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Effect of pig serum storage conditions on detection of anti-ASFV antibodies by ELISA

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SUMMARY

One of the measures used to control and prevent African swine fever spread in the Russian Federation involves testing pig and boar sera using *inter alia* serological tools based on enzyme-linked immunosorbent assay (ELISA) for anti-viral antibody detection. However, there is no unified regulatory document specifying storage conditions for sera used in the ELISA for anti-ASFV antibody detection. There are also lack of published data on the maximum admissible shelf life of the pig sera, and the effect of storage conditions on the serological status of the pig sera as for ASF is understudied. The paper demonstrates results of the experiment aimed at the determination of the effect of storage temperatures and shelf life on the serological status of ASFV seropositive and seronegative pig sera when tested by INgezim PPA Compac (Ingenasa, Spain) ELISA as well as on the possibility of false results. During the experiment and analysis of its results, the new data were obtained, and they indicated from none to non-significant effect of the simulated storage conditions on the serological status of sera used for ASFV detection, while hemolyzed sera demonstrated more significant changes proportional to hemolysis degree and storage duration. Although the results of detection of antibodies against the agents of some diseases cannot be used in case of other pathogens, this study has a substantial applied significance as it allows to specify the dependence of the valid results of ASF serodiagnosis on the storage conditions of the samples.

Keywords: African swine fever, enzyme-linked immunosorbent assay, serum, storage conditions

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Влияние условий хранения сывороток крови свиней на выявление антител к вирусу АЧС методом ИФА

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РЕЗЮМЕ

Одной из мер борьбы с распространением и профилактики африканской чумы свиней в Российской Федерации является проведение исследований проб, отбираемых от свиней и кабанов, в том числе серологическими методами с использованием иммуноферментного анализа (ИФА) для качественного определения содержания антител к вирусу. При этом в настоящее время на территории страны не существует нормативного документа, регламентирующего условия хранения проб сыворотки крови для постановки ИФА при определении содержания антител к вирусу АЧС. Также отсутствуют литературные данные о максимально допустимом сроке хранения проб сыворотки крови свиней, а влияние условий хранения на серологический статус сывороток крови домашних свиней в отношении АЧС изучено недостаточно. В статье представлены результаты эксперимента по определению влияния температурных режимов и длительности хранения серопозитивных и серонегативных в отношении вируса АЧС сывороток крови домашних свиней на их серологический статус при постановке тест-системой INgezim PPA Compac (Ingenasa, Испания) для твердофазного ИФА и вероятности получения ложных результатов. В ходе выполнения работы и анализа результатов получены новые данные, свидетельствующие об отсутствии или незначительном влиянии моделируемых режимов хранения на определение серологического статуса качественных проб сывороток крови в отношении вируса АЧС, в то время как гемолизированные пробы показали более заметное изменение, пропорциональное степени гемолиза и длительности хранения. Несмотря на то что полученные результаты по обнаружению антител к возбудителям одних болезней не применимы для других патогенов, данное исследование имеет существенное прикладное значение, позволяя установить зависимость получения достоверных результатов при серодиагностике АЧС от условий хранения проб.

Ключевые слова: африканская чума свиней, иммуноферментный анализ, сыворотка крови, условия хранения

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INTRODUCTION

African swine fever (ASF) is a contagious viral disease of domestic pigs and wild boar, manifested in susceptible animals as a hemorrhagic fever with a mortality rate of up to 100% [1]. The virus reservoirs are warthogs, bush and wild pigs, ticks of the *Ornithodoros* genus. The disease has a disastrous effect on pig farming as a whole, including establishments of all forms of ownership (starting from backyards to commercial industrial pig farms), leading to severe socio-economic consequences and compromising food security of the infected territory [2, 3].

African swine fever was first described by R. Montgomery in Kenya in 1921. Subsequently, ASF outbreaks were reported in most countries in Southern and Eastern Africa. In 1957, the porcine disease caused by the ASF virus was first registered in Europe. By mid-2020, the ASF virus was reported on the African, European and Asian continents, the Malay Archipelago and in the countries of Oceania [4].

To date, commercially available means of ASF specific prevention have not been developed. In this regard, the only way to control the disease is to implement a set of preventive measures, early diagnosis using modern and accurate methods, killing of infected and risk animals and introduction of strict restrictive measures (quarantine) [5, 6].

