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Evaluation of the cost-effectiveness of ASF detection with or without the use of on-field tests in different scenarios, in Sardinia

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ABSTRACT

African swine fever (ASF) is a highly contagious disease of domestic pigs and wild boars (WBs). Without a vaccine, early antibody and antigen detection and rapid diagnosis are crucial for the effective prevention of the disease and the employment of control measures. In Sardinia, where 3 different suid populations coexisted closely for a long time, the disease persists since 1978. The recent ASF eradication plan involves more stringent measures to combat free-ranging pigs and any kind of illegality in the pig industry. However, critical issues such as the low level of hunter cooperation with veterinary services and the time required for ASF detection in the WBs killed during the hunting season still remain. Considering the need to deliver true ASF negative carcasses as early as possible, this study focuses on the evaluation and validation of a duplex pen-side test that simultaneously detects antibodies and antigens specific to ASF virus, to improve molecular diagnosis under field conditions. The main goal was to establish the specificity of the two pen-side tests performed simultaneously and to determine their ability to detect the true ASF negative carcasses among the hunted WBs. Blood and organ samples of the WBs hunted during the 2018/2019 hunting seasons were obtained. A total of 160 animals were tested using the pen-side kit test; samples were collected for virological and serological analyses. A specificity of 98% was observed considering the official laboratory tests as gold standards. The new diagnostic techniques could facilitate faster and cost-effective control of the disease.

Keywords: African swine fever; pen-side test; ost effectiveness analysis; Sardinia; hunting season management

INTRODUCTION

African swine fever virus (ASFV; family: *Asfarviridae*; genus: *Asfivirus*) is the causative agent of African swine fever (ASF), a devastating disease of domestic and wild pigs [1-4]. ASF is particularly contagious, and it causes high mortality [5], involving various syndromes ranging from mild disease to lethal haemorrhagic fever [6]. In 2019 Beltran-Alcrudo carried out in-

Author Contributions

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depth evaluation of the great economic impact of this transboundary disease on global pig sector with regard to different aspects (reduction in the quality of livestock-derived products, socio-economic consequences, massive depopulation, and public health) [7], confirming the considerations of many earlier studies on the costs to contrast ASF spread [8-12]. In Europe, ASF was first introduced in Portugal (1957) and Spain, where it was finally eradicated. To date, the disease is present in the Russian Federations, Belgium, Hungary, Bulgaria, Latvia, Moldova, Poland, Romania, Russia, and Ukraine, and in the Asian territory, it is present in China, Mongolia, and more recently in Vietnam. So far, no outbreaks of ASF have been reported from Germany and France; the threat posed by Belgium is kept under control by national authorities [13]. In these European regions, the fundamental role of the wild boar (WB) in the disease maintenance has been recognized [6,14]. Currently, ASF is eradicated neither in Africa—where the involvement of many factors (sylvatic cycle, argasid ticks) plays a key role in the prevention and control of the infections [15]—nor in Sardinia island (Italy)—where probably owing to the introduction of food waste containing ASF-contaminated meat from the Iberian Peninsula [16], the disease has been endemic for 40 years. As soon as the disease spread to central Sardinia (June 1978), it became clear that disease control measures were not being practiced by the local population and that residents had not abandoned local cultural traditions of free-ranging and breeding [17,18]. The island is one of the few places in Europe where three different populations associated with ASF (WBs, domestic pigs, free-ranging pigs) persistently coexist [19-23]. Central areas of Sardinia, closely linked to local traditional festival, are zones whit higth consumtion of meat, sometimes without permission in an illegal context and without veterinary controls, favouring contamination by and spread of ASFV [24-28]. Indeed, the culture of breeding one or a few pigs for self-consumption is still a very common practice, mostly in combination with sheep breeding [29]. In Sardinia, thanks to the last ASF Eradication Plan 2015–2018 (PE-ASF15-18)—which focuses on incentivising good practices of swine breeding and elimination of free-ranging pigs—great progress was made. However, the lack of specific vaccine hampers the prevention and control of infection, especially in the WB population. Therefore, control measures, such as early and specific diagnosis of infection, should be implemented to prevent ASF in both WB and domestic pigs [30-33]. In this context, according to the recent European Food Safety Authority, an intensive hunting approach around the outbreak area might not be sufficient to eradicate ASF [34]. Nevertheless, in the last 2 years an increase in hunting activities in Sardinia has influenced the decrease in ASF cases [23]. Currently, based on PE-ASF15-18, the surveillance plan for wildlife includes the different management of WB inside the ASFV infected zone (IZ) [21]. The statements provided by the eradication plan established that inside the IZ all the hunted WBs must be subjected to serological and virological testing. Only the ASFV antigen (Ag) and antibody (Ab) negative carcasses could be delivered to the hunting company. During hunting season, due to logistic, economic and technical constraints, it may take several days to collect samples, deliver them to the laboratories, complete testing, and report back to veterinary officers. Compliance with all these measures complicates the relationship with hunting companies, which have to wait several days to consume the WB meat [33]. Furthermore, because the laboratory infrastructure is overloaded, examination and laboratory confirmation would require from 5 to 15 days [21,35]. Rapid serological and virological tests, such as pen-side (PS) tests, are easy to use under field condition, and they are able to detect ASFV Ags and Abs as demonstrated in-laboratory by Sastre in 2016 and subsequently, on-field by Cappai et al. in 2017 and 2018 [31,32]. However, the sensitivity and specificity of these tests and their ability to yield reliable results could be strictly influenced by the conditions under which sample collection takes place, the sample quality, the time between withdrawal of the sample and execution of the test, and the hunting company

