

Development and validation of African Swine Fever Real-time PCR kit

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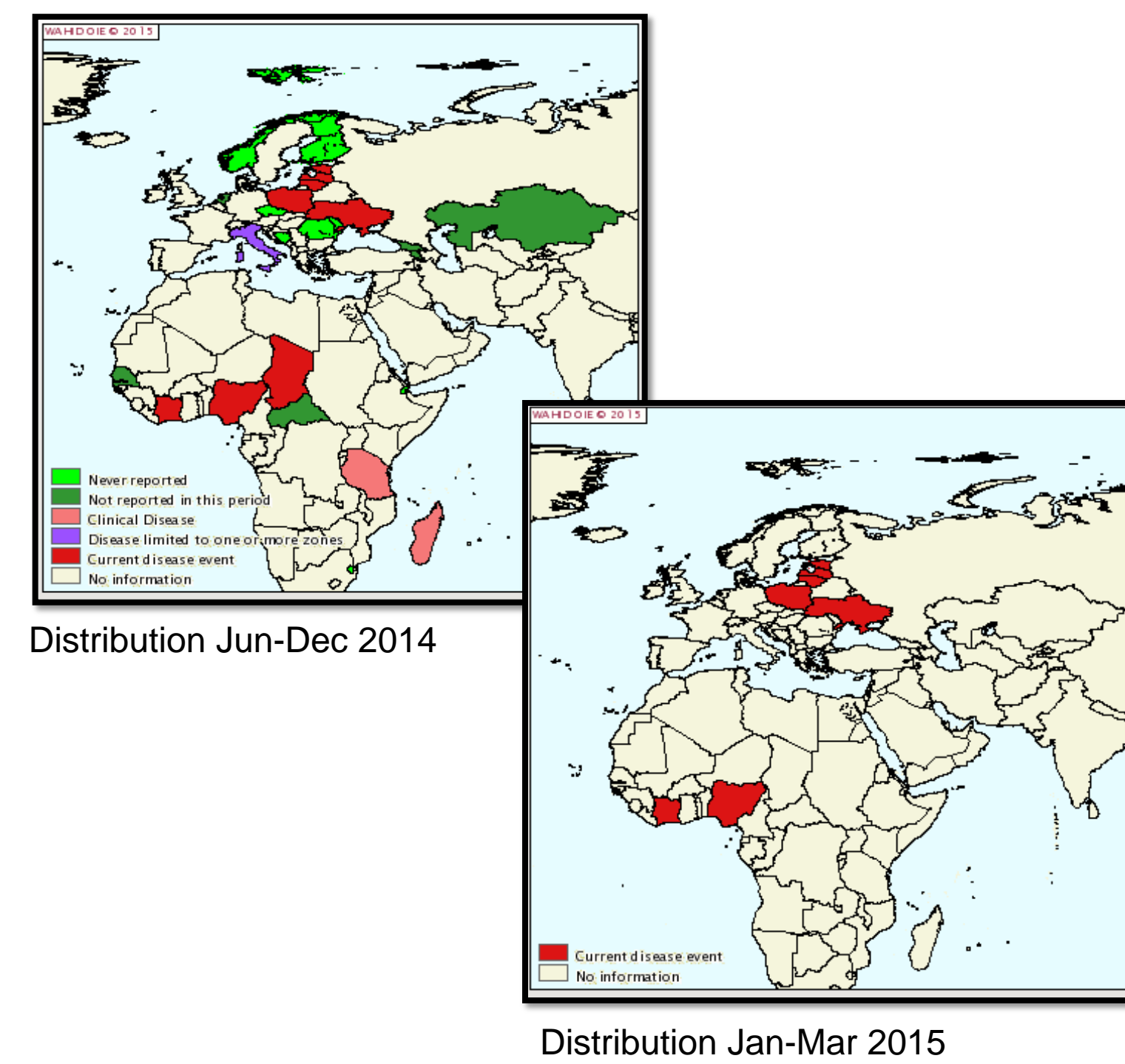
ABSTRACT

