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African swine fever Detection on Field with Antigen Rapid Kit Test

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Abstract

African swine fever (ASF) is one of the most important and complex infectious diseases affecting pigs (*Susscrofa*). Disease spread can be costly and lead to loss of exports. The presence of asymptomatic carrier pigs illegally breeding and contact between livestock and wild boar (WB) in lack of biosecurity situations are major risk factors that lead to the persistence of ASF in many areas of Sardinia Island, Italy, where the disease has been present since 1978. Important public health programs have been implemented by the Sardinian Region Authority and are characterized by strong measures to eliminate free ranging pigs and incentivize proper practices. Satisfactory results in terms of reducing the number of outbreaks and ASF prevalence have been observed. However, critical points still remain, such as the length of time necessary to obtain diagnostic results for ASF detection in WB killed during the hunting season. After a field evaluation of a commercial serological kit test, which is able to reduce cost and manpower, evaluation of an antigen Pen-Side (INGENASA©) (PS) kit test in the field was also performed to assess the prospective of future combined use. Samples from WB hunted during the 2016/2017 and 2017/2018 seasons were obtained. Four hundred animals were tested with the PS kit immediately after the hunt, and blood was collected for virological analysis to screen for the ASF virus. Sensitivity (76.5%) and specificity (98%) of the PS test were higher compared to primary results of the device. The use of the PS test can allow for rapid diagnosis and reduction of unnecessary carcass destruction. The strategy of utilizing combined independent tests (in parallel) and the interpretation of the results in the context of the ASF area is suggested by the authors as a useful tool for conducting regular monitoring during emergency situations, particularly during the final phases of disease eradication.

Keywords: African swine fever; Pen-Side; Antigen; Risk; Multiple-test; Bayesian latent class analysis

Introduction

African swine fever (ASF) is an infectious disease of domestic and wild pigs [1] caused by the African swine fever virus (ASFV), a dsDNA virus of the genus Asfivirus within the family Asfarviridae [2]. ASF is characterized by high contagiousness and mortality and involves a wide range of syndromes, from mild disease to lethal haemorrhagic fever [3]. The ASFV also replicates in Ornithodoros soft ticks, which then act as a viral reservoir, playing an important role in risk factors in africa and Iberian Paenisula [4]. In Europe, ASF was first introduced in Portugal (1957) and subsequently spread to other countries, such as Spain, where it was finally eradicated. Currently, the disease is endemic in Sub-Saharan countries and in Sardinia (Italy), causing serious economic and social damage [5,6]. After a transcontinental spread to Georgia and Eastern Europe in 2007, ASF is now present in the Russian Federation, Ukraine, Poland, Latvia, Lithuania, Estonia, Moldova, the Czech Republic, Belarus, Hungary and Romania [7]. In this region, in contrast to the epidemic situation of Africa where a sylvatic cycle involving warthogs, it is recognized that wild boar (WB) have an important role in the disease maintenance [3,8]. Economic consequences, especially due to export restriction, emphasize the importance of enforcing ASF activity and eradication programs. Even though great progress has been made in diagnosis [9,10] and in ASF immunization studies, no vaccine or treatment options are available to prevent or limit infection [11]. This current lack increases the importance of a differential ASF laboratory diagnosis to aid eradication programs. In Sardinia, the disease has been present since 1978, and the epidemiological situation is influenced by factors, such as the illegal free ranging of pigs in WB territories [12,13]. The incidence of ASF increased from 2011 to 2014, when the disease spread swiftly into territories outside the endemic area [5]. The implementation of Public Health programs is essential for controlling the disease. A new Plan of Eradication for ASF 2015-2018 ((PE-ASF-15-18) Regional Decree Number 50/17, 16 December 2014) was developed by the Sardinian Region Authority, in accordance with the European Commission. The plan established specific serological and virological measures in terms of screening activity, suspicion of disease, and slaughter for self-consumption. The surveillance plan for wildlife is geographically limited to those areas in which outbreaks of ASF occur in WB. This area is defined as the "infected zone" (IZ), and surveillance activities within this zone are different from those of the remaining Sardinian territory. Control and management of the hunting season is another important goal of the plan, and includes the

