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Evaluation of seven commercial African swine fever virus detection kits and three Taq polymerases on 300 well-characterized field samples

Marie-Eve Schoder[a,*], Marylène Tignon[a], Annick Linden[b], Muriel Vervaeke[c], Ann Brigitte Cay[a]

[a] Sciensano, Directorate of Infectious diseases in animals, Service of Enzootic, vector-borne and bee diseases, 99 Groeselenbergstraat, 1180, Brussels, Belgium
[b] Surveillance Network of Wildlife Diseases, Department of Infectious Diseases, Faculty of Veterinary Medecine, University of Liege, Avenue de Cureghem 6, 4000, Liège, Belgium
[c] Agentschap voor Natuur en Bos (ANB), Havenlaan 88 bus 75, 1000, Brussels, Belgium

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ABSTRACT

African swine fever virus (ASFV) is a complex double stranded DNA virus, responsible for a highly infectious and fatal disease in pigs and boars and for important deterioration of animal welfare. Over the last decade, the disease spread to several European and Asian countries causing unprecedented dramatic economic losses in pig industry. In the absence of a vaccine, affected countries rely on trustful diagnostic tests and adapted testing policies to set up control programs to fight against the disease. In this study, we evaluated the sensitivity and specificity of seven commercially available ASFV real-time PCR detection kits and three Taq polymerases on 300 well-characterized wild boar samples collected in Belgium during the 2018–2019 outbreak. This study confirms that all commercial kits and two Taq polymerases are suitable for ASFV detection in diagnostic laboratories. Furthermore, the use of endogenous controls is emphasized when testing field samples harvested on carcasses in an advanced stage of decomposition, in order to avoid false negative results.

1. Introduction

African swine fever virus (ASFV) is a complex double stranded DNA virus, 170–190 kbp in size, sole virus of the *Asfarviridae* family, genus *Asfivirus* (King et al., 2012). It is the causative agent of African swine fever (ASF), a disease affecting exclusively *Suidea*. ASF is a highly infectious and fatal disease, with mortality rate ranging up to 100 %, for domestic pig and boar belonging to the *Sus Scrofa* species (Dixon et al., 2004).

ASFV originates from Africa where it subclinically infects African wild pigs, such as bushpig and warthog (*Phacochoerus* and *Potamochoerus* spp.) in which it can persist for months or even years. Spreading of the disease can be very swift as transmission occurs either through direct contact with infected animals or indirect contact with contaminated fomites or ingestion of infected pork products. Furthermore, soft ticks of the *Ornithodoros* genus are an important biological vector, in the regions where they are present, such as Africa (Dixon et al., 2004). In the last decade, the global situation alarmingly deteriorated with some 50 countries worldwide affected by the disease, putting at risk the pig industry. (World Organisation for animal Health, 2019a).

The introduction of ASFV genotype II in Georgia in 2007 initiated a new epidemic situation in Eurasia (Rowlands et al., 2008). The disease rapidly spread through the Caucasus, the Russian Federation and several countries of Eastern Europe. After the Czech Republic in 2017 (Depner et al., 2017), the ASFV was introduced via an unknown source of infection into the wild boar population of Belgium during the summer 2018 (Linden et al., 2019). The situation worsened substantially in August 2018, when the Republic of China reported the first outbreak of ASF, which rapidly spread throughout the country and Southeastern Asia (FAO, 2020). The contamination from wildlife is not sufficient to explain the spread of the virus throughout the world. International trade and transport of contaminated animals, pork products or waste, as well as poor biosecurity measures in the pig production sector are arguably the underlying causes of ASFV's expansion (Chenais et al., 2019).

In the absence of a vaccine, the implementation of adapted policy instruments, such as biosecurity regulations, surveillance strategies and outbreak response policies, are important tools to prevent ASFV's dissemination in order to protect animal health. These instruments are however only effective when they rely on solid diagnosis tests for ASFV detection (Dixon et al., 2020).

Haemadsorption test, virus isolation, antigen detection by FAT (fluorescent antibody test) or enzyme-linked immunosorbent assay

* Corresponding author.
E-mail address: Marie-Eve.Schoder@sciensano.be (M.-E. Schoder).