African Swine Fever Virus (ASFV) is a notifiable, highly contagious disease that can cause significant economic losses. The disease is widely endemic in many parts of Africa, of Southern Europe and increasingly becoming a threat in Eastern Europe. As there is still no vaccine or treatment available, monitoring and controlling of the disease by means of diagnosis is the only way to control the disease and is of utmost importance. A new duplex real-time PCR kit that targets the p72 gene and an internal control has been developed and its performance for diagnosis of ASFV has been assessed. In order to demonstrate the sensitivity and specificity of the new LSI VetMAX™ African Swine Fever Virus detection kit, different internal and field studies including animal infection experiments were carried out (INIA, Spain; CVI, Netherlands; Germany). 1600 negative samples from ASFV free regions (Germany & Spain) and 33 different pathogens were tested to demonstrate specificity of the assay. About 100 ASFV positive samples from Africa and Europe were also tested. Results of the ASFV kit showed 100% sensitivity in all tested sample materials (blood, serum and tissues) and 100% specificity. No cross reaction was found with other pathogens and a serial dilution of the ASFV target sequence led to a limit of detection (LOD) of 16 genome copies per PCR reaction. The experimental LOD was 5x10³ copies per mL in serum and 1x10⁴ copies per mL in blood. The LSI VetMAX™ African Swine Fever Virus detection kit fulfills all the validation criteria of PCR characteristics and complete method, as required by the NF U47-600-2 standard.

INTRODUCTION

African Swine Fever (ASF) is a DNA virus from the *Asfarviridae* Family. ASFV infects all *Suidae* (domestic and wild animals) but is not a human health threat. The virus is found in all body fluids and tissues of infected pigs. They usually become infected by direct contact with sick animals or by ingestion of infected products. ASFV is highly resistant in the environment. ASF disease is characterized by high fever, loss of appetite, haemorrhages in the skin and internal organs and death can occur within 2 to 10 days on average. ASF cannot be differentiated from classical swine fever by either clinical or post-mortem examination. It is an economically important disease that is widely endemic in many parts of Africa and that has become a real threat in Eastern Europe (Figure 1). In order to improve ASF diagnosis, a new duplex real time PCR kit was developed.

Figure 1. Disease Distribution maps - 2014 and 2015 (OIE)



MATERIALS AND METHODS

LSI VetMAX™ African Swine Fever Virus detection kit is a TaqMan™ ready-to-use real-time PCR assay based on the simultaneous detection of ASFV and an exogenous Internal Positive Control (IPC). For the development of a reliable, sensitive and specific rPCR system, more than 450 different ASFV sequences representing the p72 protein encoding region were aligned. The isolation of viral DNA from field samples was performed with MagMax™ Pathogen RNA/DNA kit and MagVet™ Universal Isolation kit. About 1600 negative samples (blood and serum) were collected from ASFV free regions (Germany and Spain) and additionally 33 different pathogens close to ASFV or found in the same ecological niches were tested to demonstrate specificity of the assay. For validation of the sensitivity about 100 ASFV positive samples from Africa and Europe were tested. The limit of detection (LOD) was determined by serial dilution of a plasmid carrying a specific ASF sequence (pASF).

RESULTS

Table 1. Specificity of LSI VetMAX™ African Swine Fever Virus detection kit (partial data)