application of strong rules involving carcass checks and workmanship of the meat. A significant decrease in disease prevalence, both in virological and serologic prevalence, has been observed inside and outside the IZ since the application of PE-ASF-15-18 (Table 1). One of the final steps in the eradication program is the facilitation of early detection of ASF, by not only veterinarians and the authorities, but also by hunters and farmers. As previously demonstrated [14,15], the use of a field test on both WB and illegal free-ranging pigs can be a valuable tool with economic and time saving benefits. Experimental detection of ASFV-specific antibodies was first performed by Perez, et al. [16] from experimentally infected pigs, and the Pen-Side (PS) test met the sensitivity (SE) and specificity (SP) parameters (SE=99%; SP=100%) set by the World Organization for Animal Health. In 2016, Sastre, et al. [17] evaluated the performance of the PS test on field samples obtained from outbreaks in EU countries and surveillance programs. The validation of antibody testing in the field was then carried out by Cappai, et al. [14] using samples from a high ASF risk zone, where illegal pigs and WB live closely together. WB were tested with the PS test and an ELISA or immunoperoxidase monolayer assay (IPMA). On ROC curve analysis, the test was defined as moderately accurate, based on Swets agreement [18] (SE=81.8%; SP=95.9%; positive predictive value (PPV)=69.3%; negative predictive value (NPV)=97.9%). These studies confirm that the PS test offers advantage and benefits, especially in field scenario, as a rapid, economic, and simple-to-use tool with a high SP. To our knowledge, no study has been performed to evaluate the performance of the PS test for antigen detection. The aim of this work was evaluate the use of the PS test for antigen detection of ASFV in the field, and to assess any potential difficulties related to test execution. In addition to performing the PS test in 400 WB, a questionnaire was completed for each test.

Material and Methods

Animal sampling

This study was completed using samples from WB hunted during the 2016/2017 and 2017/2018 hunting seasons (CVCs) (Figure 1). The CVC spanned from 1 November to 31 January, in accordance with PE-ASF-15-18. Samples were collected by experienced veterinarians within the maximum post-mortem time of 5 hours, as suggested by Ingenasa. Data were compiled by the veterinarians for each sample, including information regarding WB sex and age, hunting geo-coordinates, climatic conditions, PS test storage, and method of test execution. Furthermore, information on hunting geo-localization was used to evaluate spatial distribution to ensure the random selection of WB. Testing was performed using the PS (INGENASA©) for antigen detection and Real Time PCR, as the gold standard. Information regarding WB sample data collection and the results of PS and PCR testing was stored in a specific password protected Microsoft Office Access database and is presented in table 2. Data consistency and accuracy were verified through extensive data checking, and any disagreements were evaluated and corrected.

Test procedure

The PS test is an immunochromatographic assay for the detection of the ASFV in blood. Blood samples must be fresh, and the reagents require storage between 4 and 25°C. On the test membrane, test and control lines are present that consist of a monoclonal antibody specific to ASFV and a control protein, respectively. The control is very important in indicating that the test has been performed correctly. To perform the test, twenty microliters of whole blood were placed into the round window and three to four drops of the running buffer were added. The results were then interpreted at ten minutes. A single blue line indicated a negative result, and a blue line along with a black line indicated a positive result. The test was considered invalid if a blue line did not appear within ten minutes.

