

Verification of the detection of African swine fever virus in food matrices with real-time PCR

Summary

In this report, we show that a diagnostic workflow that includes the Applied Biosystems™ VetMAX™ African Swine Fever Virus Detection Kit and the Applied Biosystems™ MagMAX™ CORE Nucleic Acid Purification Kit is suited for the detection of African swine fever virus (ASFV) in food matrices, including cooked sausage meat.

Test performance was compared with two World Organisation for Animal Health (OIE)-prescribed and widely used ASFV qPCR assays, the King assay [1] and the Universal Probe Library (UPL) assay [2]. In all instances, the VetMAX ASFV kit detected the virus with higher sensitivity.

Both manual and automated protocols for the MagMAX CORE kit gave good results. In a comparison of manual extraction methods, the MagMAX CORE kit provided lower C_t values than a DNA extraction kit from another supplier, indicating a higher yield of high-quality DNA.

Introduction

We conducted an assessment of a real-time PCR workflow for the detection of ASFV in food matrices, specifically sausage meat for human consumption. This workflow comprises the VetMAX ASFV Detection Kit and the MagMAX CORE Nucleic Acid Purification Kit and was originally developed to detect ASFV in porcine blood and

tissues. The MagMAX CORE kit has been designed for the purification of nucleic acid from a range of veterinary sample types. The VetMAX ASFV kit is a single-well duplex qPCR assay designed to deliver quantitative results on the presence of the ASFV genome in DNA extracts. The evaluation of the workflow has been carried out using sausage meat seeded with ASFV-containing material, at virus concentrations representative of those likely to be found in food matrices. The evaluation was performed in comparison with two OIE-prescribed ASFV qPCR assays, the King assay and the UPL assay.

Results

Sensitive ASFV detection in meat samples

The results from the performance of the VetMAX ASFV Detection Kit alongside the King and UPL assays are shown in Table 1. The MagMAX CORE kit in combination with the VetMAX ASFV kit detected ASFV in all 4 spiked meat samples (A–D), with lower C_t values than those obtained with the King or UPL assays. The mean differences in C_t between the VetMAX ASFV kit and the King and UPL assays were –1.62 and –1.41, respectively. These are statistically significant using one-way ANOVA ($P < 0.044$). The mean C_t of the exogenous internal positive control (EIPC) for the VetMAX ASFV kit was 26.98 (range 26.40–27.82), which fulfills kit criteria.

Table 1. C_t values generated using 3 qPCR assays for test samples A–D.

	Mean C_t (range)			
	A	B	C	D
VetMAX ASFV Detection Kit	32.48 (32.43–32.52)	34.63 (34.13–34.90)	35.05 (34.59–35.37)	32.52 (32.17–32.73)
King assay	33.82 (33.34–34.26)	36.23 (35.24–36.71)	36.86 (35.86–37.43)	34.23 (33.64–34.74)
UPL assay	34.20 (34.19–34.21)	36.44 (35.41–37.01)	35.66 (35.09–36.45)	34.01 (33.78–34.25)

One aliquot of each test sample was manually extracted in duplicate using the MagMAX CORE kit and another supplier's DNA extraction kit (supplier 1). The extracted DNA was analyzed using the VetMAX ASFV kit, King assay, and UPL assay. Results are presented in Table 2.

DNA extracted with the MagMAX CORE Kit was found to yield lower C_t values on average than the supplier 1 kit when using the VetMAX ASFV kit (mean difference: -0.54), King assay (mean difference: -1.69), or UPL assay (mean difference: -0.42). In all instances, the VetMAX ASFV kit yielded lower C_t values than the King or UPL assays, which were found to be significant using one-way ANOVA ($P = 0.001, 0.023, 0.044,$ and 0.004 for test samples A–D, respectively). The mean EIPC C_t for the VetMAX ASFV kit was 27.15 (range 26.69–27.47), which fulfills kit criteria. For the VetMAX ASFV kit, mean C_t values of manually extracted DNA correlated well between the MagMAX CORE and supplier 1 kits ($r = 0.985, P = 0.013$). Comparing manual and automated extraction using the MagMAX CORE kit, good correlation was found for the VetMAX ASFV kit ($r = 0.993, P = 0.007$), King assay ($r = 0.934, P = 0.066$), and UPL assay ($r = 0.959, P = 0.041$).