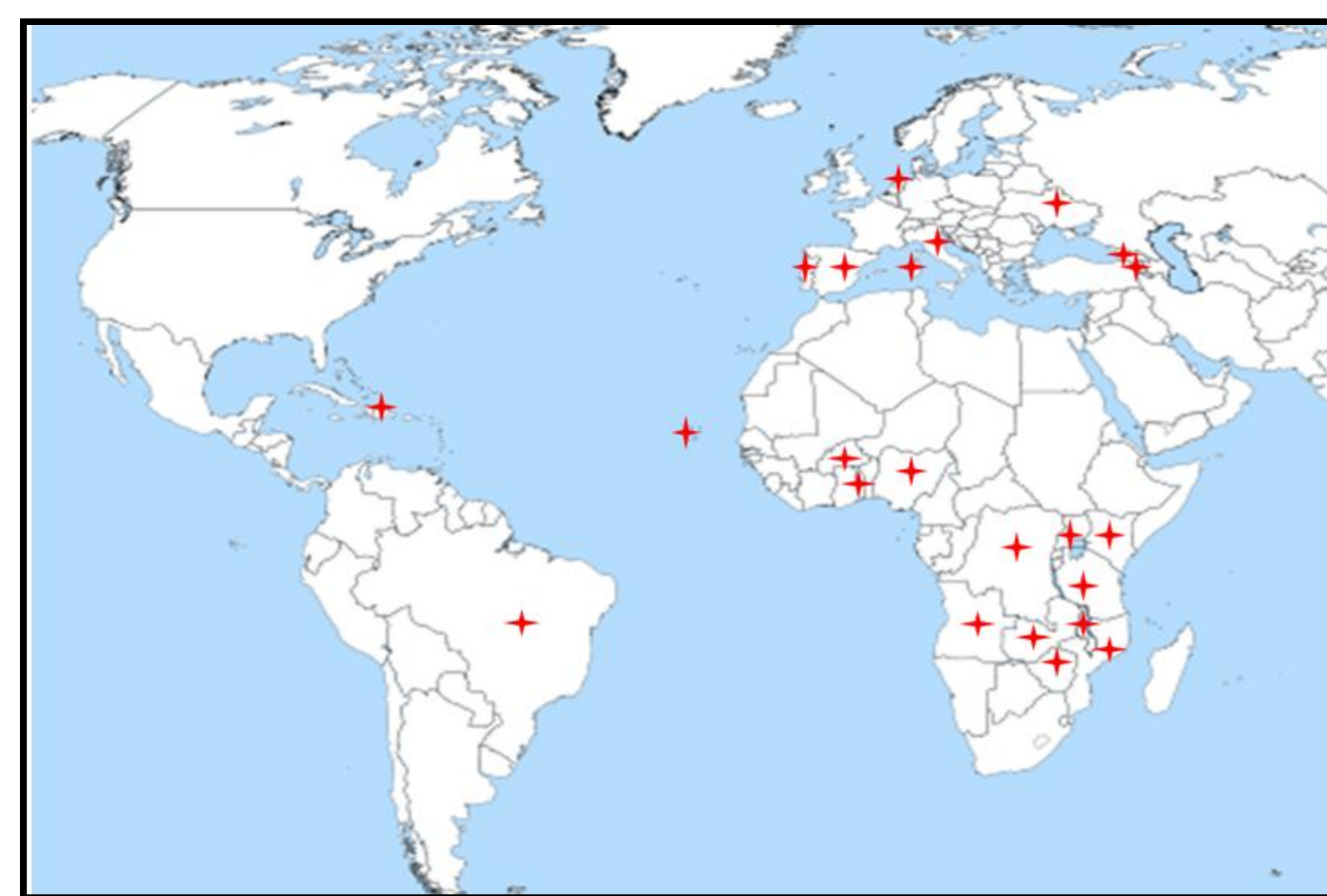
	Strain	ASFV Detection
Inclusivity	ASFV (38 reference samples from CISA-INIA)	Detected
	ASFV (20 strains from CVI)	Detected
Exclusivity	Classical Swine Fever Virus	Not detected
	Porcine circovirus 1	Not detected
	Porcine circovirus 2	Not detected
	Porcine Parvovirus	Not detected
	Herpes virus	Not detected
	PRRSV	Not detected
	Influenza H1N1	Not detected
	Mycoplasma hyopneumoniae	Not detected

The inclusivity of LSI VetMAX™ African Swine Fever Virus detection kit is evaluated on a panel of DNA isolated from 58 ASFV positive samples (organs and sera) coming from CISA-INIA, Spain and Central Veterinary Institute (CVI), Netherlands. As indicated in the table above, the kit show 100% inclusivity for the strains tested.