Statistical analyses

Descriptive analyses were performed to evaluate the baseline distribution of all collected variables and to select possible factors associated with concordance/no-concordance between tests that were then further evaluated in stratified analyses. WB ages were expressed as medians (I-III quartile) and minimum-maximum, while categorical variables were expressed by frequencies and percentages. The accuracy (i.e. SE and SP) of new diagnostic tests are usually compared against an established gold standard [19]. If the error rates of a gold standard are disregarded and the gold standard is considered perfect (100% SE and 100% SP) during the evaluation of new diagnostic tests, the accuracy of the new tests and the disease prevalence can be underestimated [20]. Although the PCR test currently considered the gold standard at Istituto Zooprofilattico Sperimentale della Sardegna (IZS-Sardegna) laboratories has excellent accuracy, its SE and SP are not 100%. In 1995, Joseph, et al. [21] proposed the use of Bayesian latent class models (LCMs) as a method to estimate the accuracy of diagnostic tests when the accuracy of the gold standard is unknown or is less than 100%. Bayesian LCMs have been increasingly used to evaluate the accuracy of diagnostic tests, and recent studies have shown that when gold standard tests have low SEs, Bayesian LCMs are useful for estimating the true accuracy of alternative diagnostic tests [22-24]. Given these premises, we decided to analyse the data collected according to two statistical methods: analysing the empirical (nonparametric) receiver operating characteristic (ROC) curves [25] and the Bayesian LCMs. The area under the curve (AUC) is widely recognized as the measure of a diagnostic test's discriminatory power and was computed using the trapezoidal rule. A Wald test was then used to compare the curves [26]. The graph of agreement charts (Figure 2) has been used as a valid alternative to the ROC curve graph for diagnostic tests, as explained by Bangdiwala, et al. [27]. The Bayesian LCM estimates accuracies of diagnostic tests and does not assume that any test is perfect. Rather, it considers that each test could be imperfect in diagnosing the true disease status. The true disease status of the patient population is then defined on the basis of overall prevalence. The model is then iterated using the Markov chain Monte Carlo (MCMC) method to estimate all unknown parameters, including prevalence and accuracy of each diagnostic test, and their 95% credible intervals [28]. Furthermore, Bayesian LCMs need to estimate true disease prevalence, and a 2 × 2 summary table of two diagnostic tests applied to one population does not provide enough data for this calculation [21,29]. Therefore, the Bayesian LCM analysis was performed by dividing the single WB population data set into multiple population data sets based on specific variables, such as sex and age, as suggested by Toft, et al. [30]. The Bayesian LCM was validated by checking for convergence of the Markov chains and fitness of the model used, as suggested by Lunn, et al. [31]. The final SE and SP were calculated using the OIE Real Time PCR as the gold standard, and the concordance of each test was evaluated using Kappa Coefficient (k). In order to assess the role of each variable related to concordance between diagnostic tests, a multivariable analysis of the factors listed in table 2 contributing to concordance between the PS test and PCR was conducted using a logistic multilevel mixed model (Equation (1)), with dichotomous outcome (concordance: yes/no). This model was chosen due to the fact that the logistic regression model is used to analyse the relationship between a dichotomous dependent variable and one or more independent variables. When the dependent variable is dichotomous, as in our case, the theoretical reference distribution should be the binomial distribution, rather than the normal distribution. In these cases, although it is equally possible to apply the simple regression

Table 1: Total WB controlled on each Hunting years and relative Sero and Virus prevalence (prev.) in Infected Zone (ZI) and not Infected Zone (NZI).

			Infected Zone (ZI)					Not Infected Zone (NZI)		
Hunting season	Number of WB hunted	WB Virus tested	Virus pos	Virus prev	WB Serum tested	pos	prev	WB Serum tested	pos	prev
2010/11	1596	626	0	0.00	754	16	2.12	785	0	0.00
2011/12	7775	3383	25	0.74	3817	143	3.75	3693	23	0.62
2012/13	6224	2363	11	0.47	3256	340	10.44	2759	13	0.47
2013/14	10419	2047	40	1.95	3431	269	7.84	4405	2	0.05
2014/15	11361	1479	9	0.61	3676	271	7.37	3947	8	0.20
2015/16	12734	2859	13	0.45	3549	240	6.76	6621	5	0.08
2016/17	15673	4106	39	0.95	4898	230	4.70	5354	7	0.13
2017/18	12561	5172	24	0.46	5177	198	3.82	5112	5	0.10

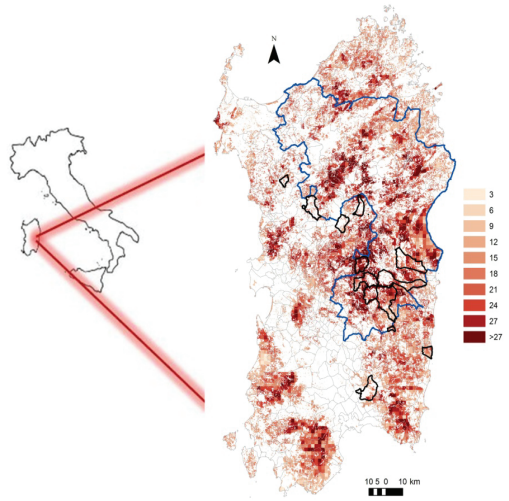

Figure 1: Wild boar density (number of animals) into the island is showed in different red-grade colors; in blue outline are delaine the WB infected area and in black the area from which the samples were collected.

