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VALIDATION OF A TEST SYSTEM FOR AFRICAN SWINE FEVER SERODIAGNOSIS USING IMMUNOBLOTTING

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Abstract

Because of the lack of a vaccine, African swine fever (ASF, caused by African swine fever virus (ASFV) of *Asfivirus* genus, *Asfarviridae* family) control strategy is based on making a rapid and early diagnosis and taking strict veterinary and sanitary measures. In the Eastern Europe countries where the infection has currently spread, highly virulent isolates are usually detected (J.M. Sanchez-Vizcaino et al., 2013). In the laboratory diagnosis, polymerase chain reaction (PCR) and direct immunofluorescence method are predominantly used. However, since 2012, researchers have observed some alteration in biological and genetic properties of a number of ASFV isolates. Therefore, serological methods may become prevalent in the laboratory diagnosis as it was during an ASF epizooty in the Iberian Peninsula in 1960-1990. We have earlier reported the development of a test system for the disease immunoblotting serodiagnosis (Rec p30-IB) based on a recombinant structural ASFV protein p30 (A.S. Kazakova et al., 2014). In this paper, the Rec p30-IB test system validation is shown. The diagnostic sensitivity of the Rec p30-IB was of 99.3 %, and the specificity was 100 %. Antibodies against p30 were detected in blood serum and organ samples taken from domestic pigs or wild boars irrespective of the seroimmunotypic membership and the virulence levels of the ASFV strains. In the blood serum samples collected from domestic pigs infected with heterologous viruses, no false-positive results were seen. In the serum of domestic pigs which were survived after intramuscular injection of attenuated strains LK-111, KK-262/C, MK-200, FK-135, PSA-1-NH and SCA 2015 VNIIVViM at 10^3 to 10^4 HAU_{50}/CPE_{50}, antibodies to p30 were detected on day 7 to 10. For organ samples from domestic pigs that had died from ASF 5 to 10 days post intramuscular infection with highly virulent strains Lisbon-57, Mozambique-78 or Stavropol 01/08 at a dose of 10^3 HAE_{50}, the antibodies to p30 were detected in 30 % of the animals. The validation results indicate that the Immunoblotting Test System for African Swine Fever Serodiagnosis (Rec p30-IB) can be used for laboratory practice and monitoring of blood sera and organ samples collected from ASFV-infected domestic pigs or wild boars.

Keywords: African swine fever, protein p30 ASFV, serodiagnosis, immunoblotting, validation

African swine fever (ASF) is a contagious septic disease characterized by fever, hemorrhagic diathesis, and high mortality rate. In acute form, 100 % of animals die within 5-10 days after manifestation of clinical traits. The disease is caused by coated large African swine fever virus (ASFV) with double-stranded DNA, the only representative of *Asfarviridae* family, which is characterized by

significant variability of biological and genetic properties [1-3]. ASFV has extremely high potential to cross-border spread. In 2007, it penetrated from Africa and Georgia, followed by Asian and Eastern-European countries (Armenia, Iran, Azerbaijan, Russia, Belarus, Ukraine, Latvia, Lithuania, Estonia, Poland) [4, 5].

Due to lack of vaccine in strategy of combat against the disease, the accent is made on rapid and early diagnostic and strict veterinary and sanitary measures. However, ASFV diagnostic is challenged by numerous pathogenesis forms and epizootic scenarios, as well as similarity of clinical and pathomorphological signs of this disease and other hemorrhagic infectious diseases, e.g. classical swine fever (CSF), acute pasteurellosis, swine erysipelas, and salmonellosis. In East-European countries, where CSF is recently spread, high-virulent ASFV isolates are usually identified [6]. For laboratory diagnostics, different variants of polymerase chain reaction (PCR) and direct immune-fluorescence method are mainly used. However, since 2012 variability of biological and genetic properties were sighted in several isolates extracted at the territory of Russia, Poland, and Baltic States [7-10]. It provides the basis to assume that pathogenicity of ASFV circulating in Eastern-Europe varied towards decrease of virulence. Thus, serologic methods could become dominant in the laboratory diagnostics as it was during CSF epizooty in 1960-1990 at Iberian Peninsula. Antibodies in blood serum and immune organs at CSF are identified 7-10 days after infection and in furtherance during a long time [11]. Their presence serves convincing testimony for diagnosing. Today, the inventory of diagnostic means for identification of antibodies is mainly presented by kits for Indirect Immune Fluorescence Reaction (IIFR), immune-enzyme analysis (ELISA) and immunoblotting assay (IB). International Epizootic Bureau (IEB) (IEB — World Organization for Animal Health, OIE, France) recommends confirming ELISA^{+} specimens by IB method since samples of field blood serums lose their reactivity in ELISA earlier than in IB, because the latter can identify linear epitops [12]. Amongst IB advantages are simplicity and objectivity of interpretation, as nitrogen-cellulose strips with absorbed virus-specific polypeptides could be kept up to 6 months at room temperature in dry atmosphere [13, 14].

