Lateral flow assays for the detection of African swine fever virus antigen are not fit for field diagnosis of wild boar carcasses

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Abstract

African swine fever (ASF) is one of the most important viral diseases of domestic pigs and wild boar. Apart from endemic cycles in Africa, ASF is now continuously spreading in Europe and Asia. As ASF leads to severe but unspecific clinical signs and high lethality, early pathogen detection is of utmost importance. Recently, "point-of-care" (POC) tests have been intensively discussed for the use in remote areas but also in the context of on-farm epidemiological investigations and wild boar carcass screening. Along these lines, the INGEZIM ASFV CROM Ag lateral flow assay (Eurofins Technologies Ingenasa) promises virus antigen detection under field conditions within minutes. In the present study, we evaluated the performance of the assay with selected high-quality reference blood samples, and also with real field samples from wild boar carcasses in different stages of decay from the ongoing ASF outbreak in Germany. While we observed a sensitivity of roughly 77% in freeze-thawed matrices of close to ideal quality, our approach to simulate field conditions in direct carcass testing without any modification resulted in a drastically reduced sensitivity of only 12.5%. Freeze thawing increased the sensitivity to roughly 44% which mirrored the overall sensitivity of 49% in the total data set of carcass samples. A diagnostic specificity of 100% was observed. However, most of the German ASF cases in wild boar would have been missed using the lateral flow assay (LFA) alone. Therefore, the antigen-specific LFA should not be regarded as a substitute for any OIE listed diagnostic method and has very limited use for carcass testing at the point of care. For optimized LFA antigen tests, the sensitivity with field samples must be significantly increased. An improved sensitivity is seen with freeze-thawed samples, which may indicate problems in the accessibility of ASFV antigen.

Introduction

African swine fever (ASF) is caused by African swine fever virus (ASFV), a large double-stranded DNA virus, and the sole member of the genus *Asfivirus* within the *Asfarviridae* family (Alonso et al., 2018). African swine fever usually causes an exceptionally high lethality in domestic pigs and Eurasian wild boar and is a notifiable disease according to the World Organization for Animal Health (OIE). Following its introduction to Georgia in 2007, ASFV spread successively through neighboring countries in the Trans-Caucasian region to several parts of Europe and Asia (Dixon, Stahl, Jori, Vial, & Pfeiffer, 2020). Since the virus reached China in 2018 (Zhou et al., 2018), millions of pigs were culled and effects on the global pork market were severe. First cases of ASF in Germany in 2020 (Sauter-Louis et al., 2020) sent another shockwave through the industry, as trade restrictions on pork took hold even as only wild boar are affected until now. With neither treatment nor a licensed vaccine available to date, strategies to fight the disease have to rely solely on strict sanitary measures, an early and effective diagnosis and the culling of affected herds (Blome, Franzke, & Beer, 2020). For the wild boar situation, fencing, adapted hunting and hunting rest practices, trapping, incentives for carcass search and removal, as well as a general reduction of the wild boar populations have been implemented (Busch et al., 2021; Chenais et al., 2019; EFSA et al., 2018).

The OIE lists conventional and real-time PCR assays, virus isolation and fluorescent antibody tests as proven and reliable diagnostic methods for the detection of virus genome or antigen (OIE, 2019). However, these

methods are rather time consuming and require laboratory conditions.

During its spread through Europe and Asia, ASF has affected countries with scarce infrastructure and limited laboratory capacities. Even if routine laboratory diagnosis is established, infected wild boar could succumb in remote forest areas, far from centralized testing facilities. With the effectiveness of disease control measures relying on a timely implementation after an outbreak (Sanchez-Vizcaino, Mur, & Martinez-Lopez, 2012) and laboratory analysis being rather cost-intensive, questions upon the utility point-of-care (POC) assays, possibly even to replace laboratory testing, have arisen. Epidemiological investigations can also benefit from the availability of an effective POC test, e.g. when culling measures take hold in an outbreak scenario and the status of individual animals or farms is of scientific interest for back tracing of transmission factors. The INGEZIM ASFV CROM Ag LFA (Sastre et al., 2016) commercialized by Eurofins Technologies Ingenasa is designed for the diagnosis of ASFV antigen in blood under field conditions, possibly suiting these scenarios.