One of the measures to control ASF spread and prevention in the Russian Federation is to conduct monitoring tests [7] and quarantine tests (before shipment and/or when introducing new animals from/to farms), including serological methods using enzyme-linked immunosorbent assay (ELISA) for qualitative measurement of the viral antibodies in porcine sera [8, 9]. To date, there are many commercial ELISA test kits. One of the kits used in the FGBI "ARRIAH" Reference Laboratory for ASF is INgezim PPA Compac (Ingenasa, Spain) kit, which, according to various authors, has a 98 to 100% specificity.

According to H. C. Bergeron et al., false-positive results may be associated with poor sample quality [10]. According to the instructions for the INgezim PPA Compac kit and the recommendations of the World Organization for Animal Health (OIE), the use of hemolyzed or contaminated samples can lead to false-positive results in ELISA. As indicated in the Guidelines of the Food and Agriculture Organization of the United Nations (FAO), sera should be tested immediately after collection or put to storage at a temperature below minus 20 °C before testing, since an accurate diagnosis can be made only when the samples are in satisfactory condition [11].

However, according to the results of L. Mur et al. [12], when testing 158 sera samples using the INgezim PPA Compac kit, among which 49 were moderately and 28 were extensively hemolyzed, 11 gave a false-positive result and 6 were inconclusive. At the same time, there

was no correlation between hemolysis and false-positive results.

Based on general recommendations, it is known, that not frozen serum should be delivered to the diagnostic laboratory during the first 24 hours and in exceptional cases – no later than the third day after blood collection [11, 13, 14].

Current veterinary and sanitary rules of the Republic of Belarus regulating the procedure of serum and blood plasma collection allow freezing of samples and transportation of frozen samples. If it is not possible to freeze the serum and plasma samples, it is allowed to store and transport them at a temperature of 2 to 6 °C within no more than 48 hours after sample collection [15].

For serological testing, it is allowed to send whole blood to the laboratory without separating the serum, provided (difficult to implement) that it will not be shaken and will not undergo hemolysis on the way [16].

In the laboratory, the serum (without a clot) is stored in refrigerators at a temperature of (5 ± 3) °C for no more than 7 days before testing. For longer storage, the serum should be frozen at minus 20 °C or lower. Re-freezing of serum is not allowed [14].

It should be noted that currently there is no regulation in the Russian Federation regulating the conditions of serum sample storage for ELISA testing when determining the level of ASFV antibodies. Such standards are available, in particular, for brucellosis, where phenol or boric acid preserved serum samples are suitable for testing for 30 days, frozen samples – for 3 days after single thawing. Cloudy, bacteria and fungi contaminated, hemolysed blood sera are not suitable for testing for brucellosis [17].

To date, only the storage conditions for organ and blood samples are available, which are stored and transported in an insulated container at a temperature of 4 to 8 °C within no more than 24 hours after collection. For longer storage, the samples should be frozen [13].

There are no published data on the maximum permissible period for porcine serum sample storage, during which 95% of samples will demonstrate unchanged serological status when tested by ELISA. This parameter was established for samples containing antibodies against some other viruses, for example, against the hepatitis D virus; the analyte stability in serum at a temperature of 4 to 8 °C is 4 weeks, from 20 to 25 °C is 5–7 days [18].

The effect of storage conditions on the serological status of domestic pig sera for ASF testing has not been sufficiently studied. There are some published works on studies of the temperature effect (50 °C, 4 °C, minus 10 °C, freezing – thawing cycle), the storage time and the hemolysis degree effect on determination of antibodies against *Erysipelothrix rhusiopathiae* in pigs and *Suid herpesvirus* 1 in wild boar in hemolyzed and not-hemolyzed sera [19, 20]. The authors concluded that the storage conditions had a negligible effect on testing of high-quality serum samples, while the hemolyzed samples showed a more noticeable change proportional to the degree of hemolysis and the duration of storage. It should be noted that the results obtained for the detection of antibodies against some viruses are not applicable for other pathogens.

The aim of the work was to determine the effect of temperature conditions and duration of storage of ASF seropositive and seronegative samples of domestic pigs on their serological status when tested using INgezim PPA Compac solid-phase ELISA test kit (Ingenasa, Spain) and the probability of obtaining false results.

This study has a significant practical value, and allows to establish the relationship between the reliable results in ASF serological diagnostics and the sample storage conditions.

MATERIALS AND METHODS

Equipment. The storage conditions (4 °C, minus 20 °C and a multiple freeze – thaw cycles) were reproduced using a laboratory refrigerator HL-340 (POZIS, Russia). The sera during ELISA test were incubated in a thermoshaker PST-60HL-4 (BioSan, Latvia). The results were read using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, Finland).