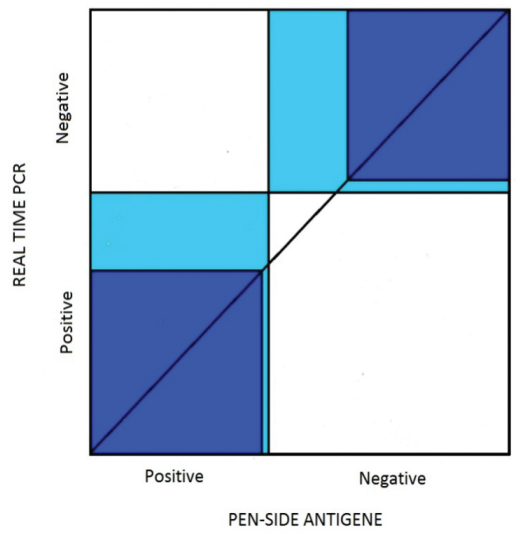

Figure 2: Final results of diagnostic PS test presented by means of agreement chart.

model, a nonlinear model would be more appropriate. After careful consideration of several potentially relevant predictors, as well as both experimental and statistical requirements (such as non-collinearity), we evaluated the variables reported in table 2 as potential covariates in our modelling analyses. Multicollinearity between variables was tested, since even when ordinary least squares assumptions are not violated, the estimation is still unbiased [32]. First, correlation coefficients between variables were calculated using Spearman non-parametric correlation coefficients. The choice of variables to be included in the final model was made on the basis of Wald's test for statistically significant results. The logistic multilevel mixed model results are presented as Adjusted Odds Ratios (OR_{adj}) calculated with a logistic regression method [33], which takes into account the effect of all the additional variables included in the analysis. All statistics tests were two-sided, with p-value<0.05 considered significant. The ROC curve analyses were performed using STATA 13.1 software (Stata Statistical Software: Release 13, StataCorp. 2013, College Station, TX, USA). The Bayesian LCM inferences were based on 50000 iterations after a burn-in for convergence of 20000 iterations. Results of the posterior estimation distributions were summarized by the median and credibility intervals. The Bayesian statistics were processed using R version 3.4.3, R to WinBUGS application version 2.1.16, and WinBUGS version 1.4.3 (Cambridge, UK) [34,35].

Results

As described in table 3, 58 hunting companies were involved from 27 different municipalities, and all samples were collected during 38 hunting days. A total of 400 WB, 182 females and 218 males, were tested with PS and Real Time PCR The age of each WB was determined via tooth analysis of the animal, and most were more than 30 months old. The average notice time of 0.85 hours (standard deviation (SD)=0.22 hours) given to the hunting company before PS test execution has been evaluated as adequate by 81.8% (324) of the veterinarians. The distance and time needed to reach the collection activity location were roughly 5 km in median (I-III quartile = 4-12 km) and 17 minutes on average (SD=0.35 minutes), respectively. All of the PS tests (400, 100%) were properly preserved, did not undergo sudden changes in temperature, and most of them were executed in a hunting company technical room (285, 71.2%). The mean temperature recorded during PS test execution was 17.7°C (SD=4.5°C), and sunny and dry was the most frequent meteorological condition (288, 72%). A mean time of 2 hours had passed between sample collection and PS test execution. The quality was defined as good for 73.3% of the samples (173), however few episodes of visceral disposal were checked (not verified = 314, 78.5%). The test execution was mainly defined as adequate (281, 70.3%). A total of 325 (81.2%) hunting companies were cooperative,

Table 2: List of the variables collected to the evaluation of the Pen-side test's performance, related to hunting, wild boar, pen-side test execution condition and pen-side test result.