Rather promising results were obtained in previous studies under laboratory conditions and when sample quality was ideal or close to ideal (Pikalo et al., 2021; Pikalo et al., 2020). Here, we aimed to assess the applicability under field conditions with a total of 237 blood samples of different origins. These investigations complement the previous studies on the lateral flow assay with a practice-orientated approach, simulating the possible application as POC test for antigen detection in carcasses when possible effects of decomposition have occurred. The results of the antigen LFA were compared to OIE listed qPCR diagnosis, and the resulting sensitivity and specificity were evaluated for assessment of the practicability of the on-site test under realistic conditions with real field samples.

Material and Methods

Sample Origin

Eighty-six EDTA blood samples were obtained from recent animal trials conducted at the Friedrich-Loeffler-Institute (FLI) with domestic pigs, wild boar and minipigs infected with different ASFV strains of genotypes I, II or X (see supporting table 1). The animal trials for strain characterization and reference material generation were approved by the competent animal welfare authority under reference number 7221.3-2-011/19. Blood was aliquoted and promptly frozen after sampling, allowing high sample quality. Additionally, eleven blood samples of shot wild boar confirmed with ASF from the affected regions in Germany were included in the study. These animals were sampled immediately after death, also ensuring close to ideal matrix quality. Eighty blood samples originated from wild boar carcasses confirmed with ASF during the German outbreak (see supporting table 2). In addition, 60 negative field samples originating from shot wild boar of the same region were included. These field samples had been sent to the NRL for investigation between September 2020 and April 2021. Blood from carcasses and shot wild boar were mainly taken by local veterinary officers at the point-of-care and sent to the FLI (samples C17-C80 and negative field samples from shot animals), or was obtained during necropsy of the wild boar cadavers directly at the high containment facilities at the FLI (samples C1-C16, see supporting tables 2 and 3). Carcass-derived blood samples were in various stages of decomposition and impaired by clotting and/or autolysis. Field samples C17-C80, negative wild boar samples, and samples H81-H91, which the NRL received prior to the start of the study or in which LFA testing could not immediately be conducted, were stored at -80° C before investigations. Samples C1-C16 could be obtained during necropsy and were tested before, and, for comparison, after freeze-thawing (see supporting table 3).

Rapid Tests

The Ingezim® ASFV CROM Ag (Eurofins Technologies Ingenasa) is a double antibody sandwich immunochromatographic assay for the detection of ASFV antigen in blood samples. The test is based on the use of two different colored latex microspheres: black microspheres that are coated with a specific monoclonal antibody (MAb) against ASFV p72, and blue microspheres, which are used as test control. On the membrane, two lines are printed: the test line (T) has a specific MAb against ASFV and the control line (C) has a specific MAb against the control protein. In case of a positive sample, the virus binds to the black beads conjugated with the anti-ASFV MAb. The immune complex then migrates through the membrane

by capillarity and is captured again by the same anti-ASFV MAb absorbed on the test line, resulting in the appearance of a black line. The presence of the control line serves as validation of the test, indicating that the immunochromatography has been performed correctly (Sastre et al., 2016). The test procedure was conducted according to the manufacturer's instructions with the exception of also including previously freeze-thawed samples in the study (see supporting tables 1 and 2), using 20 μl of blood, adding 3-4 drops of running buffer after one minute, and results were recorded after ten minutes. The outcome was interpreted either as positive or negative (see figure 1). Only valid results were counted (appearance of the control line).
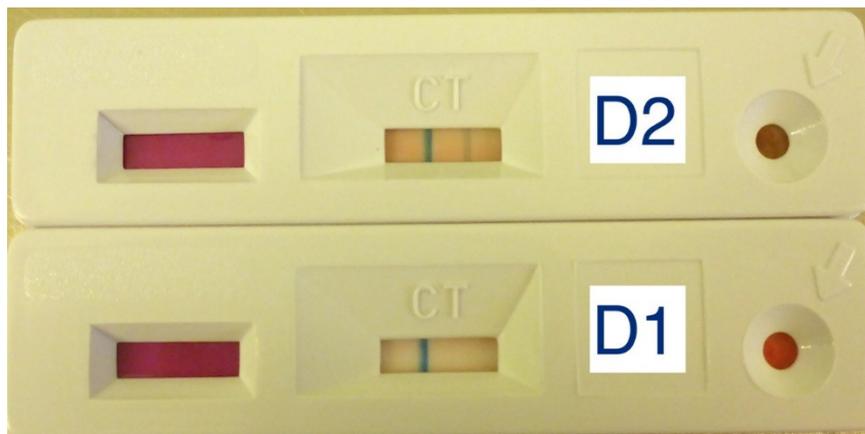
Figure 1: Exemplary antigen LFA results. D2 shows a valid positive result, D1 shows a valid negative result.