Detection of ASFV in cooked food matrices

To determine whether the real-time PCR workflow could be used to detect ASFV in cooked food matrices comparable to those suitable for human consumption, an aliquot of each test sample was heated to represent the cooking process. Each 1 g aliquot was heated to 76°C for 15 min on a heating block, then homogenized and processed by manual extraction using the MagMAX CORE kit. The results from the three qPCR assays are shown in Table 3.

The VetMAX ASFV kit was able to detect ASFV in all cooked meat samples and yielded lower C_t values on average than the King or UPL assays; however, this difference was not found to be significant using one-way ANOVA ($P = 0.34$). The King assay did not detect ASFV in the heat-treated test sample C.

The C_t values obtained from the heat-treated aliquots were higher than those obtained from raw test samples for all three qPCR assays ($-1.66, -1.67,$ and -1.44 for the VetMAX ASFV, King, and UPL assays, respectively). However, this difference was not found to be significant using a 2-sample t -test ($P < 0.144, P = 0.322,$ and $P = 0.130$ for the VetMAX ASFV, King, and UPL assays, respectively).

Table 2. C_t values generated with manually extracted nucleic acid and each of the 3 qPCR assays.

Test sample	Mean C_t			
	Extraction kit	VetMAX ASFV kit	King assay	UPL assay
A	MagMAX CORE	33.35	34.08	34.82
	Supplier 1	33.57	35.90	36.15
B	MagMAX CORE	35.06	36.25	36.58
	Supplier 1	36.03	36.43	36.31
C	MagMAX CORE	35.98	36.90	36.00
	Supplier 1	35.04	38.95	35.93
D	MagMAX CORE	32.99	33.24	33.78
	Supplier 1	32.99	36.30	33.47

Table 3. C_t values obtained from cooked meat product using manual extraction.

	Mean C_t			
	A	B	C	D
VetMAX ASFV Detection Kit	35.85	37.12	36.80	35.25
King assay	36.24	36.84	Undetected	35.49
UPL assay	37.44	37.60	36.40	35.49

Undetected: Not detected using qPCR.

Higher PCR inhibition in cooked meat samples

The VetMAX ASFV kit includes an EIPC that allows for the assessment of PCR inhibitors and the exclusion of false-negative results. EIPC C_t values from automated and manual extraction of raw and cooked testing matrices using the MagMAX CORE kit are shown in Table 4.

Table 4. EIPC C_t values for the VetMAX ASFV kit used on raw and cooked meat samples.

	Raw		Cooked
	C_t , automated	C_t , manual	C_t , manual
Mean (\pm SD)	26.98 (\pm 0.30)	27.15 (\pm 0.21)	29.38 (\pm 0.26)
Range	(26.40–27.82)	(26.69–27.47)	(28.87–29.98)

Evaluating cooked test samples, the mean C_t for the EIPC was 29.38 (range 28.87–29.98), which was significantly higher (one-way ANOVA, $P < 0.001$) than observed with raw test samples. This indicates that the cooking process introduced PCR inhibitors, which may explain the higher C_t values observed in general for all qPCR assays and the negative results from the King assay in particular.

Conclusions

In this study, we have evaluated the detection of representative levels of ASFV contamination in food matrices. In all testing matrices, each containing different levels of ASFV spiking, samples processed utilizing the entire workflow yielded detectable ASFV. The VetMAX ASFV kit showed complete agreement with the King and UPL assays, two OIE-prescribed and widely used ASFV qPCR assays. In all instances, the VetMAX ASFV kit yielded lower C_t values on average than the King or UPL assays.

In a comparison of manual extraction methods, the MagMAX CORE kit provided lower ASFV C_t values than the supplier 1 DNA purification kit. In addition, manual extraction with the MagMAX CORE kit was used to successfully detect ASFV in cooked sausage meat. The results of this evaluation indicate that the diagnostic workflow using the VetMAX ASFV and MagMAX CORE kits is suitable for detection of the ASFV genome in food matrices.