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(ELISA), as well as conventional and real-time polymerase chain reaction (real-time PCR) are the most widely used methods for ASFV diagnosis. Haemadsorption and virus isolation are sensitive and reliable confirmatory methods for the detection of infectious virus but are also laborious and not suitable for a rapid routine diagnostic. In addition, antigen detection methods are not sensitive enough in animals with low viremia levels and can be impaired in presence of antibodies (Sánchez-Vizcaíno et al., 2019). Real-time PCR is currently preferred to the gel-based conventional PCR which is more time-consuming. Indeed, real-time PCR has been recognized to be the most rapid, sensitive and reliable method by the World Organisation for animal Health (2019b). This method relies on the amplification of conserved ASFV genome regions using primers and the detection of this amplification through the fluorescent emission of a specific probe. When working with poor-quality samples (i.e. field samples from dead wildlife), false negative results, due to damaged nucleic acids or the presence of inhibitors, are avoided by controls such as the amplification of housekeeping genes (Belák and Thorén, 2001).

Several conventional and real-time PCR methods for the detection of ASFV have been described in the literature (Agüero et al., 2003; Fernández-Pinero et al., 2013; King et al., 2003) and recommended by the OIE (World Organisation for animal Health, 2019b). Tignon et al. (2011) developed a sensitive and specific real-time TaqMan PCR assay avoiding false negative results by the inclusion of an internal endogenous extraction control amplifying the swine beta-actin gene. In addition, a previous assessment of ASFV diagnostic techniques (Gallardo et al., 2015) indicated the high sensitivity of the Universal Probe Library PCR (Fernández-Pinero et al., 2013).

In parallel, the recent ASFV introduction in Eastern Europe favored to the development of numerous commercial kits easily available on the European market.

Given the existence of such a variety of commercial diagnostic tools, there is a need for validation in order to provide confidence in these tools and assure the quality of the results. The aim of the present evaluation was to estimate the sensitivity and specificity of seven commercially available ASFV real-time PCR detection kits and three Taq polymerases regarding the ASFV Belgium 2018/1 strain.

2. Materials and methods

2.1. Commercial real-time PCR kits and Taq polymerase reaction mixes for detection of ASFV

The method described by Tignon et al (2011), amplifying a 159 bp amplicon of the p72 ASFV gene and an 114 bp amplicon of the swine beta-actin gene as a positive extraction control, was used to evaluate three commercially available Taq polymerase reaction mixes:

A AgPath-ID™ One-Step RT-PCR Reagents, Applied Biosystems™, Ampli Taq Gold™ DNA polymerase
B TaqPathTM 1-Step Multiplex Master Mix (ThermoFisher), Ampli Taq™ DNA polymerase
C SsoAdvanced Universal Probes Supermix (Bio-Rad), Sso7d fusion polymerase

Furthermore, the following seven commercial real-time PCR kits for detection of ASFV, available at the time of this study on the Belgian market, were evaluated:

D Virotype ASFV 2.0 PCR kit, (Indical, Leipzig, Germany)
E Adiavet ASFV Fast Time, (Adiagen, Ploufragan, France)
F Bio-T kit ASFV, (Biosellal, Dardilly, France)
G VetMax ASFV Detection kit, (Thermofisher, Lissieu, France)
H RealPCR ASFV DNA Test, (IDEXX, Hoofddorp, The Netherlands)
I VetAlert ASF PCR Test Kit, (Tetracore, Rockville, U.S.A)
J ID Gene™ African Swine Fever Duplex (ID.vet, Grabels, France)

2.2. Random panel generation

To evaluate the sensitivity and specificity of the real-time PCR kits, we used field samples from wild boars collected during the 2018–2019 ASFV outbreak in Belgium. The evaluation was carried out using spleen samples since spleen is the most appropriate organ to use for ASFV diagnosis in dead wildlife. We subdivided the samples into four categories depending on the initial geographic locations of the animals.

For each panel, a random selection of 50 or of 100 samples was performed among the 2048 samples available, using the Excel “Rand” function as described by the USDA, Center for Veterinary Biologics (United States Department of Agriculture, 2018). To summarize the methodology, a random number is assigned to each sample, identified by a unique code. Subsequently, the random numbers linked to each sample are sorted from smallest to largest. The first 50 (or 100) samples are then selected to be part of the various panels.

