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Evaluation of different methods for preventing the African swine fever virus

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African swine fever (ASF) is a contagious disease of key importance that occurs in wild boar population and domestic pigs. To date, there is no vaccine or treatment available, hence disease surveillance and stamping out in case of an outbreak is the only way to control it. A duplex ASF-IC real-time PCR assay was optimised using field samples obtained in Lithuania and results of singleplex and duplex real-time PCR assays allowing the identification of the ASF virus were compared. The specificity and sensitivity of ASF virus detection was validated using a reference sample of positive and negative virus nucleic acid and ASF virus positive and negative samples obtained from animals in Lithuania. Results prove that duplex ASF-IC real-time PCR assay provides a rapid, sensitive, and reliable molecular tool for ASF virus detection in wild boar population and domestic pigs. In general, it was found that duplex ASF-IC real-time PCR assay proves to be faster and more sensitive than singleplex real-time PCR and therefore has a high potential to be applied in routine analysis.

Keywords: African swine fever virus detection, domestic pigs, duplex real-time PCR assays, singleplex real-time PCR assays, wild boar

INTRODUCTION

African swine fever (ASF) is a complex and lethal viral disease affecting domestic pigs and wild boar population, which also has a significant socioeconomic impact worldwide. It has a major negative effect on national, regional, and international trade in live pigs and pigs' meat (Gallardo et al., 2015a). The devastating acute form of the disease is characterized by functional and congestive-haemorrhagic disorders of the digestive and respiratory systems. Case fatality rates may be as high as 100% (Sánchez-Vizcaíno et al., 2015).

ASF is caused by a large DNA virus belonging to the family *Asfarviridae* (Dixon et al., 2005). The virus genome is 170 to 192 kbp long (Chapman et al., 2011). ASF was first described in Kenya in the 1920s as an acute haemorrhagic fever, which at that time caused case fatality approaching 100% in domestic pigs. Disease outbreaks occurred when domestic pigs came

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into close contact with wildlife species, particularly warthogs (*Phacochoerus aethiopicus* and *Phacochoerus africanus*). The source of the infection was identified as a virus carried by warthogs, which did not show clinical disease (Montgomery, 1921). ASF has been confined mainly to sub-Saharan Africa, where it is maintained in a sylvatic cycle and among domestic pigs (Costard et al., 2009). Historically, ASF outbreaks were reported in Africa, and parts of Europe, South America and the Caribbean. Since 2007, ASF has been reported in many countries across Africa, Asia, and Europe (OIE 2020). On 24 January 2014, two cases of ASF in wild boars were found in Lithuania and later in wild boars in Latvia, Estonia, and part of Poland bordering on Belarus (Gallardo et al., 2014; Pejsak et al., 2014a; Pejsak et al., 2014b). ASF spread further in Moldova, the Czech Republic, and Romania in 2017, in Hungary, China, Belgium, and Mongolia in 2018, and further to South East Asia (Mazloum et al., 2019). Since no vaccine is available against ASF, the only way to control and eradicate the disease and the spread of the virus is based on classical disease control methods, such as surveillance, rapid detection and identification of ASF virus-specific antigens or DNA and antibodies, and stamping out of infected animals (OIE, 2012, Gavier-Widen et al., 2015). To detect ASF antibodies, ELISA (enzyme-linked immunosorbent assay), indirect immunoflourescence (IIF), or the indirect immunoperoxidase test (IPT) are recommended (Gallardo et al., 2015; CISA INIA, 2019). For ASF virus genome detection by PCR, several methods can also be used, for example, OIE conventional PCR (Agüero et al., 2003), OIE real-time PCR (King et al., 2003), Universal Probe Library (UPL) real-time PCR (Fernández-Pinero et al., 2013), and duplex real-time PCR (Haines et al., 2013). Moreover, there are commercial kits for ASF detection – 'virotype ASFV PCR kit' (Qiagen), 'ID-Gene™ African Swine fever Duplex' (ID-Vet), and others.