Nucleic acid extraction and real-time PCR

Viral nucleic acids were extracted using the QIAamp® RNA Viral Mini Kit (Qiagen) according to the manufacturer's instructions. qPCR was conducted according to the protocol published by King et al. (2003) with slight modifications (addition of a heterologous internal control DNA), or with the commercial qPCR kits virotype ASFV or virotype ASFV 2.0 (Indical Bioscience) according to the manufacturer's instructions. All qPCR runs were performed on C1000 thermal cyclers with the CFX96 Real-Time System (Biorad). Results were recorded as quantification cycle (cq) values.

Screening for ASFV-specific antibodies

The ID Screen ASF Indirect Kit (ID.vet) was used according to the manufacturer's instructions to screen the field samples for ASFV-specific antibodies. The multi-antigen indirect ELISA kit detects antibodies against ASFV p32, p62 and p72 in porcine serum, plasma or blood filter paper samples.

Statistical Analysis

Results of LFAs were evaluated in comparison to results obtained in qPCR. For this purpose, qPCR was regarded as the standard for pathogen detection. Accordingly, the outcome of the LFA was rated true positive (TP), true negative (TN), false positive (FP) or false negative (FN). Diagnostic sensitivity was calculated as TP/(TP+FN)x100. Diagnostic specificity was calculated as TN/(TN+FP)x100.

Results and Discussion

Of the 97 blood samples of ideal or close to ideal quality (samples from animal trials and samples H81-H91, see supporting table 1), 79 were positive by qPCR. The INGEZIM ASFV CROM Ag LFA detected 61 positives, resulting in a sensitivity of 77.2%. No false positives occurred, hence 100% specificity was observed on this dataset. The performance was therefore in line with the study published by Sastre et al. (2016) where field samples of unimpaired quality were detected with roughly 67% sensitivity when compared to an OIE listed qPCR. Specificity was also close to 100%. Sastre et al. (2016) evaluated the LFA only with

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fresh samples, as represented by our group of samples derived from animal trials or hunted wild boar. With the ongoing circulation of ASF in European wild boar, however, virus detection in carcasses has become an important issue. Sample quality is then usually reduced due to decomposition effects, an aspect that has not yet been elucidated for the ASFV antigen LFA. All our 80 carcass-derived blood samples were obtained from ASF-positive wild boar and confirmed by qPCR with cq values ranging from 14 to 38 (see supporting table 2). Here, significant differences were observed between the samples that were previously frozen, and those that were not: in native samples tested without any modifications (C1-C16, n=16, see supporting table 3), the LFA delivered only two positive results (sensitivity of 12.5%). After freeze-thawing, testing of the same 16 samples in the LFA yielded seven positives (sensitivity of 43.75%). Surprisingly, one of the samples that had yielded a positive result in the native context was now tested negative. The increase of overall positive results is in accordance with the sensitivity of 48.75% we observed in all of the previously freeze-thawed carcass-derived samples (C1-C80), where 39 positives were detected by the LFA (see supporting table 2). Interestingly, however, we did not observe a better sensitivity after freeze-thawing in EDTA-blood samples of high quality in a previous study by our group (Pikalo et al., 2021). The positive effect of freeze-thawing is probably due to the fact that most of the virus in blood is associated with erythrocytes (Wardley & Wilkinson, 1977), and therefore, the destruction of blood cells during freeze-thawing results in a higher antigen availability for detection in the test, a process especially effective when erythrocytes are bound to clots in samples of reduced quality. No false positive reactions occurred with any sample types.

In our study, the INGEZIM ASFV CROM Ag assay could not deliver reliable results with native blood from carcasses. Particular samples with cq values as low as 15 (C4, see supporting table 3), indicating a considerable virus load in the carcass, still delivered negative results in the LFA.