The first panel, called NEG-NORTH, consisted of 50 ASFV negative samples (out of 142) collected from wild boars hunted in Flanders, North of Belgium, where no positive ASFV case has been declared. The second panel, called NEG-SOUTH, included 50 ASFV negative samples (out of 778) harvested from wild boars hunted or culled in Wallonia, South of Belgium, in the so-called “free vigilance area” for ASFV. The third panel, called POS-SOUTH, included 100 ASFV PCR positive samples (out of 416) from wild boars infected by the genotype II ASFV Belgium 2018/01 strain and found dead or culled in infected areas of Wallonia. Finally, the fourth panel, called DUBIOUS-SOUTH, included samples collected either in infected areas of Wallonia or in areas of Wallonia that had their sanitary status changed (from free to infected), before or concomitantly to the appearance of the disease. This fourth panel included 100 samples (out of 159) characterized as either positive (7) with high crossing point (Cp) values or negative (93). In real-time PCR analysis the crossing point corresponds to the number of cycles after which the fluorescence exceeds a threshold, automatically set by the instrument.

Positive or negative status of the samples were established before the study at SCIENSANO, the Belgian National Reference Laboratory, with the in-house PCR method (Tignon et al., 2011), in which the reaction mix has been replaced by AgPath-ID™ One-Step RT-PCR reagent (description at point 2.4).

2.3. Extraction

The sample pretreatment consisted in crushing approximately 15 mg of tissue in a 2 ml microcentrifuge tube containing one stainless steel bead and 1 ml phosphate-buffered saline solution 1 × . The tubes were placed in a TissueLyser at 25 Hz for 4 min. Extractions were operated on the IndiMag48 instrument using the IndiMag® Pathogen Kit (formerly known as MagAttract® 96 cador® Pathogen Kit) from INDICAL BIOSCIENCE according to the heated off-Board Lysis Protocol in the IndiMag® Pathogen handbook. Furthermore, three exogenous extraction controls (5-IPC ASFV VetMax ASFV Detection kit from Thermofisher; IC-DNA Virotype ASFV 2.0 PCR kit from Indical; IC VetAlert ASF PCR Test Kit from Tetracore) were added to the lysis buffer. A preliminary trial revealed that the addition of more than one of those exogenous control did not impact the extraction process nor did it lead to interference during the different PCRs (results not shown). To ensure the same number of freeze-thaw cycles, individual aliquots were prepared for each extracted DNA and stored at −80 °C.

2.4. PCR

The extracted samples were tested for the presence of ASFV genome using three commercial Taq polymerase reaction mixes (A, B, C) and seven commercial ASFV detection kits (D, E, F, G, H, I, J). Each PCR kit was run according to the respective manufacturer’s instructions, regarding the PCR protocol.

The three commercial real-time reaction mixes were prepared according to the manufacturer's instructions with a final primers and probe concentration of respectively 0.6 μM and 0.3μM. The primers and probes, for ASFV's p72 protein and the beta-actin house-keeping gene, used are described by Tignon et al (2011). The two primers et probe sets were run separately in singleplex runs.

The different cycling protocols were performed, in accordance to the instructions of the manufacturers, on a LightCycler® 480 Instrument. All Cp values were calculated using the 2nd derivative method, enabling comparative studies.

2.5. Data analysis

A graphical display of Cp values distribution was obtained by a boxplots analysis. Furthermore, a linear regression was drawn in Excel for each dual comparison. The correlation value (R^2) and the slope of the regression line were retrieved to measure the strength and the efficiency of the linear regression between two tests (Marill, 2004).

Finally, the sensitivity, specificity, accuracy and precision of each commercial kit were calculated as follows (Fawcett, 2006), using method A as reference:

Sensitivity = (true positives correctly identified / total number of positive samples) × 100

Specificity = (true negatives correctly identified / total number of negative samples) × 100

Accuracy = (true results correctly identified (true positive and true negative) / total number of samples studied) × 100

Precision = (true positives correctly identified / true positives and false positives identified) × 100.

3. Results

3.1. Qualitative results

The 100 samples characterized as negative in the NORTH-NEG and SOUTH-NEG panels were confirmed negative by all evaluated methods and the 100 samples characterized as positive in the SOUTH-POS panel were confirmed positive, validating the initial sample status.

In the SOUTH-DUBIOUS panel, 88 (out of 93) samples were confirmed as negative and 2 as positive (out of 7) by all evaluated methods. Ten samples gave conflicting results between methods.