The exclusivity is assessed on a panel of 33 pathogens close to ASFV (data partially shown), either because they are preferentially found in the same ecological niches, phylogenetically close, or because they have the same clinical symptoms in target species. None of the strains tested is detected.

LSI VetMAX™ African Swine Fever Virus detection kit is specific for African Swine Fever Virus and does not detect other tested pathogens.

Figure 2. Distribution of strains tested for inclusivity



Strains and field samples tested (CISA-INIA, Spain and CVI, Netherlands) allow to recover a large distribution of the virus. All of them are detected by our PCR.

Table 2. Results obtained in ASFV positive strains (partial data)

Strain	ASFV Detection
Kat 67 - DR Congo	Detected
Malawi 82	Detected
Mozambique 64	Detected
Angola 72	Detected
Dominican Republic 80	Detected
Uganda 64	Detected
608 VR13	Detected
Lerida 1975 E75	Detected
Pontevedra 1970 E70	Detected
1207	Detected
BA71-V	Detected
L60 - Portugal	Detected
Haiti 78	Detected
Sassari 88	Detected
Dominican Republic 78	Detected
Lisbon 60	Detected
Georgia 2007	Detected
Spain - OURT 88/3	Detected
Tanzania KIRT 89/1	Detected
Zimbabwe VICT 90/1	Detected

A random set of 58 ASFV strains of different origins, including also the Georgia 2007 strain, which is representative for the ongoing outbreak in the Caucasus, Russia, and neighbouring countries from 2007 to 2014 were tested.

All strains are detected and the results show a very high correlation between the Ct of LSI VetMAX™ African Swine Fever Virus detection kit and the in-house developed PCR in CVI or INIA (data not shown).