Regarding the selected appropriate primers and the probe, reaction buffers and annealing temperature, the goal was to optimize ASF duplex assay in our laboratory. Based on research conducted by Haines et al. (2013) into simultaneous detection of ASF and IC, that allows proper detection of the ASF virus and internal control in one reaction tube and a comparison of singleplex (250bp fragment) and duplex (75bp fragment) real-time PCR assays for ASF virus detection using different primers, probes, and master mix. There is an important and urgent need to develop new and rapid molecular assays for the diagnosis of ASF with high sensitivity and specificity.

MATERIALS AND METHODS

Samples. Reference samples (INIA, Spain) and ASF virus positive and negative field samples of blood (EDTA stabilized and serum), kidney, spleen, muscle, lungs, bone marrow, and heart collected in 2014–2015 from domestic pigs and wild boars from affected areas in Lithuania were used for this study. Reference samples of Koi herpesvirus, avian influenza virus, and classical swine fever were used to evaluate analytical specificity of duplex ASF-IC real-time PCR assay.

DNA extraction and real-time PCR. Total DNA was extracted from 140 µL of a wide range of samples: blood-EDTA and serum using a Viral RNA Extraction Kit (Qiagen, Germany) and homogenized tissue using a RNeasy Mini Kit (Qiagen, Germany), following the manufacturer's procedure. The final elution was done with 80–125 µL elution buffer. Extracted DNA was stored at –20°C for further analysis. Field and reference samples were tested to detect the ASF virus genome using the primer and probe sequences summarized in Table 1.

Luminaris Probe High ROX qPCR Master Mix (2x) (Thermo Fisher Scientific), Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) modified with UNG and Quantitect Multiplex PCR kit (Qiagen, Germany) were used for study. The reaction mix contained 5 µL of extracted DNA and 12.5 µL 2x reaction buffer, primers and probe for target

Table 1. **Primer and probe system used in singleplex and duplex real-time PCR assays**

Primer/Probe	Sequence	Reference
	Singleplex detection	
	ASF	
King's F	5'-CTG CTC ATG GTA TCA ATC TTA TCG A-3'	(King et al., 2003)
Kings's R	5'-AGT ACC ACA AGA TCR GCC GT – 3'	
ASF Probe 250	5'-AM-CCA CGG GAG GAA TAC CAA CCC AGT G-TAMRA-3'	
	Internal control (IC)	
IC2AI-F	5'-TCG AGG GCG ACA CCC TG-3'	(Agüero et al., 2003)
EGFP-R	5'-CTT GTA CAG CTC GTC CAT GC-3'	
EGFP-probe	5'-VIC-AGC ACC CAG TCC GCC CTG AGC-MGB-3'	
	Duplex detection	
	ASF	
ASFVp72-IVI F	5'-GAT GAT GAT TAC CTT YGC TTT GAA-3'	(Haines et al., 2013)
ASFVp72-IVI R	5'-TCT CTT GCT CTR GAT ACR TTA ATA TGA-3'	
ASFV probe	5'-FAM-CCA CGG GAG GAA TAC CAA CCC AGT G-TAMRA-3'	
	Internal control (IC)	
EGFP1-F	5'-GAC CAC TAC CAG CAG AAC AC-3'	
EGFP2-R	5'-GAA CTC CAG CAG GAC CAT G-3'	

and internal control. Primers ASF King F and ASF King R at concentration of 400 nM and probe 250 at concentration of 100 nM were used for singleplex real-time PCR. Concentration of primers and probe for ASF in duplex real-time PCR was optimized in final reaction volume of 25 µL to obtain minimal threshold cycle. Concentration of primers and probe for duplex internal control was optimised from 100 nM. Concentration of primers and probe for duplex ASF-IC real-time PCR was optimized to obtain maximum sensitivity for target and internal control in the same reaction (Tables 2, 3). Real-time PCR reaction was carried out using 96-well plates by Applied Biosystems real-time PCR system. Samples