3.2. Graphical display of crossing point values

The distribution of the ASFV PCR Cp values obtained with the different commercial kits and Taq polymerase reaction mixes for the POS-SOUTH panel (100 spleen samples collected from wild boars in the infected area of Belgium) is illustrated in Fig. 1. Each set of values displays a similar distribution. Indeed, they all show maxima and minima values around respectively, 30 and 15 Cp, asymmetrical dispersions with predominance of smaller values, median values between 18,69 and 19,52 Cp and finally similar upper and lower frontiers. Nevertheless, the Taq polymerase reaction mix C (SsoAdvanced, Bio-Rad) shows a larger dispersion in Cp values.

3.3. Dual comparative evaluation of linear regression slope and correlation

An example of linear regression is shown in Fig. 2, which presents a comparison between two methods. Here, the correlation and slope are respectively, 96,01 % and 0.90. All correlations and slopes of each dual comparison regression line of the ASFV Cp values obtained with the ten evaluated methods are gathered in Table 1.

Most dual comparisons show satisfactory correlations ranging from 85,91 % (method E / method G) to 99.15% (method H / method D), whereas method C (SsoAdvanced, Bio-Rad) shows medium correlations, between 52,16 % and 65.63 %, with the other methods.

Again, most dual comparisons show good slopes close to 1, implying similar efficiencies. However, slopes between 0,54 and 0,62 were observed with method C, indicating lesser amplification efficiency.

3.4. Precision, specificity, sensitivity and accuracy

Precision, specificity, sensitivity and accuracy of each method were calculated using the Applied Biosystems™ AgPath master mix method as reference (Table 2). The four parameters are higher than 95 % for all methods.

4. Discussion

The global ASFV situation has and is still deteriorating, with some 50 countries worldwide affected by the disease leading to a major threat to the pig industry, food security and animal welfare (World Organisation for animal Health, 2019a). In particular, the on-going epidemic in China will have a serious impact on the global pig industry. China, which hosts 50% of the world domestic pig population has already indicated a 32% decline year-on-year in the national pig population with an estimated loss of 100 million pigs so far (Driver, 2019).

The current lack of cure and vaccine is attributable to different factors: firstly, inherent difficulties brought by the complexity of the virus itself (Tulman et al., 2009), secondly, the large knowledge gap concerning ASFV infection, immunity and immune evasion (Rock, 2017) and finally the genetic diversity of ASFV strains (Malogolovkin and Kolbasov, 2019). Preventing and controlling the disease is a challenge, because in order to be effective, constant surveillance, early detection, epidemiological observations and a testing program to confirm suspected cases are required. Subsequently, biosecurity measures including preventive culling of pig holdings in the infected areas and controlling of wild animal movement by fencing and trapping must be established (European Commission, 2019). In all these areas, a quick, effective and reliable laboratory support (i.e. PCR diagnosis) is necessary to fully understand ASFV outbreak patterns and the dynamic geographic coverage in order to take action as swiftly and effectively as possible (Gallardo et al., 2019).

In this study, seven commercial real-time PCR kits for detection of ASFV and three commercial Taq polymerase reaction mixes containing three different Taq polymerases, available at the time of this study on the European market, were evaluated using spleen samples collected in the field. The data set analysis allows us to conclude that all commercial kits evaluated during this trial are sensitive, specific, precise and reliable methods for a quick diagnosis and are appropriate for the detection of genotype II ASFV isolates. Indeed, they all show similar distributions and the linear regression analysis demonstrate their analogous efficiency and correlation. In addition, their precision, specificity, sensitivity and accuracy support their reliability. Only one Taq polymerase reaction mix (C) appears to be a slightly less efficient and displays a different distribution.

Furthermore, exogenous and/or endogenous controls are essential to avoid false negative results. Indeed, endogenous controls inform about the sample's quality, which is of particular interest for field samples harvested on carcasses in advanced stage of decomposition. The absence of a positive signal from the endogenous control can be an indication that the carcass was in poor condition and draws attention to unreliable negative results for ASFV. When using an exogenous control for such samples, there is a risk of false negative result. In contrast, exogenous controls guarantee the extraction step and the absence of PCR inhibitor. Therefore, in case of ASFV detection in wild boars, the use of endogenous controls is the first choice.