While we observed increased sensitivity after erythrocytolysis by freeze-thawing (12.5% vs 44% sensitivity, samples C1 to C16, see supporting table 3), for the practical implementation of the assay in the field, of course, freezing cannot be an option due to the technical requirements not fitting a point-of-care application. Possible alternatives to freeze-thawing for erythrocytolysis could be the dilution of blood in *aqua dest* . or lysis buffer. On a very limited dataset (n=4), hypotonic lysis seemed to improve the results (3 FN native, 1 FN after water lysis, no FN after freeze-thawing; data not further shown). While both methods could be feasible under field conditions, the effects of this deviation from the manufacturer's instructions on the assay should be elucidated and could be the basis of future optimization of the assay. After all, it must be noted that even with erythrocytolysis through freeze-thawing, we could only achieve a sensitivity of roughly 50% in carcass-derived samples, a value not fit fur purpose. Considering the negative impact of immune-complexes, samples were screened for the presence of antibodies. Only seven samples were positive for ASFV-specific antibodies and three delivered doubtful results in the antibody ELISA (see supporting tables 1 and 2). Eight of these samples were positive and two were false negative in the antigen-specific LFA (see supporting tables 1 and 2). While the small number does not allow for evaluation of possible interference, the principal functionality of the test in the presence of antibodies is indicated.

In general, the LFA was more reliable using samples with cq values below 30, indicating a rather high viral load. Of those samples derived from animal trials (n=64), 56 were true positive according to the rapid test, resulting in a sensitivity of 87.5% in that group. This goes along with observations in a previous study performed in our group, when the LFA was most sensitive during the clinical phase of ASF, at the peak of viral replication (Pikalo et al., 2021). In the present study, however, it was observed that the influences of clotting and decay in the carcass-derived samples seemed be able to outweigh the effects of higher viral loads, since here no clear correlation even with very low cq values and positive results in the LFA were observed (see supporting table 2).

Taking into consideration the differences between the highly amplifying qPCR and native antigen detection by LFA, the marked lower sensitivity in the later is to be expected. Still, the possibility for point-of-care testing holds a considerable advantage and on-site assays can provide a valuable additional diagnostic tool under certain circumstances. An acceptable sensitivity of the LFA was confirmed during the clinical phase of the disease, when fresh samples can be obtained from live animals or immediately after death. Here, the

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application of a rapid test could be of value in domestic pig holdings, when ASF is clinically suspected and live animals can be picked for sampling (given a careful interpretation of negative results in the LFA and still immediate initiation of laboratory diagnosis). Furthermore, epidemiological investigations can benefit from antigen assays for the on-site analysis of infected populations, when weaknesses in sensitivity are considered. However, with a sensitivity of roughly 50%, or even well below when no erythrocytolytic procedure is applied as proposed by the manufacturer's instructions, our findings imply that the LFA has only very limited use for antigen detection in blood from carcasses after extended *post mortem* intervals. When resources are scarce and prioritization of diagnostic workflows is needed, the high specificity may allow for positive on-site results in the LFA to surrogate a laboratory confirmation. Negative results, however, must always be rated with high caution due to the low sensitivity we observed in samples of reduced quality. OIE listed methods such as qPCR remain the only safe and proven methods for the unreserved detection of an ASFV infection. Therefore, the on-site assay should be regarded as a complimentary option rather than a substitute to laboratory diagnosis for carcass testing.

Author Contributions

Conceptualization, PD and SB; Data curation, PD and JP; Funding acquisition, SB and MB; Investigation, PD; Methodology, PD, JP and SB; Visualization, PD; Writing – original draft, PD and SB; Writing – review & editing MB. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest statement

The authors declare no conflict of interest.

Ethics statement

The authors confirm adherence with the journal's ethical statements as noted in the journal's authors guideline page. Blood samples listed in supporting table 1 came from animal experiments conducted at the FLI, which were performed in accordance with EU Directive 2010/63/EC and approved by the competent authority (Landesamt fur Landwirtschaft, Lebensmittelsicherheit und Fischerei (LALLF) Mecklenburg-Vorpommern).

Data availability statement

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

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