Samples. Ten seropositive samples collected on Days 25–26 after experimental infection of pigs with the strain "ARRIAH/ASF-VERO (40)" and ten seronegative samples prepared from the blood of clinically healthy domestic pigs (pig breeding complex, Moscow Oblast) were used. The samples had no signs of hemolysis and were obtained in accordance with the "Rules for Collection of Pathological Material, Blood, Feedstuffs and Their Submission for Laboratory Testing" [16].

Test kit. Serum samples were tested using the INgezim PPA Compac solid-phase ELISA test kit (Ingenasa, Spain) in duplicate. According to the kit instructions, the status of the tested sera was expressed using coefficient of inhibition calculated by the formula:

$$x\% = \frac{\text{NC} - \text{SAMPLE OD}}{\text{NC} - \text{PC}} \times 100,$$

where NC is the value of the absorbance units (AU) of the negative control;

PC is the value of the absorbance units (AU) of the positive control;

SAMPLE OD – the value of the absorbance units (AU) of the test serum.

Interpretation of the result:

– at $x \leq 40\%$, the result is considered negative (i.e. no specific antibodies were detected in the sample);

– at $x \geq 50\%$, the result is considered positive (i.e. specific antibodies were detected in the sample);

– at $40\% > x < 50\%$ the result is considered inconclusive.

Experimental sera were stored at the following temperature conditions:

1. Storage at minus 20 °C;
2. Storage at 4 °C;
3. Storage at room temperature (from 20 to 25 °C);
4. Multiple freeze – thaw cycles (daily freezing at minus 20 °C for 23 hours, followed by thawing at room temperature for an hour).

The analysis was carried out on the start day (Day zero), on the 5th, 15th, 29th and 53rd days after the start of the experiment.

TEST RESULTS

The first test to confirm the serological status of experimental sera was performed on Day 0.

The experimental samples were divided into 4 parts and stored at different temperature conditions for 53 days. Each group of experimental sera was analyzed according to the experiment design. The results are given in the Table.

Table
Serum testing using solid-phase ELISA

Storage condition	Sample status	Serum number	Average value of coefficient of inhibition (%), ($n = 2$)					Result
			Day 0	Day 5	Day 15	Day 29	Day 53	
Freeze – thaw cycle	Seronegative	1	17.0	26.4	23.7	22.7	28.9	–
		2	16.3	25.1	25.2	20.3	35.4	–
		3	23.2	20.8	18.2	21.2	22.3	–
		4	21.6	23.5	21.4	22.8	26.1	–
		5	22.7	26.1	23.3	22.9	27.3	–
		6	25.5	24.0	28.0	24.1	31.6	–
		7	20.3	22.3	21.1	20.1	19.1	–
		8	20.5	22.8	23.8	19.7	22.6	–
		9	17.3	20.6	18.1	16.1	20.5	–
		10	23.3	23.5	26.5	21.2	32.0	–
	Seropositive	11	94.1	88.1	90.5	92.0	90.6	+
		12	100.6	96.4	98.0	97.3	96.0	+
		13	97.1	91.1	92.8	92.7	91.4	+
		14	98.3	90.6	91.0	91.8	90.2	+
		15	88.1	93.7	95.0	94.9	92.3	+
		16	98.3	93.8	93.9	95.0	91.8	+
		17	86.7	85.4	83.8	87.2	85.5	+
		18	97.0	94.5	94.7	96.8	93.5	+
		19	94.8	91.6	94.7	93.5	89.9	+
		20	99.8	98.6	99.1	99.3	95.5	+
at minus 20 °C	Seronegative	1	17.0	20.8	19.9	18.8	25.4	–
		2	16.3	20.3	21.3	22.7	26.0	–
		3	23.2	24.0	22.2	24.2	29.4	–
		4	21.6	20.9	23.2	20.3	33.8	–
		5	22.7	16.5	20.0	19.8	7.1	–
		6	25.5	19.5	20.0	21.8	24.4	–
		7	20.3	21.1	22.2	23.4	28.7	–
		8	20.5	20.0	24.4	22.9	35.4	–
		9	17.3	10.2	11.4	15.8	15.9	–
		10	23.3	20.0	25.4	20.1	28.7	–
	Seropositive	11	94.1	89.2	90.9	91.8	89.2	+
		12	100.6	96.2	96.2	97.6	94.9	+
		13	97.1	89.9	91.2	91.7	89.7	+
		14	98.3	89.8	90.5	92.6	90.9	+
		15	88.1	93.3	94.2	95.3	92.6	+
		16	98.3	94.3	95.3	95.8	93.2	+
		17	86.7	82.9	80.5	83.0	81.9	+
		18	97.0	91.2	90.5	92.8	91.3	+
		19	94.8	91.6	93.8	94.9	93.1	+
		20	99.8	98.2	97.4	98.4	95.3	+