Table 2. **Optimization of ASF duplex primer concentration. Ct value at different primer concentrations**

ASF primer concentration, nM	Ct			Average	Standard deviation
	Session 1	Session 2	Session 3		
900	29.1 ± 0.3	28.6 ± 0.05	28.6 ± 0.07	28.8	0.3
800	28.7 ± 0.07	28.8 ± 0.1	28.7 ± 0.06	28.7	0.09
700	29.2 ± 0.3	29.0 ± 0.2	28.8 ± 0.06	29.0	0.27
600	29.5 ± 0.04	29.1 ± 0.2	28.9 ± 0.1	29.1	0.28

Table 3. **Optimization of ASF duplex probe concentration. Ct value at different probe concentrations**

Probe concentration, nM	Ct			Average	Standard deviation
	Session 1	Session 2	Session 3		
100	28.9 ± 0.2	29.6 ± 0.09	29.6 ± 0.2	29.3	0.3
200	28.5 ± 0.07	29.4 ± 0.1	29.4 ± 0.07	29.1	0.4
300	28.4 ± 0.06	29.2 ± 0.2	29.5 ± 0.1	29.0	0.5
400	28.4 ± 0.04	28.8 ± 0.2	29.2 ± 0.3	28.8	0.4

were amplified using the following conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and at 60°C (singleplex ASF assay) for 1 min. Duplex ASF-IC real-time PCR annealing and extension temperature (58–63°C) was optimised. Repetitiveness of the method was assessed using diluted reference ASF samples by repeating three times on different days. Data analysis was performed by following criteria: if a fluorescent signal is lower than a value of 38 ct then the samples would be proved as positive, and if a fluorescent signal is equal to 38 ct or higher, then these samples are repeated several times for confirmation.

Statistical analysis. The measured variables were tested for normality using the Kolmogorov-Smirnov test, and the hypotheses on data normality were not accepted. Therefore, the nonparametric Mann-Whitney U test was used. For all hypotheses, statistically significant differences were accepted at a significance level of $P = 0.10$ (Forthofer et al., 2007; Cuadra-Sánchez et al., 2012).

RESULTS

Optimization of concentration of ASF primers and probe thermo-cycling conditions and buffers for duplex ASF-IC real-time PCR. Regarding the selected appropriate primers and probe, reaction buffers and annealing temperature, the goal was to develop a duplex real-time PCR assay for ASF and IC simultaneous detection that allows the proper detection of ASF virus and internal control in one reaction tube which does not affect the sensitivity of the method.

For optimization of ASF duplex primer concentration, was selected to perform the tests using primer concentration 600–900 nM (Haines et al., 2013). Diluted reference ASF samples were used for the tests (Table 2).

The lowest Ct value was obtained at 800 nM ASF primer concentration. Also, using this concentration of primers, the standard deviation between tests was the lowest expecting the most repetitive results. Statistical analysis of primer concentration was performed using the nonparametric Mann-Whitney U test. Almost all measurements of different high concentrations of primers showed statistically significantly differences at a significance level of $P = 0.10$.

ASF probe concentration for duplex study varied from 100 nM to 400 nM (Table 3).

By using the nonparametric Mann-Whitney U test for low concentrations, clearly defined tendencies were not observed. Differences between probe concentrations were altered without any consistent pattern. Studies with different combinations of probe concentration for the detection of the ASF virus determined that probe concentration had no influence on the results. The lowest selected probe concentration was 100 nM.

Similar studies were carried out for internal control using different (100–300 nM) primer and probe concentrations, and the data showed that there were no influences on results. The selected concentration was 100 nM (data not shown).

The primer melting temperature for DNA matrices can be calculated using two methods: according to the formula Tm = 2 (A+T) + 4(G+C) (Von Ahsen et al., 2001), or

by using the Thermo Fisher online calculator (Thermo Fisher Scientific). Comparing the results with different temperatures in all three sessions showed that clearly defined tendencies were not observed (Table 4).