Materials and methods

Preparation of samples for evaluation

A pig that was experimentally infected with ASFV genotype II (Georgia 2007/1 isolate) was euthanized at 8 days postinfection, having reached the humane endpoint. This experimental infection study was performed within a high-containment animal isolation facility in the United Kingdom (UK) in accordance with all local and internationally recognized animal husbandry regulations. Three sample types were obtained from this animal: loin, leg bone, and loin meat juice.

In addition, an ASFV genotype II isolate obtained from Hong Kong (Ref-SKU: 016V-03743, available through the European Virus Archive – Global (EVAg) website (european-virus-archive.com) was used in the experiments.

Sausage meat (containing >72% pork meat) was purchased from a UK supermarket and was used as the testing matrix. Four different test samples (A–D) were prepared by spiking the matrix with ASFV-containing material. The samples were tested for the presence of ASFV using the King assay [1] to determine ASFV levels. The results are shown in Table 5.

To prepare test samples A–D, 9.5 g of sausage meat was added to a labeled 50 mL centrifuge tube to which 500 μ L of spiking material was added. The material was mixed manually for 2 min, and then 1 g of the material was distributed equally to 10 labeled 15 mL centrifuge tubes. All tubes were stored at -80°C until processing.

From our experience involving previous testing of ASFV-contaminated food products, we know that ASFV is typically detected at C_t values ranging between 34 and 38. We therefore diluted the test samples using sterile PBS to obtain comparable ASFV levels matching the real-life situation in subsequent experiments.

Table 5. Test samples prepared for evaluation.

Test sample	Spike	C_t
A	ASFV Hong Kong	19.79
B	Loin meat	28.55
C	Meat juice	27.31
D	Bone marrow	20.62

Processing of meat samples—homogenization

Approximately 1 g of sausage meat was added to a sterile mortar in addition to a small quantity of sterile sand. The material was ground using a sterile pestle to produce a paste. 5 mL of sterile PBS was added, and the suspension was transferred to a 15 mL centrifuge tube. The suspension was centrifuged at 3,000 x *g* for 5 min, following which the supernatant was removed and stored at 4°C until DNA extraction.

Automated extraction of ASFV DNA using the MagMAX CORE kit

Supernatant (200 µL) from the homogenate was added to a 2 mL microcentrifuge tube, followed by 450 µL of lysis solution (prepared by mixing 450 µL of MagMAX CORE Lysis Solution and 5 µL of VetMAX ASFV Internal Positive Control). The tube was vortexed for 3 min to create the lysate, and 600 µL was added to the designated well of a deep-well plate containing 30 µL of bead/PK mix (20 µL of MagMAX CORE Magnetic Beads and 10 µL of MagMAX CORE Proteinase K). Binding solution (350 µL) was added, and the plate was transferred to a Thermo Scientific™ KingFisher™ Flex Purification System for automated extraction. The “non-heated” script was selected, and following completion of the program, ASFV DNA was eluted into 90 µL of elution buffer. DNA extracts were stored at –20°C until further analysis.

Manual extraction of ASFV DNA using the MagMAX CORE kit

Supernatant (200 µL) from the homogenate was added to a 2 mL microcentrifuge tube to which 450 µL of lysis solution was added. The tube was vortexed for 3 min to create the lysate, and 600 µL was added to a tube containing 30 µL of bead/PK mix as previously described for automated extraction. Binding solution (350 µL) was added, and tubes were placed on an orbital shaker for 10 min. The tubes were then placed in a magnetic stand for 1 min, following which the supernatant was aspirated and discarded. The beads were washed using the magnetic stand, with 500 µL of MagMAX CORE Wash Solution 1 followed by 500 µL of MagMAX CORE Wash Solution 2. The resulting magnetic bead pellet was dried for 5 min prior to eluting ASFV DNA in 90 µL of elution buffer. DNA extracts were stored at –20°C prior to further analysis.