Storage condition	Sample status	Serum number	Average value of coefficient of inhibition (%), ($n = 2$)					Result
			Day 0	Day 5	Day 15	Day 29	Day 53	
at 4 °C	Seronegative	1	17.0	19.8	20.1	10.5	26.6	–
		2	16.3	19.9	19.1	11.2	29.8	–
		3	23.2	12.3	21.2	15.8	16.5	–
		4	21.6	19.3	22.3	13.0	23.5	–
		5	22.7	20.2	20.8	11.2	27.4	–
		6	25.5	16.5	19.7	13.4	26.9	–
		7	20.3	19.6	20.4	15.7	16.2	–
		8	20.5	20.5	20.1	14.1	26.1	–
		9	17.3	17.0	11.1	4.3	23.6	–
		10	23.3	17.0	21.5	13.5	25.9	–
	Seropositive	11	94.1	87.3	89.0	89.3	87.2	+
		12	100.6	93.3	95.9	96.8	94.3	+
		13	97.1	93.5	93.4	90.3	92.0	+
		14	98.3	90.2	90.2	88.6	88.1	+
		15	88.1	93.7	95.0	95.6	92.4	+
		16	98.3	92.5	95.3	93.8	91.3	+
		17	86.7	84.2	81.7	80.3	83.7	+
		18	97.0	93.1	93.4	94.4	92.8	+
		19	94.8	93.1	93.0	94.9	90.8	+
		20	99.8	96.2	96.8	97.9	93.5	+
at 20 °C	Seronegative	1	17.0	13.1	17.0	19.8	11.2	–
		2	16.3	18.8	20.9	20.6	13.0	–
		3	23.2	19.3	14.0	17.0	19.1	–
		4	21.6	18.6	19.9	22.1	20.8	–
		5	22.7	16.6	22.0	21.9	10.2	–
		6	25.5	19.4	18.1	16.6	15.4	–
		7	20.3	18.7	20.9	17.7	18.9	–
		8	20.5	18.9	20.3	21.1	19.2	–
		9	17.3	10.2	17.2	17.3	6.3	–
		10	23.3	17.3	22.0	18.0	18.8	–
	Seropositive	11	94.1	89.1	89.1	90.5	87.5	+
		12	100.6	97.9	97.7	98.7	92.6	+
		13	97.1	91.3	91.2	94.6	88.1	+
		14	98.3	88.3	88.4	90.9	85.9	+
		15	88.1	95.4	95.2	95.5	92.4	+
		16	98.3	95.2	95.5	95.8	92.3	+
		17	86.7	81.0	83.4	85.5	79.2	+
		18	97.0	92.3	95.3	95.7	90.8	+
		19	94.8	92.6	93.5	93.7	91.7	+
		20	99.8	96.9	97.8	98.7	94.0	+

"+" – positive result; "–" – negative result.

The obtained data demonstrate that when the coefficient of inhibition changed during the experiment, the serum status in solid-phase ELISA remained unchanged.

Following the general recommendations on storage of serum samples for laboratory diagnostics [11, 14, 15], the reference values were considered to be the results obtained by the testing of sera stored frozen. The variation of the values depended on the technical conditions of the method performance (temperature, humidity, kit storage time, etc.).

The dynamics of changes in the coefficients of inhibition of seronegative and seropositive samples is shown in Figure 1. From the presented data, related to negative sera, it can be seen that:

– the average coefficient of inhibition at the beginning of the experiment was 20.8%;

– for sera stored at minus 20 °C, the average coefficient of inhibition was 21.5%, the minimum – 7.1% (Day 53), the maximum – 35.4% (Day 53);

– for sera subjected to the freeze – thaw cycle, the average coefficient of inhibition was 22.8%, the minimum value was 16.1% (Day 29), the maximum value was 35.4% (Day 53);

– for sera stored at 4 °C, the average coefficient of inhibition was 19%, the minimum – 4.3% (Day 29), the maximum – 29.8% (Day 53);

– for sera stored at 20 °C, the average value was 18.3%, the minimum value was 6.3% (Day 53), the maximum value was 25.5% (Day zero).