Therefore we can state that the analysis of temperature changes based on the nonparametric Mann-Whitney U test did not result in significant outcomes. After the experiment of the optimisation of primer annealing temperature, the optimal primer annealing temperature of 62°C was selected only by the assessment of the profile of the amplification curves.

Three different reaction buffers for singleplex and two different reaction buffers for duplex real-time PCR were used for ASF virus amplification from positive samples with primers listed in Table 1. Summary data in the Box and Whisker Plot are presented in Figure.

Table 4. **Optimization of primer annealing temperature. Ct value at different primer annealing temperatures**

Temperature, °C	Ct			Average	Standard deviation
	Session 1	Session 2	Session 3		
58	29.4 ± 0.1	28.9 ± 0.3	28.9 ± 0.2	29.1	0.3
59	28.9 ± 0.2	28.6 ± 0.2	28.7 ± 0.1	28.8	0.2
60	28.8 ± 0.1	28.7 ± 0.2	28.5 ± 0.2	28.6	0.2
61	28.6 ± 0.07	28.4 ± 0.1	28.3 ± 0.3	28.5	0.2
62	28.6 ± 0.1	28.3 ± 0.2	28.2 ± 0.1	28.4	0.1
63	28.8 ± 0.2	28.7 ± 0.2	28.7 ± 0.1	28.7	0.2