collaboration [33]. In the context of upcoming disease eradication programs in countries such as Sardinian, rapid field tests could be applied to detect the true negative ASF carcasses and deliver them, since the positive carcasses need laboratory confirmation as define by the ASF diagnostic manual (Commission Decision 2003/422/EC). This study intended to evaluate the simultaneous usage of PS tests for ASFV Ag and Ab detection under field conditions, to improve molecular diagnosis on field, and to perform a specific economic evaluation in terms of time, costs, and benefits of the use of rapid kit tests.

MATERIALS AND METHODS

Test procedure

This study was carried out using samples from animals hunted during the 2018/2019 wild boar hunting season (WB-HS). The WB-HS spans from 1 November to 31 January in accordance with PE-ASF-15-18. As suggested by the company that manufactures the PS test kits (INGENASA, Spain) and considering these factors influencing the agreement between PS and laboratory tests [33], all the samples were collected by experienced veterinarians within a maximum post-mortem time of 5 h, inside the hunting company technical room, within a short time (less than 30 min), ensuring the collection of an adequate sample quality. Ag test is an immunochromatographic assay for the detection of ASFV in blood samples. The test is based on the use of two different coloured latex microspheres: black microspheres that are coated with a specific monoclonal antibody (mAb) to ASFV, and blue microspheres, which are used as test control. On the membrane, two lines are printed: the test line has a specific mAb to ASFV and the control line has a specific mAb for the control protein. If the case of a positive sample, the virus binds to the black beads conjugated to an anti-ASFV mAb. The immune complex then migrates through the membrane by capillarity and is captured again by the anti-AFSV mAb absorbed on the test line, resulting in the appearance of a black line. The presence of the control line serves as validity of the test, indicating that the immunochromatography has been performed correctly. Ab test is based on the technique of immunochromatography, a migration technique that uses purified VP72 protein of ASFV, and is able of detecting specific Abs against ASFV in porcine serum samples. The diagnostic device consists of two windows: Sample window, that contains VP72 protein of ASFV and a protein control, coated to coloured latex particles; Result window, that contains a test line (T) formed by the VP72 protein of ASFV and a control line (C) formed by a mAb specific for the protein control. If the test sample contains Abs against the VP72 protein of ASFV, they will react with the red particles conjugated to VP72 protein. The latex-protein-Ab complex migrates through the membrane and reacts again with the VP72 protein adsorbed in the test line resulting in the appearance of a red/pink. The appearance of a blue line in the control area (C) indicates that the chromatography has been correctly performed. The entire tests procedure was completed in 10 min (INGENASA Test procedure).