Variables	Description	Abbreviation	Category
x_1	Number of municipalities	Municipalities	Hunting
x_2	Number of hunting companies	Hunting companies	Hunting
x_3	Number of hunting days	Hunting days	Hunting
x_4	Latitude	Lat	Hunting
x_5	Longitude	Long	Hunting
x_6	Sex (female/male)	Sex	Wild boar
x_7	Age of the hunted animals	Age	Wild boar
x_8	Time of notice given to hunting company before pen-side test execution (hours)	Item A-notice time	Hunting
x_9	Appropriateness of time notice related to withdrawal activities organization (yes/not)	Item B-notice enough	Hunting
x_{10}	Distance needed to reach the withdrawal activity place (km)	Item C1-distance test place	Test condition
x_{11}	Time needed to reach the withdrawal activity place (hours)	Item C2-time to test place (hours)	Test condition
x_{12}	Pen-side test preservation (yes/not)	Item D-pen-side test preservation	Test condition
x_{13}	Location of pen-side test execution (hunting company technical room, partial shelter, open field, auto)	Item E-test execution place	Test condition
x_{14}	Any temperature variations to which the test was submitted (yes/not)	Item F-test temperature variations	Test condition
x_{15}	Recorded temperature during the test execution (°C)	Item G-test execution temperature	Test condition
x_{16}	Meteorological conditions (sunny dry, humidity, raining, thunderstorm, snow)	Item H-meteorological conditions	Test condition
x_{17}	Quality of sample used for pen-side test (good, sufficient, not sufficient)	Item I-sample quality	Test result
x_{18}	Time elapsed between sample withdrawal and pen-side test execution (hours)	Item J-visceral disposal safety	Test result
x_{19}	Evaluation of visceral disposal in safety way (yes/no/not verified)	Item K-time between withdrawal and test	Wild boar
x_{20}	Global evaluation of the pen-side test execution (adequate, adequate with problems, not adequate)	Item L-adequate test execution	Test result
x_{21}	Hunter behavior (collaborative/not collaborative)	Item M-hunter behavior	Hunting
x_{22}	Any inspection in other hunting companies (yes/not)	Item N-inspection in other hunting companies	Hunting
x_{23}	Result of the Pen-side test (positive/negative)	Pen-side test result	Test result
x_{24}	Result of Real Time PCRtest (positive/negative)	Real Time PCRtest result	Test result

while 75 (18.8%) were not, and 324 (81%) other hunting company checks were possible. As reported in table 4, 44 Real Time PCR tests were positive and 356 Real Time PCR tests negative for ASFV, while 35 PS tests were positive and 365 PS tests negative. Based on ROC analysis (AUC=0.82, standard error (SE)=0.036, 95% CI [0.749-0.892], p <0.05), the PS test was moderately accurate (SE=65.9%, 95% CI [50.0-79.1]; SP=98.3%, 95% CI [96.2-99.3]; positive predictive value=82.8%, 95% CI [65.7-92.8]; and negative predictive value =95.9%, 95% CI [93.2-97.6] Sweets, 1988). The Bayesian LCM was applied for the two populations of young (0-6 and 6-18 months old) and old (18-30 and >30 months old) WB tested. Tables 5a and 5b describe the PS and Real Time PCR test results in the contingency table used to perform the Bayesian analysis. Setting Real Time PCR as the perfect gold standard and using non-informative prior Beta (0.5, 0.5) distribution for Real Time PCR test's SE and SP, the PS test detected 85.9% of true positives (PPV=85.9%, 95% CI [70.3-96.4]) and 97.4% of true negatives (NPV=97%, 95% CI [95.0-99.3]), with SE =76.5 (95% CI=59.0-92.1) and SP=98.6 (95% CI=96.9-99.7). As described by Berger, et al. [36], Bayesian LCM result estimates are reliable only when the chains in the Bayesian LCM converge properly. If the two chains do not converge, the parameters estimated by the model are unreliable. As shown in figure 3, the SE and SP chains converge, and the frequencies predicted by the Bayesian LCM fit with the observed data. Therefore, it is possible to affirm the goodness of the model's fit. Furthermore, the goodness of fit for the Bayesian LCM should be evaluated based on agreement between "frequency observed" and "frequency predicted" using Bayesian p-value and posterior predictive distribution of each profile. The Bayesian p-value is the probability that replicated data (predicted frequency) from the Bayesian model were more extreme than those from the observed data. A Bayesian p-value close to 0 or 1 indicates

Table 3: Baseline descriptions of all variables involved in Pen-side test's performance evaluation, related to hunting, wild boar, pen-side test execution conditions and pen-side test results, expressed as median (Median) and quartiles (I-III quartile), frequency (n) and percentage (%), minimum-maximum.