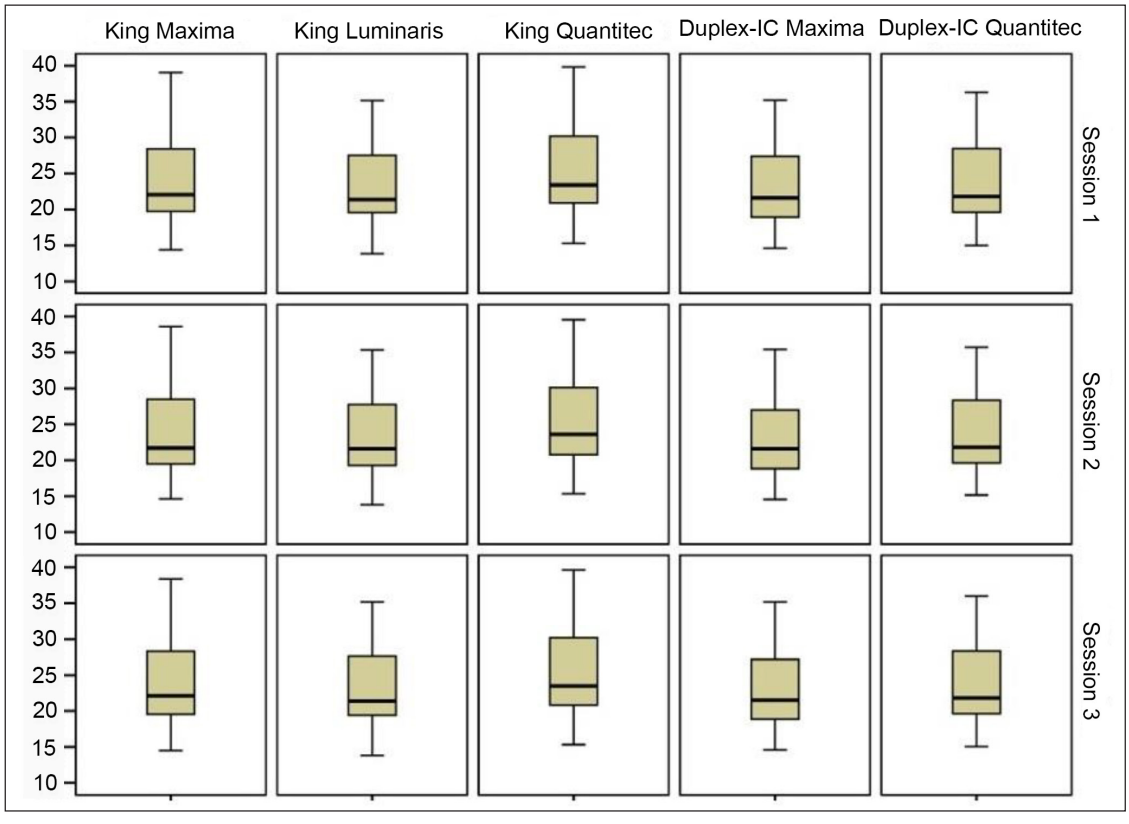

Figure. Real-time PCR results depending on ASF primers, buffers, and different real-time PCR sessions

Statistically significant differences in all three sessions were observed at a significance level of $P = 0.10$ using ASF King Quantitect Multiplex PCR kit (Qiagen) vs. Duplex ASF-IC Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific). In all other cases there were no significant differences ($P > 0.10$). Study results confirmed that the reaction is more efficient if synthesized fragments are shorter. Shorter fragments were denatured effectively in each reaction cycle.

Buffers Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) and Quantitect Multiplex PCR Master Mix kit (Qiagen, Germany) were selected for further tests. Using these buffers, the synthesized ASF virus DNA fragment determined lower Ct values. These cases were observed to have statistically significant differences. Non-specific amplification curves were obtained from duplex assays with buffer Luminaris Probe High ROX qPCR Master Mix (2X).

Analytical specificity. The nucleic acid extracted from positive ASF reference samples, positive reference samples of Koi herpes virus (KHV), Avian influenza virus (AIV), and classical swine fever virus (CSF) were used to evaluate analytical specificity of duplex ASF-IC real-time PCR assay. ASF reference samples were all positive. The nucleic acid from the reference samples of Koi herpes virus, Avian influenza virus, and classical swine fever were all negative. Results and Ct values are presented in Table 5.

Comparison of singleplex and duplex assay. For confirmation of validated duplex real-time PCR assay, ASF virus positive samples were tested in duplex ASF-IC real-time PCR and ASF real-time PCR (without IC) using Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) (Table 6).

The nonparametric Mann-Whitney U test showed that there were no statistically significant differences between measurements of strongly positive samples for ASF real-time PCR and duplex ASF-IC real-time PCR with P-value, $P = 0.10$.

Duplex ASF-IC real-time PCR is more sensitive and faster than singleplex ASF real-time PCR in detection of the ASF virus in blood, bone marrow, heart, kidney, liver, spleen, lungs, muscle, and meat products of domestic pigs and wild boars. Comparing singleplex real time PCR and duplex real-time PCR results, the difference in Ct value is 0.55–8.4, which means that 0.3–70.5 times more of the reaction product is synthesized. The internal control for ASF was detected in negative samples; this confirms the absence of inhibition in the sample. The ASF virus and the internal control were detected in one tube.

Table 5. **Analytical specificity for duplex AS-IC real-time PCR assay. Average of the crossing point (threshold cycle) for duplex ASF-IC real-time PCR using different master mixes**

Samples	Ct value of real-time PCR	
	Maxima™ Probe qPCR Master Mix	Quantitect Multiplex Master Mix
ASF PC 17	28.8	29.9
ASF PC 19	25.7	26.7
ASF PC 29	23.5	24.5
KHV PC	not detected	not detected
AIV PC	not detected	not detected
CSF PC	not detected	not detected

Table 6. **Sample analysis with singleplex and duplex real-time PCR. Comparison of clinical sample detection using duplex ASF-IC and singleplex ASF analysis**