IEBB recommends for ELISA and IB tests cytoplasmic soluble antigen produced in ASFV infected cell line of monkey's kidney raised in presence of swine serum [14, 15]. To secure production of diagnostic test systems, virus-specific antigens based on recombinant (chimer) immune dominant proteins of ASFV are developed [16-22]. Such antigens increase sensitivity and specificity of diagnostics by decreasing the frequency of false positives caused by substances of cell culture which inevitably contaminate antigens prepared on its basis [15].

Earlier, we have reported on development of a test system for serological diagnostic of African swine fever by immune blotting, prepared based on recombinant ASFV protein p30 [23]. In present paper, we have for the first time presented data on its validation.

Our objective was estimate of validity of immune blotting test for ASFV detection based on use recombinant protein p30.

Techniques. Specimens were collected from Large White pigs weighting 30-50 kg of Animal Sector of Federal Research Center of Virology and Immunology, and from wild boars of hunting farm Pokrovskoye, Vladimir Region. ASFV virulente strains were Lisbon-57 (L-57, seroimmunotype I), Congo-49 (C-49, seroimmunotype II), Mozambique-78 (M-78, seroimmunotype III), Stavropol 01/08 (seroimmunotype VIII), ASFV attenuated strains were KK-262/C (seroimmunotype II), MK-200 (seroimmunotype III), FK-135 (seroimmunotype IV), PSA-1-NH (seroimmunotype IV), CKA 2015 VNIIVViM (seroimmunotype VIII) (all strains) (State Collection of Microorganisms of the Federal Research

Center of Virology and Immunology) [24, 25; Patent RF No 2439152, 2012].

Swine antisera against causative agents of CSF, transmissible gastroenteritis (TGE), and Aujeszky's disease were produced in Diagnostics and Monitoring Laboratory of the Federal Research Center of Virology and Microbiology. Reference negative blood serum sample included 28 specimens from domestic pigs and wild boars (Smolensk, Voronezh, Tver, Pskov, Rostov, and Volgograd regions), 25 specimens from healthy domestic pigs and wild boards (vivarium of the Federal Research Center of Virology and Microbiology), 480 specimens from domestic pigs from ASF-free pig breeding farms (Voronezh Region). Reference positive blood serum sample included 42 specimens from domestic pigs (farming units of Volgograd and Tver regions), 59 specimens from experimentally infected domestic pigs (Federal Research Center of Virology and Microbiology, collected at different times after intramuscular inoculation with virulent or attenuated ASFV strains, seroimmunetypes I-IV, VIII) and from wild boars. $ASFV^+$ blood sera were also received from the reference laboratory of CISA-INIA (Centro de Investigaciyn en Sanidad Animal, Instituto Nacional de Investigaciyn y TecnologНa Agraria y Alimentaria, Spain). Total 18 spleen specimens from wild boars of Smolensk Region were reference $ASFV^-$; 11 spleen specimens from wild boars of Smolensk Region, 18 spleen specimens from domestic pigs of Tula Region, and 10 specimens from experimentally infected pigs (Federal Research Center of Virology and Microbiology) were reference $ASFV^+$. Absence or presence of anti-ASFV antibodies in each reference specimen were confirmed by indirect immune fluorescence assay (IIFA), the reference test recommended by IEB. Blood sera and 10 % tissue suspension processing, hemadsorption, detection of antibodies to ASFV by IIFA and ELISA were performed as per GOST 28573-90 (Moscow, 2005). If required, blood serum was incubated during 30 minutes at 56 °C prior to tests to inactivate infectious virus.

In IIFA, commercial Kit for Differential Immune Fluorescent Diagnostic of African swine fever, classical swine fever, and Aujeszky's disease (Federal Research Center of Virology and Microbiology) and cell culture of African green monkey's kidney CV-1 (Collection of cell cultures of the Federal Research Center of Virology and Microbiology) infected by avirulent non hemadsorping ASFV (strain 691/88, State Collection of Microorganisms of the Federal Research Center of Virology and Microbiology) were used with luminescent microscopy (Eclipse E200, Nikon Co., Japan) to read the results.

ELISA test was performed with commercial ASF-IFA Ab/Ag kit (Federal Research Center of Virology and Microbiology) [26]. For comparison, we used ELISA tests for antibodies to ASFV — Ingezim PPA Compac 1.1.PPA.K.3 based on virus protein vp73 and monoclonal antibodies (Inmunologia y Genetica Aplicada S.A., Spain), and also ID Screen® African Swine Fever Indirect based on mixture of recombinant proteins p32, p62, and p72 (IDvet Genetics, France).