Figure 3. PCR efficiency of LSI VetMAX™ African Swine Fever Virus detection kit

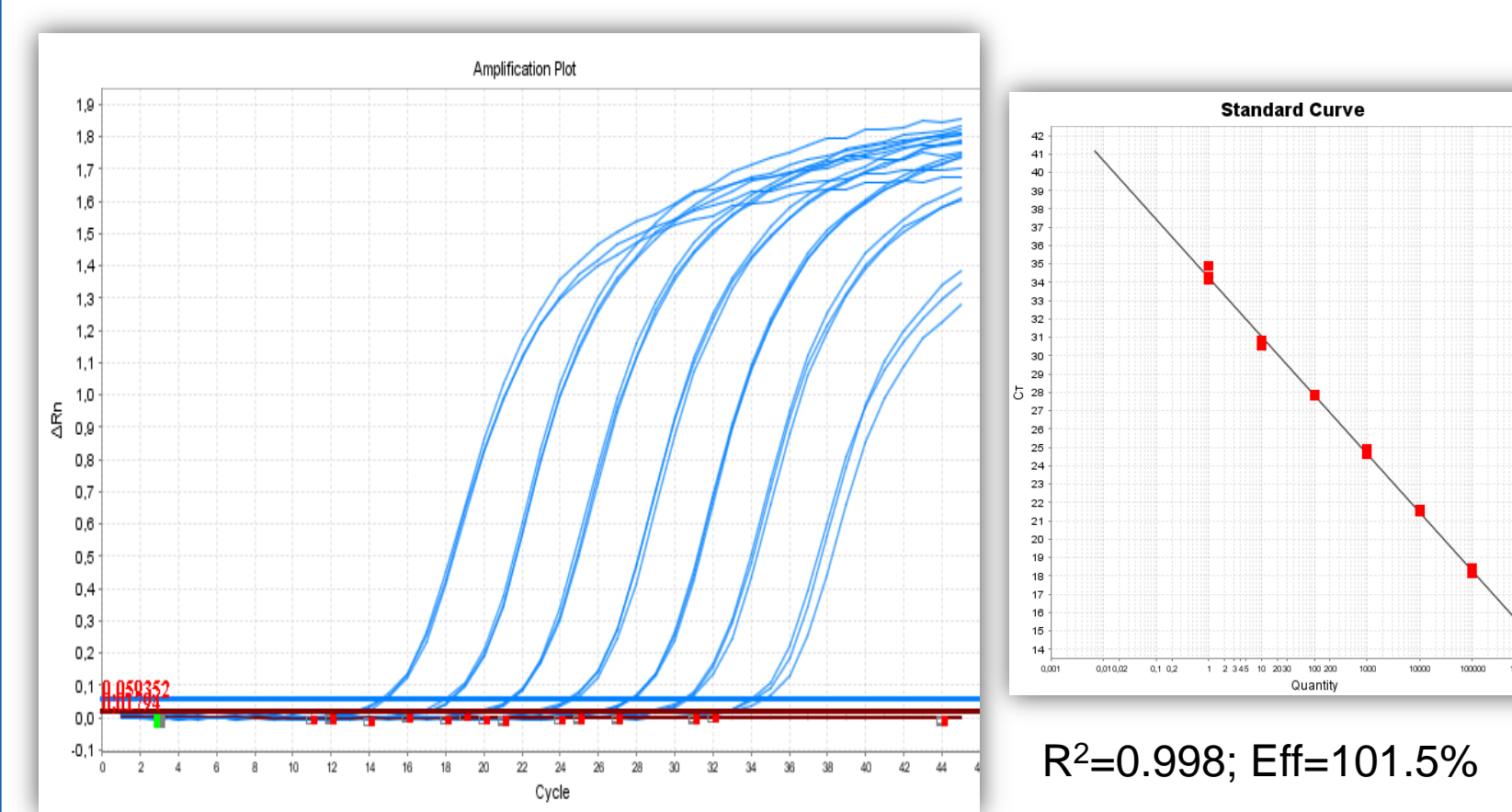


Table 3. Characteristics of LSI VetMAX™ African Swine Fever Virus detection kit according to AFNOR Standard for veterinary PCR (NF U47-600-2)

Characteristics	ASFV validation
Analytical specificity (Table 1)	100%
Efficiency (Figure 3)	Close to 100%
Limit of detection	16 copies per PCR
Repeatability	CV<3.01 %
Intermediate precision	CV<4.09 %
Robustness	Unaffected by all parameters tested
Experimental LOD - Serum	5x10 ³ cp per mL
Experimental LOD - Blood	1x10 ⁴ cp per mL

The PCR efficiency of LSI VetMAX™ African Swine Fever Virus detection kit, assessed from serial dilutions of a quantified ASF plasmid (pASF) until signal extinction, tested in triplicate, is close to 100% (Figure 3).

The limit of detection (LOD), evaluated on a quantified ASF plasmid, is estimated to be 16 copies of nucleic acids per PCR.

Repeatability and intermediate precision are evaluated at: coefficients of variation less than 4.09%.

For robustness, variations of temperature of hybridization (59° C, 60° C and 61° C), time of hybridization (54 sec, 60 sec and 66 sec), mix volume (18µL, 20µL and 22µL) and nucleic acid volume (4.5µL, 5µL and 5.5µL) do not affect the ASF PCR.

To determine the experimental limit of detection, serial dilutions of quantified plasmid are prepared to spike negative matrix at different concentration levels. The detection limit of MagMax™ Pathogen RNA/DNA method was estimated at 5x10³ copies per mL in serum and 1x10⁴ copies per mL in blood in individual samples.

Pooled assays are also performed by evaluating one positive sample among 5 or 10 samples. Serial dilutions of quantified plasmid are prepared to spike negative matrix as for individual tests. Then this positive sample is diluted in 4 or 9 negative samples to mimic pooled samples. The results of experimental LOD obtained show the same results as when tested individually (Table 4).

