Part 3. Emergent diseases and biosafety

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THE INTERLABORATORY TESTING OF THE TEST-SYSTEM FOR DETECTION OF THE AFRICAN SWINE FEVER VIRUS DNA USING REAL-TIME PCR 'SUIDNATESTASF'

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Summary. African swine fever (ASF) is a highly contagious viral disease of pigs that produces a wide range of clinical signs and lesions that closely resemble those of classical swine fever, and associated with the hemorrhagic lesions in pigs of different species.

Keywords: African swine fever, virus, PCR

ASF is enzootic in many African countries and the Mediterranean island, Sardinia. In June 2007, ASF was confirmed for the first time in Georgia in the Caucasus region. Since its introduction into Caucasus region, African swine fever virus (ASFV) has spread rapidly into vast areas of western and southern Russia, where it is currently (2013) circulating out of control in domestic and wild pig populations. The virus has spread to the edges of Europe, with outbreaks reported in both the Ukraine and Belarus in 2013 (OIE, 2015). Since 2014–2015 it has been widely distributed in Ukraine (both wild and domestic cases, almost 111 cases were reported by CPS of Ukraine) (CPSU news-line at www.consumer.gov.ua). Also the disease has been distributed in Poland, Latvia, and Lithuania.

Disease surveillance both in wildlife and the domestic animals is one of the most significant ways for the disease control. Multiple measures were development using serological and molecular techniques, sufficient for the virus detection (OIE, 2012).

PCR, as the direct virus detection mean is appropriate for surveillance and diagnostics of the disease. Several PCR techniques were developed and described using conventional and real-time PCR protocols. PCR allows to detect ASF agent, using primers from a highly conserved region of the genome, to detect and identify a wide range of isolates belonging to all the known virus genotypes, including both non-haemadsorbing viruses and isolates of low virulence.

Two validated PCR procedures are described and consist of a sample preparation followed by the test procedure in OIE Terrestrial Manual (OIE, 2012). These procedures serve as a general guideline and a starting point for the PCR protocol. Optimal reaction conditions (incubation times and temperatures, models and suppliers of equipment, concentrations of assay reagents such as the primers and dNTPs) may vary so the described conditions should be evaluated first.

Application of the real-time PCR allows providing an effective detection of the virus in multiple type and ranges of samples with parallel reduction of the cross-contamination risks in laboratory conditions (Stegniy and Gerilovych, 2014).

National Scientific Center 'Institute for Experimental and Clinical Veterinary Medicine' and State Scientific and Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise were developed *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* due the reason of enhancement of ASF control measures in Ukraine.

The aim of the presented research is to study sensitivity, specificity, repeatability and reproducibility of this diagnostics kit.

Materials and methods. During commission trials evaluated appearance, activity, specificity, sensitivity of components of *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* and reproducibility.

5 positive samples derived from infected pigs from Sumy and Chernigiv regions were used (blood and

spleen). Their DNA extracts were diluted 1:100–1:10000 (samples # 1–20).

For the study of the taxonomic specificity the DNA extracts from PCV2 (isolate Poltava), PPV (isolate Sumy), PRV, Aujeszky's disease virus UNDIEV18V and *M. hyopmeumonia* (SB1 isolate) were used from the collection of NSC 'IECVM' (samples # 21–25).

Also the eukaryotic porcine DNA (# 26–28) and PK15 cells DNA (# 29–30) were used.

The polymerase chain reaction was managed under the recommended conditions described in the manual to *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'*. All testing was done without changes of the recommended features of reaction. To estimate the repeatability of testing this has been performed tree times.

Table 1 – Amplification cycle for ASF virus detection

Stage	Temperature	Length	Step	Number of cycles
1	95°C	3 min	Activation of the DNA-polymerase	1
2	95°C	10 sec	DNA denaturation	45
3	58°C	30 sec	Primer annealing/ elongation	

Results. Tests were conducted to verify the commission sensitivity and specificity of the test system and reproducibility obtained when using the results.

At the beginning of the commission were checked for completeness PCR test systems. All components necessary for operation and instruction were available.

In assessing the appearance found that the test system consists of the following components:

DNA extraction kit (kit # 1):

- 'extraction buffer' (lysis buffer) — 1 flack — 15 (30) ml, transparent non-colored liquid;
- sorbent solution — 1 (2) tube — 1.5 ml, opalescentic fluid with white color;
- 'washing buffer' — 1 flack — 50 (100) ml, transparent non-colored liquid;
- 'washing buffer ethanol' — 1 flack — 50 (100) ml, transparent non-colored liquid;
- 'solution № 4' for the final washing — 1 flack — 30 (60) ml, transparent non-colored liquid;
- TEbuffer — 1 flack — 20 (40) ml, transparent non-colored liquid.

Kit for PCR amplification (kit # 2):

- 'RTPCR MasterMix' — 1 (2) tubes — 0.5 ml — transparent non-colored liquid;
- probe solution (10 pM/μl) — 1 tube — 0.02 or 0.04 ml — transparent rose liquid;
- primer solution (20 pM/μl) — 1 tube of each — 0.125 (0.25) ml — transparent non-colored liquid;
- deionized water — 1 (2) tubes — 1.25 ml — transparent non-colored liquid;
- positive control template (for 5 or 10 reactions) — 1 tube — 0.05–0.1 ml — transparent non-colored liquid.