The change in the difference in the inhibition coefficients from the reference samples for negative sera is shown in Figure 2.

It was established that during the whole study, the coefficient of inhibition for negative sera subjected to the freeze – thaw cycle was higher than the reference values, but did not exceed 4.2%, and on average differed by 1.7%; this indicator of sera stored at 4 °C was lower than the reference values, but did not exceed 8.7%, and on average differed by 3.1%. The inhibition coefficient of sera stored at 20 °C was lower than the reference values, but did not exceed 10.2%, and on average differed by 4%.

Based on the data obtained during the testing of porcine seropositive samples (Fig. 1), it can be seen that:

– the initial coefficient of inhibition was on average 95.5%;

– for sera stored at minus 20 °C, the average value was 92.8%, the minimum – 80.5% (Day 15), the maximum – 100.6% (Day zero);

– for sera subjected to the freeze – thaw cycle, the average coefficient of inhibition was 93.4%, the minimum value was 83.8% (Day 15), the maximum value was 100.6% (Day zero);

– for sera stored at 4 °C, the average coefficient of inhibition was 92.5%, the minimum – 80.3% (Day 29), the maximum – 100.6% (Day zero);

– for sera stored at 20 °C, the average coefficient of inhibition was 92.7%, the minimum was 79.2% (Day 53), the maximum was 100.6% (Day zero).

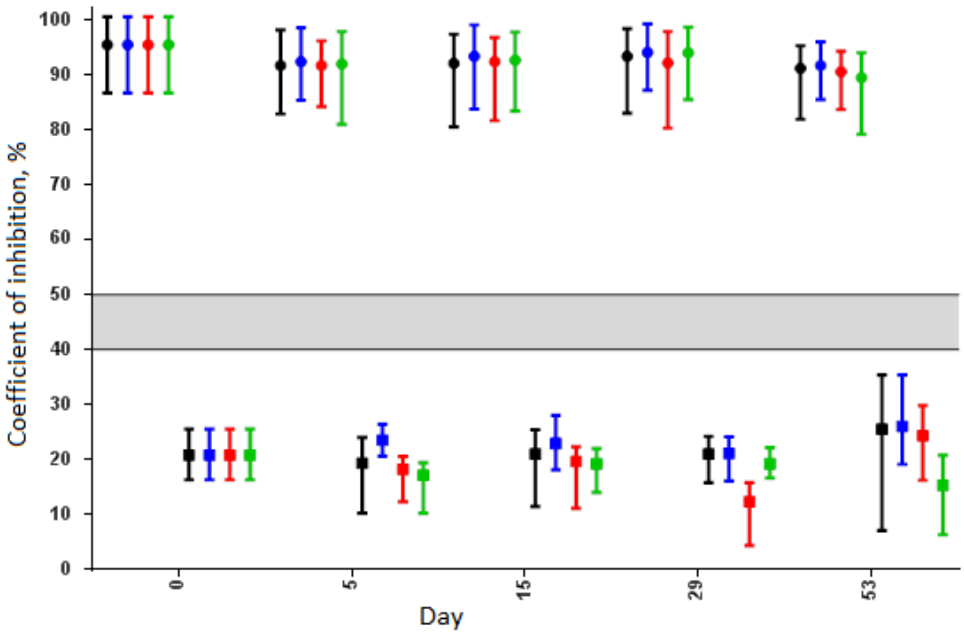


Fig. 1. Dynamics of inhibition coefficients for seronegative and seropositive samples. Lines in case of 40% and 50% inhibition are the negative/positive cut-off.

The difference in the coefficients of inhibition of seropositive samples stored at minus 20 °C is shown in Figure 3.

As it can be seen, the coefficient of inhibition of seropositive samples subjected to the freeze –thaw cycle was higher than the reference values throughout the entire study period, but did not exceed 1.3%, and on average differed by 0.8%.

The coefficient of inhibition of sera stored at 4 °C was higher than the reference value (maximum by 0.3%) for 15 days; later it became lower than it (maximum by 1.2%), and on average differed by 0.4%.

The coefficient of inhibition of sera stored at 20 °C was higher than the values of the reference samples until the Day 29 (maximum by 0.7%), and then lower by 1.8%, and on average differed by 0.1%.

In the group of seropositive samples stored at 4 °C, the coefficient of inhibition for some samples during the study varied from 4.3 to 23.6%. However, the status of the samples did not change throughout the experiment.

DISCUSSION

The simulation of various storage conditions of experimental pig sera, positive and negative for ASFV antibodies, provided the data on the absence of changes in their serological status over time.

