not tested for the presence of specific antibodies and 5 were negative to serological assays. Therefore, most seropositive samples (30/44) were not reactive in the LFIA, suggesting a chronic infection with a low viral load. Moreover, the presence of endogenous antibodies to ASFV could inhibit the binding of the ASFV antigen by the anti-p30 mAb, because of competition for the same epitope or because of a masking effect, thus lowering the analytical sensitivity of the LFIA.

Considering the different tissues analysed, the LFIA showed highest sensitivity in kidneys (21/22), followed by other tissues (2/2 lymph nodes, 1/1 bladder, 1/1 gut, 0/1 lung), while spleen samples provided a lower sensitivity (30/66). Furthermore, the sensitivity increased up to 100% in in kidneys

(21/21) and other tissues (4/4) and to 85.7% in spleens (3035) when samples with ascertained seropositivity and samples not characterized by serological methods (and thus possibly seropositive) were excluded from the analysis. In fact, 27 out of the 36 false negative spleens were ascertained to contain endogenous anti-ASFV antibodies and 2 were not tested for antibodies. Finally, 1 out of the 5 remaining false negative spleen samples turned to be positive when the LFIA result was observed at 30' (**Table S3**). The overall sensitivity was 59.1% (CI95% 49.1-69.1) and 63.7% (CI95% 53.8-73.6) for the LFIA (n=93) and RPA (n=91), respectively. Excluding the seropositive samples, the sensitivity of the LFIA remarkably increases to 91.7% (CI95% 84.7-98.7) by 15' readout, and to 92.2% (CI95% 85.6-98.8) when the result was observed after 30' (**Table S3)**, and so the k-values and agreement as well (**Table S5)**. Concerning the categories of samples, both rapid tests gave the best performance in samples belonging to passive surveillance (positive animals found dead), which correspond to an expected very high viral load in the main target tissues. Samples from active surveillance of domestic pigs were mainly collected from herds with an active outbreak or from animals with suspected clinical signs. It is therefore expected a good performance as well, albeit slight lower. Active surveillance in wild boar or free ranging pig were conducted during hunting activity or slaughtering. Presence of antibody positive animals in these subsets is a distinctive feature of endemic ASF in Sardinia and likely correspond to a chronic infection with very low viral load in the target tissues. Performances of both rapid tests were unsatisfactory in these categories, with the LFIA more prone to the presence of endogenous antibodies. The RPA test maintained a certain degree of reactivity with samples with higher Ct values, but the presence of interfering substances probably reduces the overall expected potential, at least based on number of target copies as determined using plasmid DNA. Retesting a subset of tissues with high viral load and preliminarily negative to RPA turned positive when an alternative extraction method was used (Qiagen DNeasy blood and tissue) (data not shown).

The concordance among the rapid methods and the reference conventional RT-PCR was estimated by the Cohen's kappa score and as the percentage of agreement (rate of concordant results) including negative and positive tissue samples (**Table 4**). The overall concordance among methods was moderate for both the RPA and LFIA, with a slight lower agreement among RPA and conventional RT-PCR than from LFIA and the reference method, explainable by the higher specificity of the LFIA. Moreover, when excluding samples with ascertained presence of anti-ASFV antibodies or which were not characterized by serological methods (thus could contain antibodies) the concordance among the LFIA and the RT-PCR was almost perfect, suggesting again that the LFIA was mainly suitable to detect the virus in animals with a severe disease, not seroconverted.

Stability of the LFIA device

The long-term stability of the devices, preferably without temperature control, is of utmost importance for their on-field applicability. Therefore, we studied the stability of the LFIA response over time. To this aim, two experiments were conducted: an accelerated stability assessment (7 days at 37°C) and a real stability assessment (3 months at room temperature). The devices were tested by using the positive and negative controls at the day 0 (just produced) and after storage. No false positive results occurred when analysing the negative control and consistent colour signals, measured at the test line, were recorded after three months of storage at room temperature or because of the thermal stress (**Figure S12**). We concluded that the LFIA device was acceptably stable over time without requiring a specific temperature of storage and that it was insensitive to (limited) thermal stress, which is particularly useful for envisaging its on-field application.

Conclusions

In conclusion this work showed that the two rapid methods are both valid as powerful tools for the on-field monitoring of the ASF infection. The RPA method, though slightly more sensitive for high Ct values, requires several pre-treatment steps and the use of a portable equipment, which still limit its practical applicability for on-field diagnosis and increase cost analysis. The antigen LFIA was easily adapted for on-field application thanks to the fact that it requires just a simple and practical preparation of samples. As expected, the best performances were found in samples from passive surveillance and samples with high viral load Ct values <27. However, due to limited number of tissues available in this subset, a more detailed study will be necessary to confirm these findings.

Author Contributions

S.C.: Investigation, Visualization, Writing-Original Draft, Writing-Review and Editing. B.C.: Investigation, Visualization, Writing-Original Draft, Writing-Review and Editing. G.M.D.M.: Supervision. F.F.: Supervision. S.D.G.: Validation. L.A.: Conceptualization, Supervision, Resources, Writing-Review and Editing. S.R.: Conceptualization, Supervision, Resources, Writing-Review and Editing. C.B.: Supervision. P.P.A.: Validation D.S.: Validation. F.D.E.: Validation. A.S.: Validation. A.O.: Validation. F.D.N.: Methodology, Formal Analysis. T.S.: Formal Analysis. V.T.: Formal Analysis, M.C.: Formal Analysis. All authors have read and agreed to the published version of the manuscript.

Electronic Supplementary Information (ESI) available:

Probe preparation, Full-factorial design optimizing the probe, Table S1: results of the FF-DoE, Table S2-3-4: Sensitivity and k-Cohen of LFIA after 30', Figure S1: Salt-induced aggregation test, Figure S2: Visible spectra of AuNPs and conjugates of the FF-DoE, Figure S3-4: sensitivity and boxplot analysis of RPA, Figure S5-8: false positive cleaning process, Figure S9: RPA-LFIA 30' comparison, Figure S10: boxplot analysis of sample populations, Figure S11: Stability

Conflicts of interest

There are no conflicts to declare.

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