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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, especially immunochromatographic assays, 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. The later topic was the starting point for our present study. In detail, we evaluated the performance of the commercially available INGEZIM ASFV CROM Ag lateral flow assay (Eurofins Technologies Ingenasa) with selected high-quality reference blood samples, and with blood samples from wild boar carcasses collected under field conditions 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 testing of blood samples from carcasses without any modification, resulted in a drastically reduced sensitivity of only 12.5% with the given sample set. Freeze thawing increased the sensitivity to roughly 44% which mirrored the overall sensitivity of 49% in the total data set of wild boar carcass samples. A diagnostic specificity of 100% was observed. In summary, the antigen 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 that could be overcome, to a certain extent, with assay modifications.

KEYWORDS
African swine fever virus, antigen lateral flow assay, point-of-care test, sensitivity, specificity

1 | 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 et al., 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. Within the European Union, the abundant wild boar population was most severely affected and plays a major role in the epidemic (EFSA et al., 2018). First cases of ASF in Germany in 2020 (Sauter-Louis et al., 2021) sent another shockwave through the pig industry, as trade restrictions on pork took hold even though 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 reliable diagnosis, and the culling of affected herds (Blome et al., 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).

With the effectiveness of disease control measures relying on a timely implementation after an outbreak (Sanchez-Vizcaino et al., 2012), and laboratory analysis being rather resource intensive, questions regarding the utility of point-of-care (POC) assays, possibly even to replace laboratory testing, have arisen. These tests could help diagnosing the disease in remote areas with scarce infrastructure and limited laboratory capacities but also aid epidemiological investigations on outbreak farms during the culling procedure. Moreover, screening of wild boar carcasses prior to their safe removal without the need of sophisticated laboratory diagnosis could save time and resources and was discussed on high level.

One of the assays that could suit these scenarios is the INGEZIM ASFV CROM Ag LFA (Sastre et al., 2016), commercialized by Eurofins Ingenasa. This immunochromatographic assay is designed to detect ASFV antigen in blood samples under field conditions and showed rather promising results in previous studies under laboratory conditions (Pikalo et al., 2020; Pikalo et al., 2021). Taking the question regarding wild boar carcass testing as a starting point, we aimed to assess the applicability with samples of reduced quality, although the assay was not originally designed for this purpose. A total set of 237 blood samples of different origins was therefore investigated. 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.

2 | MATERIAL AND METHODS

2.1 | 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 Table S1). 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, 11 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 Table S2). 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 Tables S2 and S3). Carcass-derived blood samples were 'field-like' 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 Table S3).

2.2 | 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 (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 Tables S1 and S2). The outcome was interpreted either as positive or negative (see Figure 1). Only valid results were counted (appearance of the control line).

2.3 | 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. Subsequently, qPCR was conducted according to the protocol published by

FIGURE 1 Exemplary antigen LFA results. D2 shows a valid positive result, D1 shows a valid negative result

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.

2.4 | Screening for ASFV-specific antibodies

The ID Screen ASF Indirect ELISA 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.

2.5 | 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 $TP/(TP + FN) \times 100$. Diagnostic specificity was calculated as $TN/(TN + FP) \times 100$. Confidence intervals were calculated of share values.

3 | RESULTS AND DISCUSSION

To obtain a broader picture on assay performance, the study comprised experimental samples of high quality and the targeted blood samples of wild boar carcasses. Of the 97 blood samples of ideal or close to ideal quality (samples from animal trials and samples H81–H91, see Table S1), 79 were positive by qPCR. The INGEZIM ASFV CROM Ag LFA detected 61 positives, resulting in a diagnostic sensitivity of 77.2% [95% confidence interval (68%, 86%)]. 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%. With the ongoing circulation of ASF in European wild boar, however, virus detection in carcasses as initial test or screening prior to save removal, 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 Table S2). 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 Table S3), the LFA delivered only two positive results [sensitivity of 12.5% (0%, 25%)]. After freeze-thawing, testing of the same 16 samples in the LFA yielded seven positives [sensitivity of 43.75% (19%, 68%)]. Unexpectedly, 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% (38%, 60%) we observed in all of the previously freeze-thawed carcass-derived samples (C1–C80), where 39 positives were detected by the LFA (see Table S2). 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 Table S3), 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–C16; see Table S3), 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 ($n = 4$) that does not allow for statistical significance, 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 for 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 Tables S1 and S2). Eight of these samples were positive and two were false negative in the antigen-specific LFA

(see Tables S1 and S2). 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% (79%, 96%) 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 was observed (see Table S2).

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 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. Under field conditions, pre-testing could help directing resources, for example, succession of removal, treatment of surroundings, or disinfection measures. Negative results, however, must always be interpreted 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

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 Table S1 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 für 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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SUPPORTING INFORMATION

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

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