Statistical analysis

The sample size was calculated on the basis of the sample size tables for ROC studies proposed by Obuchowski in 2000 and determined for a study with 5% type I error rate and 80% power [36]. The total sample size was establish as 144 WBs considering the following: the aim of this study, the previous results obtained on-field [31,33], the need to determinatethe specificity at a false-negative rate ≥ 0.10 (sensitivity fixed at ≥ 0.90), the number of observers as six (i.e., veterinarians and lab technicians), the variability among observers as small, the level of tests accuracy as high (specificity of 0.80 at a false-negative

rate of 0.10), the suspected difference in specificities between the molecular diagnosis tests at a false-negative rate of 0.15, and a ratio of 4:1 between sick and healthy WB. Considering a "drop out" rate of 10%, 15 WBs were added to the sample size. Each of the 160 sampled wild animals tested with the PS tests (INGENASA), was simultaneously tested for ASFV with real-time polymerase chain reaction (PCR) technique as ASFV Ag detection gold standard, and using enzyme-linked immunosorbent assay (ELISA) test (INgezim PPA Compac–Ingenasa), which is confirmed by using the immunoblotting test (IB), as ASFV Ab detection gold standard [37], as described in the EU Diagnostic Manual for ASF (Commission Decision 2003/422/EC). According to the manufacturer's instructions, serum and organ samples were frozen at −20°C and −80°C, respectively and the reagents were stored at 4°C–25°C until the procedures were carried out. The data on WB sample collection and the results of on-field and in-laboratory tests were stored in a specific password protected MS Excel Spreadsheets and were analysed using the statistical software Stata, release 13 (StataCorp LP, USA; 2013). Consistency and accuracy were verified through extensive data checking, and any disagreements were evaluated and corrected. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the PS tests were estimated individually and in combination using respective 95% confidence intervals [38]. Sensitivity was calculated as the proportion of positive cases identified using the index test among the true-positive cases. Specificity was calculated as the proportion of negative cases identified using the index test among the true-negative cases. PPV was calculated as the proportion of true-positive results among all the positive results obtained using the PS tests. NPV was calculated as the proportion of true-negative results among all the negative results obtained using the PS tests. A WB was considered ASF positive if at least one of the 2 tests is positive. The choice of both tests performed on each animal was based on the proprieties of in parallel tests, which are able to give rapid diagnosis with a decrease in the number of false negative with respect to a single test, because in our case was fundamental the ability of the procedure to exclude any possible false-negative samples [39]. Furthermore, the simultaneous application of tests is recommended in the case of tests with low sensitivity. The results were interpreted in key of 'OR.'

RESULTS

The results of laboratory and PS tests are presented in **Tables 1** and **2**. Among the 160 tested WBs, none were found to be ASFV positive; only 3 (1.9%) were identified as positive for ASFV AB using ELISA tests, which was confirmed using IB. Two of these three were identified as ASFV positive using AB PS test. With regard to the negative samples, 157 WBs were negative when tested with the gold standard test for Ab detection, and 154 (98%) of these were identified as negative using Ab PS test. All the samples (n = 160) were ASFV Ag negative based on PCR analysis; however, 4 of these (2.5%) were ASFV positive based on the PS test for Ag detection. Although it was not possible to calculate the sensitivity of ASFV Ag detection (no positive cases), specificity and sensitivity of the PS tests that were performed simultaneously, were calculated. As reported in **Table 3**, ASFV Ab (97.5%) and Ag (98.1%) detection showed similar specificity, and Ag (66.7%) detection showed moderate sensitivity. When these tests were performed simultaneously (in parallel), the global specificity decreased to 95.5%, while global sensitivity remained 66.7%. These results indicate the ability of these 2 tests to correctly identify healthy animals. In terms of probability, 95.5% of the animals tested negative using PS tests were effectively free from ASF. On the contrary, 66.7% of the WBs that were tested positive using both the PS tests were effectively ASFV infected.

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Table 1. The contingency table showing the detection of African swine fever virus Ag using the PS tests, compared to that using PCR technique

Ag PS test	PCR technique	
	Positive	Negative
Positive	0 (0%)	4 (2%)
Negative	0 (0%)	156 (98%)
Total	0 (0%)	160 (100%)

Ag, antigen; PS, pen-side; PCR, polymerase chain reaction.

Table 2. The contingency table showing the detection of African swine fever virus Ab using the PS tests, compared to that using ELISA test + IB

Ab PS test	ELISA test + IB	
	Positive	Negative
Positive	2 (1.4%)	3 (2%)
Negative	1 (0.6%)	154 (96%)
Total	3 (2%)	157 (98%)

The results are expressed as percentages (%).
Ab, antibody; PS, pen-side; ELISA, enzyme-linked immunosorbent assay; IB, immunoblotting test.