Type of sample	Singleplex ASF Real-time PCR	Duplex ASF-IC Real-time PCR	
	ASF Ct	ASF Ct	IC Ct
Blood	22.3	21.8	28.9
	24.3	18.8	No Ct
	31.4	28.4	27.6
	27.3	24.9	27.5
	21.6	21.5	28.4
	24.5	19.6	Not detected
	34.3	30.6	27.5
	38.1	35.4	27.5
	30.2	25.8	28.5
	15.3	15.1	Not detected no Ct
	26.5	25.4	28
	19.8	20.1	Not detected
Bone marrow	29.6	28.4	27.6
	33.6	31.5	27.9
	24.4	23.4	27.6
Heart	27.6	28.4	26.9
	32.5	32.7	27.6
	25.2	26.4	27.8
	24.2	25.4	27.5
	32.5	33.2	27.9
Kidney	26.6	25.6	27.2
	20.7	19.9	31.6
	21.2	21.9	27.1
	26.4	27.7	27.2
	25.9	26.6	27.2
	24.4	25.4	27.7
	34.9	35.0	28.2
Liver	18.9	16.9	Not detected
	35.6	32.5	27.5
	22.1	19.1	Not detected
	20.9	21.7	27.5
	18.4	19.8	Not detected
	22.5	23.5	29.1
	20.69	21.6	33.5
	22.3	22.8	28.2
Tissue	23.2	21.6	30.8
	25.6	24.5	27.6

Type of sample	Singleplex ASF Real-time PCR	Duplex ASF-IC Real-time PCR	
	ASF Ct	ASF Ct	IC Ct
Lungs	20.5	21.5	32.8
	24.8	25.5	27.5
	20.4	21.7	28.9
	21.5	22.5	27.7
	24.7	25.5	27.2
Lungs/ spleen	24.2	23.9	27.5
	20.4	19.9	32.1
	21.2	18.7	Not detected
Meat product	Not detected	31.6	30.8
	Not detected	34.5	29.9
	30.6	28.1	27.7
Muscle	36.8	36.0	27.6
	34.4	33.1	28.9
	39.7	33.5	29.8
Spleen	19.5	18.5	Not detected
	30.5	28.9	27.5
	18.5	17.8	Not detected
	19.4	18.7	Not detected
	26.9	25.6	27.4
	23.5	22.3	28
	17.7	16.7	Not detected
	21.3	20.7	29.8
	20.4	19.7	36.4
	20.8	20.7	31.1
	35.3	33.1	28.1
	21.7	21.5	30.4
	18.2	17.5	Not detected
	22.6	21.8	28.5
	22.4	21	35.3
	18.6	16.6	Not detected
	20.8	20.1	34.2
	17.8	18.9	Not detected
	20.2	21.5	28.7
	18.3	19.7	Not detected
	21.7	22.7	27.6
	22.7	23.4	27.4