The PCR master mix was prepared using number of samples amount plus one under proportion per sample:

№	Component	Final concentration	1× for reaction (μl)
1	Water for PCR		6.7
2	RT-PCR Master Mix-Path ID	1×	10
3	Probe ASF probe (10 pM/μl)	250 nM	0.5
4	Primer ASVF1 (20 pM/μl)	400 nM	0.4
5	Primer ASFV2 (20 pM/μl)	400 nM	0.4
	DNA (template or control)		2

The results of the sensitivity testing is the ability to identify all encrypted obviously positive samples, it was found that the test system is able to detect DNA of the African swine fever virus in both positive clinical samples, including their dilution 1:100–1:10000 with Ct value 19–28.4. This is equal to the minimum titer of virus in the pork organism, we recorded an infected animals (in the nasal swabs and blood samples) in our early works.

The specificity of the test system proved amplicon in the absence of any size of Ct value or Ct value over 38 in samples of pig's tissues from non-infected animals and culture intact cells of PK15 line. In addition, primers designed did not hybrydizated with DNA samples from other viruses, including DNA extracts from PCV2 (isolate Poltava), PPV (isolate Sumy), PRV, Aujeszky's disease virus UNDIEV18V and *M. hyopmeumonia* (SB1 isolate) (Table 2).

It was also marked by complete coincidence test results using *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* in three repetitions under similar conditions and using different thermocyclers same type.

Table 2 – Testing results for the *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* with the panels of positive, negative and heterogenic samples

№	Material	1st repeat result	2nd repeat result	3rd repeat result
1	*Sample 1*	Ct 18	Ct 18.2	Ct 18
2	*Sample 1 1:100*	Ct 22	Ct 22.1	Ct 22.2
3	*Sample 1 1:1000*	Ct 26.3	Ct 26.4	Ct 27.2
4	*Sample 1 1:10000*	Ct 28.2	Ct 27.4	Ct 27.6
5	*Sample 2*	Ct 19.2	Ct 19.6	Ct 19.8
6	*Sample 2 1:100*	Ct 21.2	Ct 22.4	Ct 22.2
7	*Sample 2 1:1000*	Ct 24.5	Ct 24.6	Ct 24.5
8	*Sample 2 1:10000*	Ct 28.2	Ct 27.8	Ct 27.7
9	*Sample3*	Ct 19.6	Ct 18.8	Ct 18.9
10	*Sample 3 1:100*	Ct 22.4	Ct 22.3	Ct 22.6
11	*Sample 3 1:1000*	Ct 27.3	Ct 27.2	Ct 27.2
12	*Sample 3 1:10000*	Ct 29.2	Ct 29.7	Ct 29.7
13	*Sample 4*	Ct 22.2	Ct 22.4	Ct 22.4
14	*Sample 4 1:100*	Ct 24.5	Ct 24.6	Ct 24.3
15	*Sample 4 1:1000*	Ct 27.2	Ct 27.3	Ct 28.2
16	*Sample 4 1:10000*	Ct 31.4	Ct 31.2	Ct 30.9
17	*Sample 5*	Ct 19.4	Ct 19.6	Ct 19.4
18	*Sample 5 1:100*	Ct 21.8	Ct 22.1	Ct 22.3
19	*Sample 5 1:1000*	Ct 25.5	Ct 25.6	Ct 25.5
20	*Sample 5 1:10000*	Ct 29.2	Ct 29.8	Ct 29.7
21	PCV-2 (isolate Poltava)	N/d	N/d	N/d
22	PPV (isolate Sumy)	N/d	N/d	N/d
23	PRV	N/d	N/d	N/d
24	Aujeszky's disease virus UNDIEV-18V	N/d	N/d	N/d
25	*M. hyopmeumonia* (SB1 isolate)	N/d	N/d	N/d
26	Pig intact 1	N/d	N/d	N/d
27	Pig intact 2	N/d	N/d	N/d
28	Pig intact 3	N/d	N/d	N/d
29	PK15 cells DNA	N/d	N/d	N/d
30	PK15 cells DNA	N/d	N/d	N/d

Conclusions: 1. Specificity of the *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* developed by National Scientific Center 'Institute for Experimental and Clinical Veterinary Medicine' and State Scientific-Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise has the appropriate level of sensitivity and specificity, aligned with the detection of ASF virus DNA in clinical samples and their 1:100–1:10000 dilutions, in Ct values of 18–31.4, with its absence of Ct value over 38 in the 'negative samples'. The developed kit doesn't demonstrate the false positive reactions with heterogenic DNA samples of DNA extracts from PCV2 (isolate Poltava), PPV (isolate Sumy), PRV, Aujeszky's disease virus UNDIEV18V and M.hyopmeumonia (SB-1 isolate) were used from the collection of NSC 'IECVM'.

2. The sensitivity of the system can be considered satisfactory, since positive results were positive for samples with low concentrations of specific DNA.

3. Primers, probe and other components of the proposed test kits are highly sensitive, specific and comply with TU.

4. Reproducibility and repeatability of African swine fever virus DNA detection method using the ASFV target gene amplification for that is the basis of the PCR test system *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* has the appropriate level, in all three repetitions differences were observed results.

The perspectives for further application of the results. As the commission test *Test-system for detection of the African swine fever virus DNA using real-time PCR 'SuiDNATestASF'* developed by National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' and State Scientific-Research Institute for Laboratory Diagnostics and Veterinary-Sanitary Expertise indicators of quality, such as sensitivity, specificity and reproducibility fit the requirements. In this regard, this test system can be recommended for interestablishmental testing and using in practice of the State veterinary laboratories for the detection of DNA of African swine fever virus.

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