Table 3. Individual and combined (sequential or in parallel) accuracies of Ag PS test and Ab PS test in detecting ASFV Ag and ASFV Ab in the blood samples of WBs from the areas endemic for ASFV in Sardinia

Accuracy	Ag PS test	Ab PS test	Parallel testing
Sensitivity	66.7 (9.4–99.1)	-	66.7 (9.4–99.1)
Specificity	98.1 (94.5–99.6)	97.5 (93.7–99.3)	95.5 (91.0–98.2)
PPV	40.0 (5.2–85.3)	-	22.2 (2.8–60)
NPV	99.3 (96.4–99.9)	100 (96.3–100)	99.3 (96.4–99.9)

The results are expressed as percentages (%) and 95% confidence interval.
Ag, antigen; PS, pen-side; Ab, antibody; ASFV, African swine fever virus; WB, wild boar; PPV, positive predictive value; NPV, negative predictive value.

DISCUSSION

As indicated in the last Sardinian eradication plan, the epidemiological surveillance of ASF in WBs is enforced through the regulation of WB hunting all over the territory. The carcasses of the hunted boars that need to be tested for ASF should be stored with care at the expense of the hunting companies until the results of serological and virological tests are obtained. Subsequently, the carcass are destroyed if they test positive or released if they test negative for ASF. The understandable complications involved in the management of this process lead to numerous delays from the time of delivery of the samples to that of obtaining the results, and this aspect translates into an inevitable difficulty in collaboration with the hunters [21,31-33,40]. In the context of eradication, because the on-field application of the PS tests in the Sardinian territory could identify the true-negative WBs with highest accuracy, they facilitate fast delivery of carcasses and confirmation of positives with conventional laboratory techniques.

On the base of this assumption, cost-effectiveness and time efficiency were analysed (**Tables 4** and **5**) considering two possible scenarios: the first scenario (scenario A: actual hunting management) illustrates the costs sustained by Sardinian government during the last WB-HS (18-19), including the laboratory and veterinarian costs. The second one (scenario B: hunting management using PS tests) would estimate the costs of managing the same hunting season using the two PS tests simultaneously and confirming the possible positive WB with conventional laboratory tests.

As illustrated in **Table 4**, during the last hunting season 12,728 WBs have been serologically and virologically tested for ASFV. The same animals could be subjected to ELISA (screening

Table 4. Table showing the laboratory tests performed, corresponding waiting period (days) for laboratory results, and total number of WBs showing positive results, during the hunting season of 2018/2019 in Istituto Zooprofilattico Sperimentale della Sardegna

Laboratory test	WBs tested	No. of tests	Total positive	Day waiting period*
ELISA	10,554	10,671	549	3.26 ± 2.60 (0–40)
IB	574	575	111	4.16 ± 2.48 (1–18)
PCR	5,603	6,502	5	2.99 ± 2.33 (0–40)
Total	12,728†	17,748	116‡	-

WB, wild boar; ELISA, enzyme-linked immunosorbent assay; IB, immunoblotting test; PCR, polymerase chain reaction. *The values of day waiting period are expressed as mean ± standard deviation (range); †The total number of WBs tested is not equal to the sum of animals tested using ELISA, IB, and PCR, because ELISA is a screening test performed before IB. The same animal could be tested using ELISA alone (negative ELISA result) or using ELISA and IB (positive ELISA result); ‡The total number of positives were calculated considering that each WB could serologically be defined as ASF positive only if it is IB positive.