The tested serum samples were stored at various temperature conditions (minus 20 °C, 4 °C, 20 °C, freeze – thaw cycle) for 53 days. Despite the differences in the coefficients of inhibition of negative and positive samples during the study from the reference values, a qualitative result was obtained by solid-phase ELISA, which suggests no or insignificant effect of the applied storage conditions on the results of the study.

The obtained data are consistent with the results of other authors who conducted similar studies in detection of antibodies to other infectious agents. In the work of E. J. Neumann and K. N. Bonistalli, serum antibodies were significantly more stable than previously thought, and the optical density values were stable even in case of gross violation of the storage temperature conditions [19, 20].

However, the methodology used in this study has some limitations. The test kit of a single manufacturer was used in the work, the serum antibody titers were not determined, and weakly positive samples were not tested.

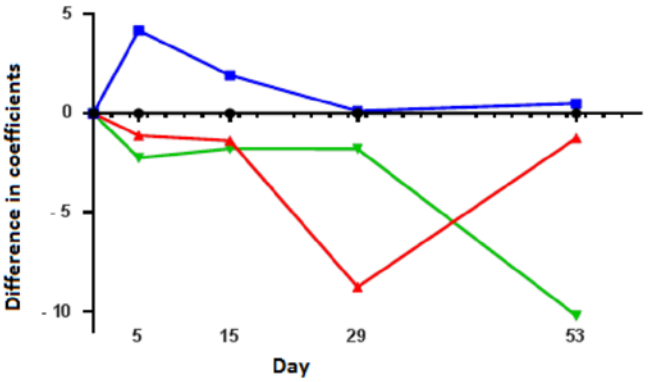
Therefore, to determine the reason of false results, it is necessary to conduct further studies and to analyze the preservation of serum samples for ASF testing, given that biological samples obtained from animals are not homogeneous in their composition, especially in the presence of pathogenic microflora.

CONCLUSION

The studies conducted showed that storage of porcine serum samples in simulated temperature conditions did not affect the status of positive sera with an initially high coefficient of inhibition and negative sera tested by solid-phase ELISA using the INgezim PPA Compac test kit (Ingenasa, Spain) during 53 days.

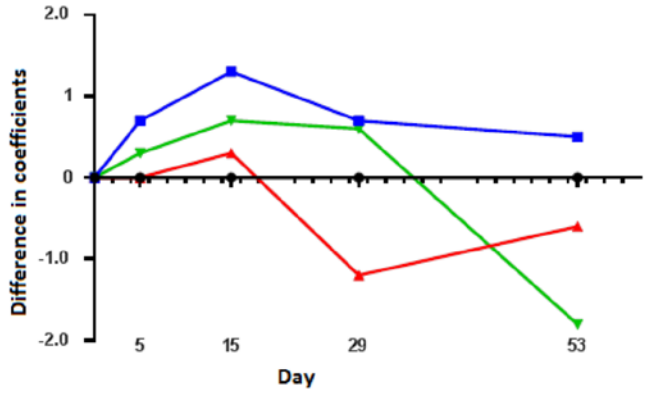
Sera of domestic pigs collected in accordance with the current rules can be stored under the above conditions for up to 53 days (observation period), provided that they are subsequently tested using an appropriate kit. This will simplify their transportation for a longer time if freezing is impossible. Also, if additional tests are needed, the repeated testing of a single sample becomes possible during long-term storage.

However, until the effect of storage conditions on the status of sera during ASF testing using other kits and methods (Western blotting, immunoperoxidase, immunochromatographic assays, ELISA using a complex antigen,



Conditions/day	0	5	15	29	53
minus 20 °C	0	0	0	0	0
freeze/thaw	0	4.2	1.9	0.1	0.5
4 °C	0	-1.1	-1.4	-8.7	-1.2
20 °C	0	-2.2	-1.8	-1.8	-10.2

Fig. 2. Changes of inhibition coefficients of seronegative sera as compared to reference sera (storage at minus 20 °C)



Conditions/day	0	5	15	29	53
minus 20 °C	0	0	0	0	0
freeze/thaw	0	0.7	1.3	0.7	0.5
4 °C	0	0	0.3	-1.2	-0.6
20 °C	0	0.3	0.7	0.6	-1.8

Fig. 3. Changes of inhibition coefficients of seropositive sera as compared to reference sera (storage at minus 20 °C)

etc.) has not been studied, the general recommendation remains the same – to deliver samples to the laboratory for research as soon as possible.

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