DISCUSSION

ASF has emerged in several European countries (EFSA, 2020). Since it is complicated to control the Asian wild boar population and its movements, ASF is progressively spreading among the animals in this area (Guberti et al., 2019; FAO, 2020). Although large pig farms are better protected by biosecurity, ASF-infected wild boars contaminating the environment pose a threat (Gavier-Widen et al., 2015). The real-time PCR is the main diagnostic tool when the viral isolates are lethal to animals. However, due to the presence of strains of reduced virulence that result in lower mortality, the ASF disease can be diagnosed by using serological tests (Gallardo et al., 2015b). Laboratory testing of wild boars and domestic pigs is essential for active and passive surveillance and prevention of new ASF outbreaks and implementation of effective control programmess (Guinat et al., 2017). Both singleplex and duplex real-time PCR assays enable detection of the ASF virus and can be used as more sensitive test methods for the detection of the ASF virus (King et al., 2003; Haines et al., 2013). Therefore, a specific duplex (simultaneous detection of ASF and IC) real-time PCR assay was analysed using different real-time PCR Master Mix: Luminaris Probe High ROX qPCR Master Mix (Thermo Fisher Scientific), Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific), and Quantitect Multiplex PCR kit (Qiagen, Germany). Specific primers and the probe used resulted in amplifying a 250-bp region and 75-bp region of the p72 gene. Amplification results demonstrated that the duplex real-time PCR assay is able to reliably detect sensitivity and is much quicker when the most suitable means are used: Master Mix which is Maxima Probe/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific) and Quantitect Multiplex PCR kit (Qiagen, Germany). However, duplex ASF-IC real-time PCR assay optimization of primers, probe concentrations, and annealing temperature ensures optimal efficiency in real-time PCRs. For the detection of the ASF virus, the optimal primer concentration for duplex ASF is 800 nM and optimal probe concentration was 100 nM while the optimal primer annealing temperature was observed to be 62°C. The study was carried out using different primer concentrations for internal control. Study results showed that concentration of primer for internal control did not influence the detection of ASF virus. Real-time PCR assay parameters were optimized; the efficiency of the real-time PCR was highly sensitive to the incidence of PCR inhibitors, which are sometimes present in DNA extracts, depending on the type of product to be analysed, including blood, bone marrow, heart, kidney, liver, spleen, lungs, muscle, and meat products. The results show that for the detection of the ASF virus, the highest Ct value was recorded in meat products and muscle, while in all other organs and tissues it was comparable, but differences were observed between singleplex and duplex real-time assays detection. Duplex real-time PCR assay is more sensitive to the Ct value margin, which varies from 0.5 Ct to almost 8.5 Ct. If the sample is strongly positive for ASFV DNA, the difference is negligible, and if the sample is weakly positive then the differences between singleplex and duplex real-time PCR assays are more significant.

CONCLUSIONS

Duplex real-time PCR can complement the current methods that are used for the detection of the ASF virus. Duplex ASF-IC real-time PCR assay proved to be specific, more sensitive, and replicable. This makes the method particularly useful for the early stage of ASF virus infection or for testing the disintegrated samples in the shortest possible time. Duplex ASF-IC real-time PCR assay offers the possibility to save time and reduce the costs of analysis, and therefore has a high potential for application in routine analysis.

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SKIRTINGŲ AFRIKINIO KIAULIŲ MARO VIRUSO NUSTATYMO METODŲ ĮVERTINIMAS

Santrauka

Afrikinio kiaulių maro (AKM) suvaldymui iki šiol nėra jokios vakcinos ar gydymo, todėl ligos stebėjimas ir ankstyva diagnostika yra vienintelis būdas ją kontroliuoti. Labai svarbu yra įdiegti naujus ir greitus molekulinius AKM diagnozavimo metodus, pasižyminčius dideliu jautrumu ir specifiškumu. Šio tyrimo tikslas buvo parinkus tinkamus reakcijos pradmenis ir zondus, reakcijos buferius ir pradmenų prilydimo temperatūrą, optimizuoti daugybinės realaus laiko PGR metodą, kuriuo galima aptikti AKM virusą ir užtikrinti vidinę kontrolę, bei palyginti AKM viengubos (250 bp AKM fragmentas) ir AKM-IC daugybinės (75 bp AKM fragmentas) realaus laiko PGR metodus. Buvo patvirtintas daugybinės realaus laiko PGR metodo specifiškumas ir jautrumas. Gauti rezultatai rodo, kad AKM-IC daugybinės realaus laiko PGR metodas yra greitesnis ir jautresnis nei realaus laiko PGR (juo nustatomas tik 250 bp AKM fragmentas) nustatant AKM virusą naminių kiaulių ir šernų kaulų čiulpų, širdies, inkstų, kepenų, blužnies, plaučių, maisto produktų ir kt. mėginiuose. Po tyrimų Ct vertės pagal šiuos metodus skyrėsi per 0,55–8,4 ciklo, tai reiškia, kad daugybinės realaus laiko PGR metodu sintetinama 0,3–70,5 karto daugiau reakcijos produkto.

Raktažodžiai: afrikinio kiaulių maro viruso aptikimas, naminės kiaulės, daugybinė realaus laiko PGR, tikslinė realaus laiko PGR, šernai