Table 5. Total cost (€) of hunting season management based on scenario A (actual hunting management) and scenario B (hunting management using Ag PS test and Ab PS test), considering laboratory or on-field test costs and veterinarians' remunerations

Scenarios	Type of tests	Costs of lab/field tests (€)	Cost for positive sample test	Remuneration for veterinarians (€)	Total costs for managing hunting season (€)*
Scenario A†	ELISA	41,296.8‡	1,701.9§	358,293.2‖	542,546.1
	IB	8,625¶	1,165**		
	PCR	134,331.3††	103.3‡‡		
Scenario B§§	Ag PS test	43,911.6‖‖	16,544.7¶¶	114,857.5***	219,225.4
	Ab PS test	43,911.6†††			

Ag, antigen; Ab, antibody; PS, pen-side; ELISA, enzyme-linked immunosorbent assay; IB, immunoblotting test; PCR, polymerase chain reaction; WB, wild boar.
*The total cost of the hunting season has been calculated as sum veterinarian cost and test cost; Scenario based on †actual hunting management and §§possible hunting management using PS tests; ‡The total cost of the ELISA tests performed during the 2018/2019 WB-HS has been calculated as the product of 3.87 € (cost of single exam) and 10,671 (the number of exams performed); §The total cost of ELISA tests in the animals tested positive using ELISA has been calculated as the product of 3.87 € (cost of single exam) and 549 (the number of animals tested positive; **Table 4**); ‖The total veterinarian cost in scenario A has been calculated as the product of 28.15 € (cost for testing a single animal) and 12,728 (number of WBs tested); ¶The total cost of the IB tests performed during the 2018/2019 WB-HS has been calculated as the product of 15.00 € (cost of single exam) and 575 (the number of exams performed); **The total cost of IB in the animals tested positive using IB has been calculated the product of 15.00 € (cost of single exam) and 111 (the number of animals tested positive; **Table 4**); ††The total cost of real-time PCR analyses performed during the 2018/2019 WB-HS has been calculated as the product of 20.66 € (cost of single exam) and 5,603 (the number of exams performed); ‡‡The total cost of real-time PCR in the animals tested positive using real-time PCR has been calculated as the product of 20.66 € (cost of single exam) and 5 (the number of animals tested positive; **Table 4**); ‖‖.†††The total cost of PS tests (Ag and Ab) has been calculated as the product of 3.45 € (cost of single PS kit) and the number of WBs hypothesised to be tested during the hunting season 2018/2019; ¶¶The total cost for the conventional testing of the animals that showed positive results based on PS tests has been calculated as the sum of §, **, and ‡‡ [(549 × 3.87 €) + (111 × 15.00 €) + (5 × 20.66 €)]; ***The total veterinarian cost in scenario B has been calculated as the product of 9.02 € (cost for testing a single animal) and 12,728 (number of WBs tested).

tests) alone (negative results) or ELISA with IB (positive ELISA results) for confirmation. Sometimes, when the results are ambiguous, the tests could be performed again. Consequently, the total number of WBs tested could be different to the sum of WBs subjected to ELISA (n = 10,671), IB (n = 575), and real-time PCR (n = 6,502). A total of 116 positive WBs were detected during the last WB-HS; 5 of them were virologically positive for ASF based on real-time PCR results, while 111 were tested with ELISA and ASF seropositivity was confirmed using IB. Furthermore, after recent enforce of Sardinian ASF laboratories, the average time required for obtaining results in our laboratory is 3 days for ELISA (mean ± standard deviation [SD], 3.26 ± 2.60), 4 days for IB (4.16 ± 2.48), and 3 days for real-time PCR (2.99 ± 2.33). Currently, in Sardinia the overall cost (including kit test, laboratory infrastructure, and staff) is calculated to be about 3.87 euros (€) for ELISA, 15.00 € for IB, and 20.66 € for real-time PCR. Considering the total number of ELISA, IB, and real-time PCR analyses performed during the last WB-HS (10,671, 575, and 6,502, respectively) the total laboratory test cost for the last year was 184,253.1 €, which is the sum of 41,296.8 € (ELISA), 8,625 € (IB), 134,331.3 € (real-time PCR).

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The cost of a single PS test kit is about 3.45 €. Therefore, the total cost of purchasing 400 PS test kits for our institute was 2,767.0 € (tax included). Assuming that all the hunted WBs had been subjected to both PS tests for the detection of ASFV Ag and Ab, the total cost would be 88,044.7 €. Considering that each hunted animal would require simultaneous testing (with ASFV Ag and Ab detection), the cost of testing each animal would be 6.90 €. Considering that the specificity is 95.5% (1 − SP = 4.5%), among the ASFV negative animal (12,728 WBs − 116 positive WBs = 12,612 WBs), 4.5% (568 false positive [FP] WBs) should be subjected to ELISA and real-time PCR laboratory test without IB confirmation due to false positivity. The total cost incurred due to these animals could be calculated as follows:

$$FP_{cost} = (568WB * 3.80\,€_{ELISA}) + (568WB * 20.06\,€_{PCR}) = 13{,}552.5\,€$$