Manual extraction of ASFV DNA using supplier 1 kit

Supernatant (140 µL) from the homogenate was added to a 2 mL microcentrifuge tube containing 560 µL of lysis buffer and was incubated at room temperature for 10 min. Absolute ethanol (560 µL) was added, and the tube was mixed by pulse vortexing for 15 seconds. Then, 630 µL of the mixture was added to a microspin column. The column was centrifuged at 6,000 x *g*, and the filtrate was discarded. The same process was repeated with the remaining 630 µL of lysate. Wash buffer 1 (500 µL) was added to the spin column, which was centrifuged at 6,000 x *g*. The filtrate was discarded, and the process repeated. Then 500 µL of wash buffer 2 was added to the spin column, which was centrifuged at 12,000 x *g* and the filtrate discarded. ASFV DNA was eluted into 50 µL of elution buffer, and DNA extracts were stored at –20°C prior to further analysis.

ASFV qPCR assays

Real-time PCR assays using the VetMAX ASFV kit were performed according to the instructions for use (Pub. No. MAN0010783). Briefly, 5 µL of extracted DNA was added to a well containing 20 µL of VetMAX ASFV qPCR master mix. Cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and then 45 cycles of PCR, with each cycle consisting of 95°C for 15 sec and 60°C for 1 min. Analysis was performed on the Applied Biosystems™ 7500 Fast Real-Time PCR System using fast ramp rates.

The King and UPL assays for the detection of the ASFV genome in porcine blood and tissues are accredited by the United Kingdom Accreditation Service (UKAS) to ISO/IEC 17025 requirements and are referenced in the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* [3]. The King and UPL assays were performed in accordance with NVR-SOP-20 and NVR-SOP-29, respectively. For the King assay, 18 µL of reaction mix was prepared using 10 µL of Applied Biosystems™ Path-ID™ qPCR Master Mix, 400 nM forward and reverse primers, and 250 nM probe. For the UPL assay, 18 µL of reaction mix was prepared using 10 µL of Path-ID qPCR Master Mix, 400 nM forward and reverse primers, and 100 nM probe. Both assays were performed using 2 µL of DNA extract. Cycling conditions were as follows: 95°C for 10 min and then 45 cycles of PCR, with each cycle consisting of 95°C for 15 sec and 60°C for 1 min. Analysis was performed on the 7500 Fast Real-Time PCR System using fast ramp rates.

Evaluation of ASFV detection system and statistical analysis

From each of the test samples A–D, aliquots 1–3 were extracted in duplicate using either the automated procedure with the MagMAX CORE kit and KingFisher Flex Purification System, the manual procedure with the MagMAX CORE kit, or the manual procedure with the supplier 1 kit. Each DNA extract was then analyzed in duplicate using the VetMAX ASFV kit, the King assay, or the UPL assay.

To assess whether the diagnostic workflow with the VetMAX ASFV kit can be used on processed food matrices, an aliquot of each test sample was heated at 76°C for 15 min on a heating block. These aliquots were manually extracted in duplicate, and DNA extracts were analyzed in duplicate using the VetMAX ASFV kit, the King assay, or the UPL assay. C_t values from all qPCR assays were then compared, and statistical analysis was performed using Minitab™ statistical software version 17 (Minitab Inc., PA, USA).

References

1. King DP, Reid SM, Hutchings GH et al. (2003) Development of a TaqMan PCR assay with internal amplification control for the detection of African swine fever virus. *J Virol Methods* 107(1):53–61.
2. Fernandez-Pinero J, Gallardo C, Elizalde M et al. (2013) Molecular diagnosis of African Swine Fever by a new real-time PCR using universal probe library. *Transbound Emerg Dis* 60(1):48–58.
3. OIE (2018) Chapter 3.8.1: African swine fever (infection with African swine fever virus). In: *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, 8th Edition.

Ordering information

Product	Quantity	Cat. No.
VetMAX African Swine Fever Virus Detection Kit	100 reactions	A28809
MagMAX CORE Nucleic Acid Purification Kit	100 reactions	A32700
	500 reactions	A32702
Path-ID qPCR Master Mix	100 reactions	4388643
	500 reactions	4388644
7500 Fast Real-Time PCR System, desktop	1 instrument	4351107
KingFisher Flex Purification System with 96 Deep-Well Head	1 instrument	5400630

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