We observed the failure of the exogenous control for sixteen samples with the VetAlert ASF PCR Test Kit (Tetracore). For this kit, the

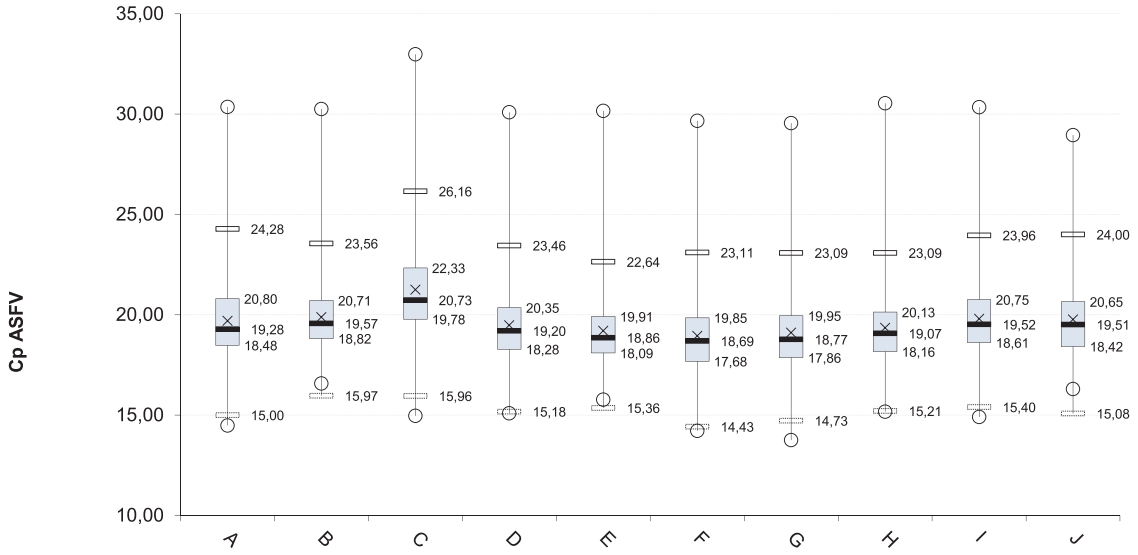

Fig. 1. Boxplots of the SOUTH-POS panel ASFV real time PCR Cp values, obtained with the various commercial kits and Taq polymerase reaction mixes. Rectangular box : Q1 (25 % of value) to Q3 (75 % of values), black band : median (50% of values), cross (x) : average value, white band : lower and upper frontiers [lower : Q1-1.5*(Q3-Q1)]; upper : Q3 + 1.5*(Q3-Q1)], white dots : min/max values.

quantitative 2nd derivative method should have been replaced by the fit point calculation method, enabling to the crossing points to be in the manufacturer's expected range. However, this calculation method was rejected as it doesn't allow comparative analysis. In addition, the endogenous extraction control failed for four samples with the SsoAdvanced Universal Probes Supermix (Bio-Rad) Taq polymerase reaction mix, possibly the consequence of inhibitors in the DNA extraction, inhibiting the Taq polymerase. This Taq polymerase displays a greater sensitivity to inhibitors on the tested panel and is therefore not recommended for field samples in poor conditions. Finally, exogenous PCR controls provided by Indical and Tetracore kits are seemingly inhibited by the amplification of the ASFV target and the endogenous control. Such results have theoretically no impact on the final diagnosis, but their validation is an issue in terms of accredited testing.

Furthermore, it is important to emphasize that each commercial PCR kit is validated with a specific extraction protocol, recommended by the producer. In our study, for comparison purposes between the different PCR performances, all samples were extracted using the same extraction method; this may have influenced the final results and the performances for some of the PCR kits.

Hence, commercially available reagents for ASFV diagnosis (*i.e.* kits and reaction mixes) are as robust and sensitive as the Belgian NRL's method described by Tignon et al., 2011. In that respect, this study provides confidence in these reagents, as they show accurate, specific and sensitive results.

Ultimately, out of a 300 samples panel, 188 were trustfully diagnosed as negative and 102 as positive. Nevertheless, the statuses of ten samples remained uncertain as they could not be confirmed by all the evaluated methods. The Cp values that were obtained for those samples are at the limit of detection (between 35 and 40 Cp).