VARIABLES	(min-max) mean (SD); n (%); median [I-III quartile]
Municipalities	n=27
Hunting companies	n=58
Hunting days	n=30
Latitude	8,881531-9,72585
Longitude	40,193249-40,648545
Sex	
Male	218 (54.5%)
Female	182 (45.5%)
Age	
0-6 months	33 (8.2 %)
6-18 months	68 (17 %)
18-30 months	83 (20.8 %)
>30 months	216 (54 %)
Item A-notice time (hours)	0.85 (0.22)
Item B-notice enough	
Yes	324 (81.8%)
Not	0 (0%)
Partially	73 (18.2%)
Item C1-distance test place (km)	5 (4-12)
Item C2-time to test place (minutes)	17 (0.35)
Item D-pen-side test preservation	
Yes	400 (100%)
Not	0 (0%)
Item E-test execution place	
Hunting company technical room	285 (71.2 %)
Partial shelter	73 (18.3 %)
Open field	20 (5 %)
Auto	22 (5.5 %)
Item F-test temperature variations	
Yes	0 (0%)
Not	400 (100%)
Item G-test execution temperature (°C)	17.7 (4.5)
Item H-meteorological conditions	
Sunny dry	288 (72%)
Humidity	92 (23%)
Raining	20 (5%)
Thunderstorm	0 (0%)
Snow	0 (0%)
Item I-sample quality	
Good	173 (43.3%)
Sufficient	154 (38.4%)
Not sufficient	73 (18.3%)
Item J-time between withdrawal and test (hours)	2 [0.1-3]
Item K-visceral disposal safety	
Yes	78 (19.5%)
Not	0 (0%)
Not verified	314 (78.5%)
Item L-adequate test execution	
Yes	281 (70.3%)
Problematic but adequate	119 (29.7%)
Not	0 (0%)
Item M-hunter behavior	
Collaborative	325 (81.2%)
Not collaborative	75 (18.8%)
Item N-inspection in other hunting companies	
Yes	324 (81%)
Not	76 (19%)

Table 4: The contingency table based on the Pen-side (PS) test and compared to the Real Time PCR tests for African swine fever to obtain an indication of the occurrence of false positives and false negatives, used to fit the receiver operator characteristics curve.

Diagnostic tests result	Real Time PCR positive	Real Time PCR negative	Total
Pen-side positive	29 (82.8%)	6 (17.2%)	35
Pen-side negative	15 (4.1%)	350 (95.9%)	365
Total	44	356	400

Table 5a: The contingency table for young (0-6 and 6-18 months) WB population based on the Pen-side (PS) test and compared to the Real Time PCR tests for African swine fever to obtain an indication of the occurrence of false positives and false negatives, used to fit the Bayesian latent class model.

Diagnostic tests result	Real Time PCR positive	Real Time PCR negative	Total
Pen-side positive	16 (%)	1 (%)	17
Pen-side negative	8 (%)	91 (%)	99
Total	24	92	116

Table 5b: The contingency table for old (18-30 and >30 months) WB population based on the Pen-side (PS) test and compared to the Real Time PCR tests for African swine fever to obtain an indication of the occurrence of false positives and false negatives, used to fit the Bayesian latent class model.

Diagnostic tests result	Real Time PCR positive	Real Time PCR negative	Total
Pen-side positive	13 (%)	5 (%)	18
Pen-side negative	7 (%)	259 (%)	266
Total	20	264	284