Considering the founded sensitivity simultaneously, 66.7% of the 116 ASFV positive animals (n = 77) should be confirmed using conventional laboratory tests; this cost could be calculated as follows:

$$VP_{cost} = (77 * 3.80\,€_{ELISA}) + (77 * 20.06\,€_{PCR}) + (77 * 15.00\,€_{ELISA}) = 2{,}992.2\,€$$

The cost incurred for PS tests would be 104,589.4 €, which is the sum of 88,044.7 € (cost of both ASFV Ag and Ab PS tests) and 16,544.7 € (cost for checking positive WBs using laboratory tests).

The veterinarian costs for collecting a single blood or organ sample or for performing a single PS test under field conditions have been calculated using personal data from Local Sanitary Agency (ATS). Considering that collection of samples from 1,000 WBs takes 12 hunting days and that 13 expert veterinarians have been employed, the number of WBs sampled by a veterinarian/day could be calculated as:

$$WB_{sample} = (\frac{1000\,WB}{12\,days})/13\,vet = 6.41\ \text{WB/day}$$

Furthermore, considering that the remuneration of each veterinarian is 30.08 €/h and that a work day consists of 6 h, the cost of sampling each WB could be calculated as follows:

$$WB_{cost} = \frac{(30.08\,€ * 6\,h)}{6.41\,WB/day} = 28.15\,€/WB$$

With regard to the PS tests described above, each veterinarian is calculated to be able to test in parallel 20 WB/day. Considering that the remuneration of each veterinarian per hour is the same (30.08 €/h), and that a work day consists of 6 h, the cost of sampling each WB could be calculated as follows:

$$WB_{cost} = \frac{(30.08\,€ * 6\,h)}{20\,WB/day} = 9.02\,€/WB$$

Considering the total number of WBs to be tested (n = 12,728), the total cost of sampling all the WBs by an expert veterinarians is about 358,293.2 €, while the total cost of testing 12,728 animals using the PS tests simultaneously would be 114,857.5 € (**Table 5**). Therefore, considering the possible scenarios A (actual hunting management) and B

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(hunting management using PS tests), 2 different cost analyses could finally be carried out. The total cost of managing the latest WB-HS (18-19) in Sardinia was approximatively 542,546.1 € considering the costs related to laboratory tests and the remunerations of expert veterinarians. On the contrary, the total cost incurred for hypothetically managing the same hunting season by simultaneously using the PS tests for ASFV detection would be 219,225.4 € considering both kit test-related costs and veterinarians' remunerations. Apart from the money that is clearly saved (323,320.7 €), the other great advantage of using the PS tests is that they can be performed directly under field conditions and there is an apparent decrease in the waiting period because the PS tests provide immediate results. Furthermore, if influencing factors (place of execution, sample quality, and time between withdrawal and test execution) are kept under control, the results of PS tests are independent of operators and locations of execution [35], making these tests very versatile and suitable for on field application. This could considerably improve the collaboration between hunting companies and veterinary services. However, considering the possible bias generated by the not directly calculation of the sensitivity of the in parallel tests due no ASFV positive WBs have been found, further evaluations are necessary. Similar low sensitivity values (76.5%) were observe in a previous study conducted in Sardinia by Cappai et al. [33], generating a 20%–30% risk of deliver false-negative carcasses among the total ASFV positive carcasses. On the other hand, the use of a rapid test performed directly on field could reduce the waiting period. In fact, animals diagnosed as negative using a test with a high specificity (specificity of PS tests = 95.5%) could be released immediately for consumption, saving time and money. In an endemic area for ASF, such as the Sardinian territory, the early detection of the disease is a fundamental requirement for the employment of an eradication program. Furthermore, the PS tests can also be used for the detection of ASF in new areas, such as hunting reserves that have not previously been subjected to surveillance. A complete change in the management and detection of disease during the hunting season would be desirable in the future, and the PS tests could be a valide instrument for this goal, with regard to saving time, money, and number of samples subjected to testing.

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