Table 4. Results obtained for the experimental limit of detection

Matrices	MagMAX™ Pathogen RNA/DNA Kit		
	Individual analysis	Analysis of pool of 5	Analysis of pool of 10
Serum	5x10 ³ cp/mL	5x10 ³ cp/mL	5x10 ³ cp/mL
Blood	1x10 ⁴ cp/mL	1x10 ⁴ cp/mL	1x10 ⁴ cp/mL

Pooled tests show the same experimental limits of detection (LOD) than individual tests. Furthermore this method enables to increase the analysis capacity in labs during outbreaks and reduce cost per analysis.

LSI VetMAX™ African Swine Fever Virus detection kit fulfills the validation criteria of PCR characteristics and complete method required by the NF U47-600-2 standard.

Table 5. Results obtained from field studies

Blood, Serum	Other methods		
	Positive	Negative	Total
LSI VetMAX™ African Swine Fever Virus detection kit	21	1542	1563

The results show a correlation of 100% between both methods on positive blood and serum assays and show diagnostic specificity at: Sp = 1542 / (1542+0) = 100%.

5.2. Tissues

Tissues	Other methods		
	Positive	Negative	Total
LSI VetMAX™ African Swine Fever Virus detection kit	51	6	57

The results show a correlation of 100% between both methods on negative tissues assays and show sensitivity at: Se = 51 / (51+0) = 100%.

For field studies, 1620 samples from various origins were tested. These field samples included various matrices (serum, blood and organs) at different levels of viral load (negative, low, medium, and high positive samples). 45 samples identified as positive or negative for ASF coming from a European Union Reference lab for ASFV (CISA-INIA, Valdeolmos, Spain); 1140 sera collected in a German slaughterhouse, a region free of ASFV; 400 blood samples collected on young pigs from weaning herds by a Spanish company, a region free for ASFV; and 36 samples from animal experiments carried out with 3 different ASFV strains from Central Veterinary Institute (CVI, Netherlands) were all evaluated.

Overall, in this assay, LSI VetMAX™ African Swine Fever Virus detection kit shows a diagnostic sensitivity of 100% on tissues and diagnostic specificity of 100% on blood and serum.

CONCLUSIONS

LSI VetMAX™ African Swine Fever Virus detection kit is a real-time PCR kit allowing the simultaneous detection of ASFV and an exogenous positive control in blood, serum and tissues samples.

The kit fulfills all the validation criteria for PCR characteristics and complete method required by the French standard (NF U47-600-2) "Requirements and recommendations for the development and validation of qRT-PCR in Animal Health".

The specificity, evaluated on different strains showed no cross-reactions with closely related pathogens. This kit had an efficiency close to 100% and its PCR limit of detection was 16 copies per PCR (95% confidence interval).

The experimental LOD was 5x10³ copies per mL in serum and 1x10⁴ copies per mL in blood regardless of the test (individual or pool assays). Test results on about 100 positive ASFV samples/strains and about 1600 negative samples showed 100% sensitivity on tissues and 100% specificity on blood and serum.

ASFV has significant economic impact and high mortality rate. The recent outbreaks of ASFV close to EU borders calls for a sensitive, reliable and specific real-time PCR such as the one described in this work.

LSI VetMAX™ African Swine Fever Virus detection kit provides a useful tool for an early detection of the ASF virus in various matrices from pigs and wild boars in order to guarantee the free status of pigs for trade. It helps enable control of the spread of disease and monitors circulating virus following outbreaks.

REFERENCES

- NF U 47-600-2 – Animal health analysis methods – PCR-Part 2: Requirements and recommendations for the development and the validation of veterinary PCR. (<http://www.afnor.org>)

ACKNOWLEDGEMENTS

- CISA-INIA, Valdeolmos, Spain
- CVI, Netherlands

TRADEMARKS/LICENSING

- LSI VetMAX™ African Swine Fever Virus detection kit (Cat. no. A28809)
- MagMax™ Pathogen RNA/DNA kit (Cat. No. 4462359)
- MagVet™ Universal Isolation kit (Cat. No. MV384)

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