that the observed result would be unlikely to be seen in replication of the data if the model was true. This means that when the Bayesian *p*-value is close to 0.5 or exactly 0.5, the Bayesian model describes the observed data very well. Values of frequency predicted should be close to values of frequency observed. Table 6 shows the *p*-values obtained from the Bayesian LCM for both young and old populations. Since all *p*-values are equal or close to 0.5, this affirms a good fitness of the final model. Figures 3a-h presents the histograms of the predictive posterior distribution of predicted frequency, and the red line represents the observed frequency of each test result profile. In each of the figures, the dataset was replicated 20000 times and selected only 2000 times (thin sampling =10) to assess the probability of observed frequencies, assuming the model was true. Finally, all variables collected during sampling were evaluated as possible explicative variables in order to detect any factors involved in discordance between diagnostic tests. The multilevel logistic model's results are reported in table 7, including four explicative variables: Item E-test execution place (open field, hunting company technical room, partial shelter, or auto); Item I-sample quality (good, sufficient, or not sufficient); Item J-time between collection and test (hours); Item L-adequate test execution (yes or problematic yet adequate); and Item M-hunter behaviour (collaborative or not collaborative), excluding all those not statistically significant. All possible interactions between variables were evaluated, and no statistically significant interaction terms were found. The results obtained by multivariable analyses performed to explore the variation of the effect size for the considered factors

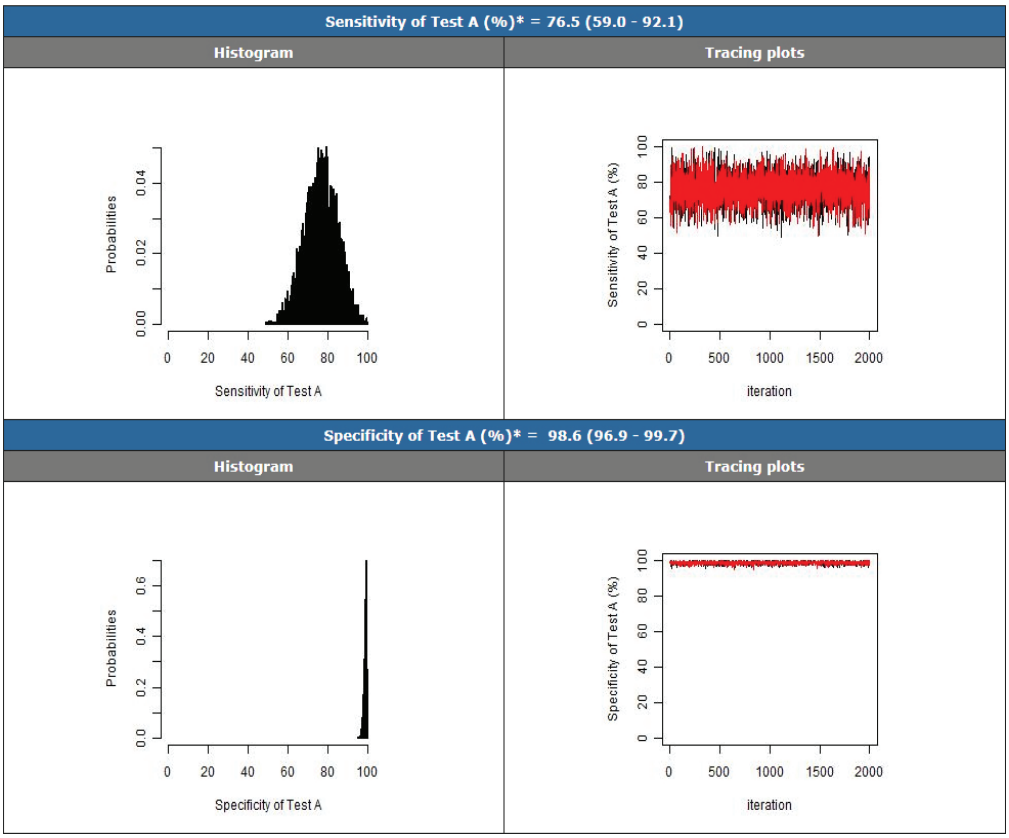

Figure 3: Output of Bayesian LCM models in term of Sensitivity and specificity of antigen detection PS test.

Table 6: Agreement between "frequency observed" and "frequency predicted" using Bayesian LCM p-value and posterior predictive distribution of each profile.