A previous assessment of ASFV diagnostic techniques (Gallardo et al., 2015) also underlined the necessity to combine the ASFV virological detection with a serology screening, using enzyme-linked immunosorbent assay (ELISA) or indirect immunoperoxidase test (IPT), in order to detect animals that were previously infected by the virus but survived the infection. Indeed, it has been shown that an indefinite fraction of animals can survive the infection. Whether they can be healthy carrier and transmit the disease is still under debate (Eblé et al.,

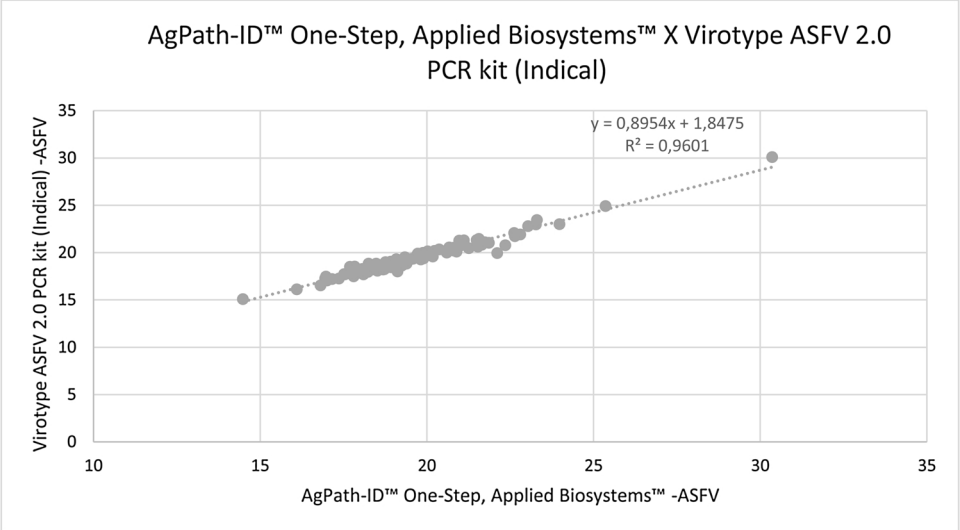

Fig. 2. Example of linear regression. Linear regression of the ASFV real time PCR Cp values of the AgPath-ID™ One-Step RT-PCR Reagents Master mix and the Virotype ASFV 2.0 PCR kit (Indical) method. R^2 corresponds to the correlation and the coefficient "a" of the linear function (y = ax + b) to the slope.

Table 1
Correlation (light grey) and slope (dark grey) of each dual comparison regression line of the ASFV Cp values obtained with the different methods (A, B, C, D, E, F, G, H, I., J).

	A	B	C	D	E	F	G	H	I	J
A		91,21	56,01	96,01	89,62	95,49	93,86	94,75	95,42	80,30
B	1,06		55,43	93,98	93,77	91,08	84,23	94,72	90,69	89,31
C	0,62	0,54		59,72	65,63	61,95	52,16	60,22	59,24	70,31
D	0,90	0,95	0,58		95,14	98,48	93,12	99,15	98,57	84,49
E	0,86	0,93	0,61	0,97		93,59	85,91	96,35	93,0	89,38
F	0,91	0,96	0,60	1,01	0,99		92,53	97,46	98,24	82,31
G	0,99	1,06	0,61	1,08	1,05	1,06		91,74	93,25	77,87
H	0,90	0,95	0,59	1,01	1,00	0,98	0,86		98,11	86,44
I	0,90	0,94	0,58	1,00	0,98	0,98	0,86	0,98		83,34
J	1,03	1,06	1,17	0,97	0,99	0,97	1,04	0,99	0,96	

Table 2
Precision, Specificity, Sensitivity and Accuracy of the different kits compared to method A (AgPath-ID™ One-Step RT-PCR Reagents, Applied-Biosystems™).

	Precision	Specificity	Sensitivity	Accuracy
A	100,00%	100,00%	100,00%	100,00%
B	100,00%	100,00%	100,00%	100,00%
C	98,11%	98,96%	97,20%	98,33%
D	97,22%	98,45%	98,13%	98,33%
E	100,00%	100,00%	99,07%	99,67%
F	99,07%	99,48%	99,07%	99,33%
G	97,22%	98,13%	98,45%	98,33%
H	97,22%	98,13%	98,45%	98,33%
I	98,17%	100,00%	98,96%	99,33%
J	98,11%	98,96%	97,20%	98,33%
Average	98,38%	99,14%	98,67%	98,96%

2019; Ståhl et al., 2019).

5. Conclusion

Considering the unpredicted introduction of ASFV in Belgium and in the Czech Republic as well as the constant spread of the disease across Eastern Europe, all countries have to be ready to react rapidly to this threat that can hit unexpectedly. The ASFV real-time PCR reagents, evaluated during this study, have proven to be suitable for diagnostic laboratories working on ASFV detection.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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