Immunoblotting assay (IB) was performed with Test System for African Swine Fever Serodiagnosis by Immunoblotting Method (Rec p30-IB) (five experimental series, Federal Research Center of Virology and Microbiology).

Mean (M) and standard errors of the mean (±SEM) were calculated. Statistical indicators of test effectiveness (sensitivity and specificity) were determined as described [26] at 95-99 % confidence interval.

Results. Rec p30-IB test kit includes immunostrips, positive and negative control sera, protein A conjugate with horseradish peroxidase, chromogenic substrate and stock solutions required for analysis.

The rate of accurately identified truly positive cases (as sensitivity estimate) was assessed for Rec p30-IB using a panel of blood serum and organ specimens positive for anti-ASFV antibodies as shown by IIFA ("golden standard"

test). The rate of truly negative reads accurately identified by Rec p30-IB for blood serum and organ specimens which were ASFV⁻ in IIFA were considered as specificity estimate. Reproducibility we assessed by comparison of data obtained by two researchers working independently with two experimental series of Rec p30-IB. During tests, we used blood serum and organ specimens from domestic pigs and wild boars sampled both in field and under laboratory conditions.

1. Immunoblotting assay (IB) **and indirect immunofluorescent assay** (IIFA) **of specimens from healthy and ASFV experimentally infected**

Specimens	IIFA		Total
	positive	negative	
Infected (IB positive):			
blood serum	100	0	139
organs	39	0	
Healthy (IB negative):			
blood serum	1	53	72
organs	0	18	
Total	140	71	211

A total of 100 blood specimen out of 101 ones positive in IIFA (including 42 field specimens and 58 specimens from experimental animals) were also positive in IB. All 39 out of 39 $IIFA^+$ specimens of organs were IB^+. Hence, diagnostic sensitivity of Rec p30-IB makes 99.3 % at confidence interval of 95 to 99 %. Antibodies to p30 were detected in blood serum of domestic pigs and wild boars regardless of seroimmunotype and virulence of ASFV strains. Out of 53 blood specimens negative in IIFA, 53 were IB^-, and out of 18 $IIFA^-$ organ specimens all were IB^-. Hence, diagnostic specificity of Rec p30-IB is 100 % (Table 1). Out of 480 blood samples from domestic pigs which were negative in test with validated commercial ASF-IEA Ab/Ag kit, all were IB^- with Rec p30-IB. In 12 blood serum specimens from domestic pigs infected by heterologous viruses (DIC syndrome, CSF, and Aujeszky's disease), antibodies were not detected by IB method, i.e. no false positives were registered. For comparison: reported sensitivity and specificity of ELISA and immunoblotting with baculovirus recombinant p30 and field sera of European domestic pigs are within 96-99 % [18, 27]. Estimates of Rec p30-IB testify on highly reliable results of anti-ASFV antibody identification in blood serum and organs of domestic pigs and wild boars.

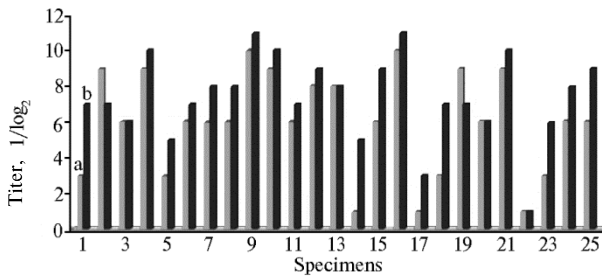

Fig. 1. Analytic sensitivity of indirect immunofluorescence assay (IIFA, a) **and immunoblotting test** (IB, b) **in identification of blood antibodies to ASFV after inoculation:** specimens NoNo 1-7, 10, and 17-25 — inoculated with SKA 2015 VNIIVViM; 8, 9 — with MK-200; 11, 12 — with KK-262/C; 13 — with FK-135 (domestic pigs); 14-16 — with MK-200 (a wild boar) (laboratory test).

We compared effectiveness of Rec p30-IB and ASFV-specific commercial tests offered on the market (Inmunologia y Genetica Aplicada S.A., Spain; IDvet Genetics, France) for 10 ASFV⁻ specimens (9 of intact non-infected and clinically healthy domestic pigs and 1 of a wild boar) and 10 $ASFV^+$ specimens (9 of domestic pigs and 1 of a wild boar experimentally inoculated with attenuated and virulent ASFV strains). Additionally, we tested 4 positive blood sera received from the reference laboratory CISA-INIA (Madrid). The obtained results show 100 % sensitivity and specificity of Rec p30-IB.