Diagnostic tests result	Pen-side	Real Time PCR	Observed	Predicted	Bayesian p-value
Young WB population	Positive	Positive	16	16	0.547
	Positive	Negative	1	1	0.499
	Negative	Positive	8	7	0.396
	Negative	Negative	91	91	0.515
Young WB population	Positive	Positive	13	12	0.463
	Positive	Negative	5	4	0.437
	Negative	Positive	7	8	0.537
	Negative	Negative	259	258	0.493

on diagnostic tests concordance highlighted that the performance of the test in a safe place, such as the hunting company technical room, improved the concordance in contrast to the execution in the open field with statistical significance (OR_{adj}=9.423 [95% CI=3.645-24.361], p<0.0001). A sample of good quality increased the probability of concordance between tests by three times, in comparison to low sample quality (OR_{adj}=2.975 [95% CI=1.602-5.527], p=0.001). An increased time span between sample collection and PS test execution was considered a significant risk factor, and analysis confirmed its tendency to hamper the concordance between tests by 30% when the time was longer than one hour (OR_{adj}=0.702 [95% CI=0.583-0.859], p<0.0001). The general test execution conditions evaluated as adequate by the veterinarians increased the concordance between Real Time PCR and PS test by approximately five times (OR = 5.217 [95% CI=2.049 -13.291], p=0.001). Furthermore, collaboration of the hunting companies with the veterinarian operations increased the concordance by 10 times (OR_{adj}=10.425 [95% CI =4.041-26.898], p<0.0001), with statistically significant results.

Discussion and Conclusion

The goal of this study was to validate the PS test for antigen detection of ASFV in the field. The efficiency in terms of SE and SP of the PS test compared to those of the Real Time PCR test defined the test as moderately accurate. The large number of samples used for validation also provides an overview of the actual situation of ASF in Sardinia and strengthen the results obtained. In an endemic ASF area, such as the Sardinian territory, the early detection of the disease is a fundamental requirement for an eradication program. As previously

Table 7: Results of logistic multilevel regression model using agreement between pen-side test and Real Time PCRtest as outcome, including statistically significant explicative variables. Results are reported as Adjusted Odds Ratio (ORadj), 95% confidence intervals (95% CI), p-values.

VARIABLES	OR_{adj} [95% CI]	p-value
Item E-test execution place		
Open field	Ref	
Hunting company technical room	9.423 [3.645-24.361]	<0.0001
Partial shelter	1.812 [1.113-2.947]	0.018
Auto	4.214 [2.353-7.541]	<0.0001
Item I-sample quality		
Goodt	2.975 [1.602-5.527]	0.001
Sufficient	1.038 [1.010-1.068]	0.008
Not sufficient	Ref	
Item J-time between withdrawal and test (hours)	0.702 [0.583-0.859]	<0.0001
Item L-adequate test execution		
Yes	5.217 [2.049-13.291]	
Problematic but adequate	Ref	0.001
Not	(none)	
Item M-hunter behavior		
Collaborative	10.425 [4.041-26.898]	<0.0001
Not collaborative	Ref	

demonstrated Cappai, et al. [14], the time taken to obtain laboratory results of WB samples is a risk factor involved in the increase of ASF outbreaks and disease spread. The use of a diagnostic device with a rapid response is essential for the surveillance and eradication program in areas of contact between WB and free range pigs. The results in terms of SP of the test demonstrate its important role in diagnosing WB as ASFV negative. The statements provided by the eradication plan established that inside the IZ, all hunted WB must be submitted for serological and virological testing. Until the test results are completed, all WB carcasses must be secured. The use of a rapid test performed directly in the field could reduce this waiting period. In fact, animals diagnosed as negative on a test with a high SP could be immediately released for consumption, allowing for a gain in time and money. In cases of positive results, measures and restrictions can be adopted immediately. However, the situation in field application could be improved considerably through the use of multiple tests (PS antigen and PS antibody) applied under a parallel test interpretation, since independent tests assess different indicators of disease [37]. The aim of using both tests in this study was to enhance the operational effectiveness of the control activities and to identify areas where further investigation is needed. The variables considered for each WB tested, those linked to test operation, and those connected to company cooperation are strictly correlated with good test results. These results support the use of this test only by trained and prepared operators. This aspect should be considered in order to achieve the test objective and to ensure the feasibility of the project in terms of cost, since most of the testing is carried out during the hunting season. A complete change in the management of disease testing during the hunting season in the future would be desirable, with operators distributed around the territory to guarantee the control and testing of the carcasses on the day of hunting, even on holidays, using the PS test.

Conflict of Interest

The authors declare no conflict of interest.

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