For analytical sensitivity evaluation, we compared IIFA and Rec p30-IB performance for detection of anti-ASFV antibodies in 2-fold dilutions of positive blood serum specimens from domestic pigs and a wild boar inoculated with attenuated ASFV strains of seroimmunotypes II-IV and VIII (Fig. 1). Among 25 exam-

ined positive specimens, the antibody titers with IB exceeded those with IIFA in 19 specimens, were the same in 4 specimens and lower in 2 ones. In 10 organ specimens from domestic pigs inoculated with strain MK-200, the antibody titers determined with Rec p30-IB were 4-8 times higher compared to IIFA. This testifies on higher analytic sensitivity of IB as compared to IIFA.

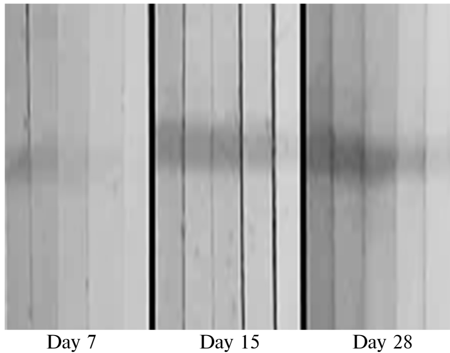

Fig. 2. Titration of blood anti-ASFV antibodies (1:8-1:128) **7 to 28 days after intramuscular inoculation of Large White domestic pigs with strain CKA 2015 VNIIVViM** (10^6 HAU_{50}, immunoblotting test).

For reproducibility estimate, Rec p30-IB should be evaluated either by independent researchers or with different series of the kit. In this experiment, two experts got the same reads for 4 negative and 4 positive specimens. The same results were for 8 negative and 19 positive blood sera of domestic pigs and wild boars tested with two Rec p30-IB series.

Antibodies to p30 are detected from day 7 to day 10 in blood serum of domestic pigs survived after inoculation with attenuated strains LK-111, KK-262/C, MK-200, FK-135, PSA-1-NH, CKA 2015 at 10^3-10^4 $HAU_{50}/TCID_{50}$. On days 15 and 28, the antibody titers rise and intensity of IB-bands increases (Fig. 2).

IB test revealed specific antibodies in 30 % of organ specimens from domestic pigs which died from ASF on days 7-10 after intramuscular inoculation with high virulent strains L-57, M-78 and Stavropol 01/08 at 10^3 HAU_{50}.

As relapses and remissions in chronic ASF occur periodically, it is important whether the serodiagnosis is always correct. In our experiment, we first inoculated pigs with attenuated strain KK-262/C (106 HAU_{50}), and then, 28 days after, infected these animals with virulent strain K-49 (103 HAU_{50}) for simulation of ASF relapse. Specific antibodies induced by strain KK-262/C were detected on days 3 and 5 after K-49 inoculation and not revealed on day 7 (Table 2). From days 12 to 17 of surveillance antibody titers of 1:16 to 1:256 were detected by IB assay. These results testify that in chronic ASF, antibodies may not be detected during remissions. This leads to false negatives in serodiagnosis. Therefore, due to occurrence of low virulent isolates, both serologic and PCR methods are needed for laboratory diagnosis.

2. Blood antibodies in Large White domestic pigs after inoculation with attenuated ASFV strain KK-262/C followed homologous virulent strain K-49 (n = 3, M±SEM, immunoblotting assay)

Days after infection with K-49	Antibody titer, log_2			
	animal No 1	animal No 2	animal No 3	animal No 4
3	6.7±0.6	5.7±0.3	6.3±0.3	6.3±0.6
5	6.3±0.3	5.7±0.6	5.7±0.3	6.3±0.3
7	0.0	0.0	0.0	0.0
12	5.3±0.6	6.3±0.6	5.3±0.3	7.3±0.3
17	6.7±0.3	6.7±0.6	3.7±0.6	7.7±0.6

Note, the collection of the Federal Research Center of Virology and Microbiology involves over 100 ASFV accession from Africa, Latin America, Europe, while abroad, the total number of deposited samples are 500. In our studies of blood of domestic pigs and wild boars infected by virulent, low virulent, attenuated, hemadsorbing and non-hemadsorbing ASFV strains and isolates referred to 5 seroimmunotypes out of known 9, there were no cases when positive serums did not react with recombinant p30.

Therefore, the proposed immunoblot-based test for African swine fever serological diagnosis in blood and biopsy material is effective for laboratory practice and ASF monitoring of domestic pigs and wild boars. Immunoblotting (IB) assay is reliable and does not need complex technical equipment. Given recommendations of the International Epizootic Bureau (OIE — World Organization for Animal Health, Paris, France), IB method should be approved in ASF diagnosis scheme adopted in the Russian Federation.

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