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DEVELOPMENT AND IMPROVEMENT OF ASF SEROLOGICAL DIAGNOSTIC TECHNIQUES

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SUMMARY

Due to the lack of effective tools of ASF specific prevention it is evident that early diagnosis is one of the most important and resultative ways of the disease control. However, contemporary diagnosis is a complex component of any effective surveillance system. Latest scientific achievements facilitated not only highly specific and sensitive but also rapid methods of laboratory diagnosis. Nevertheless, further development, improvement and expansion of ASF diagnosis techniques including rapid tests is a topical task of a great concern. The research is devoted to development of rapid test methods for rapid detection of antibodies to ASFV in blood sera of infected animals as well as to analysis of their use effectiveness. The following methods were suggested: immunoperoxidase monolayer assay using fixed cell line (ASFV permissive CV-1 cell-line infected with the virus strain ASF/ARRIAH/ CV-1) and latex agglutination test using ASFV p30 recombinant protein. The performed research demonstrated the effectiveness of the applied techniques for ASF serological diagnosis. Latex agglutination test and immunoperoxidase monolayer assay give rapid and high quality test results (within 1–2 hours). The advantage of the specified methods as compared to ELISA is their simplicity and the possibility of use in conditions of limited technical support.

Key words: African swine fever, latex agglutination test, immunoperoxidase monolayer assay, CV-1 continuous cell line, p30 recombinant protein, virus antigen.

INTRODUCTION

Effective means of disease-specific prevention with respect to such a dangerous disease as African swine fever (ASF) have not been developed so far; therefore, the presence of ASFV-specific antibodies is indicative of previous or ongoing infection [6]. In this regard, timely diagnosis is of particular importance in the system of ASF control measures. Diagnosis allows agent detection, containment and infection outbreak eradication. It is also a rather efficient tool for studying the characteristics of disease pathogenesis [2, 9]. Taking into account the fact that the cases when virus isolates characterized by reduced virulence and decreased lethality for pigs are detected are becoming more frequent, there is a growing need for the development of new ASF serological diagnostic techniques, as well as the improvement of existing ones. Rapid and reliable detection of ASF agent and specific antibodies to it is crucial for the timely implementation of measures aimed to prevent the spread of the disease. Summarizing the above, it can be concluded that there is a need for improved serological diagnostic methods, including rapid diagnosis techniques.

As for immunological testing, diagnostica based on passive agglutination assay are used. They are rapid immunochemical tests that combine high specificity and sensitivity with simplicity and rapid result, and do not require sophisticated devices for result recording. Such methods are widely used in medicinal and veterinary practice [4, 5]. Latex agglutination test (LAT) systems comprise the suspension of polymer latex microparticles carrying specific bioligands on their surface that can affinely bind with a detectable component (antigen/antibody) forming easily visible agglomerates [1, 8].

Immunocytochemical analysis techniques for antibody detection are based on the specificity of antigen-antibody reaction and the sensitivity of a light microscope. The immune complex is detected using anti-species anti-

bodies conjugated to enzyme labels. Immunoperoxidase monolayer assay (IPMA) is an immunocytochemistry technique where fixed cells of the permissive cell line infected with the relevant virus are used to detect specific antibodies. The advantages of the method are as follows: the preparation of IPMA plates is simple, and they can be stored for an extended period of time; the reaction results are examined with an inverted light microscope which is suited for field conditions; staining result is stable, and stained specimens can be stored for a long time [7].

Currently, there are no commercial test kits and test systems for anti-ASFV antibody detection based on latex agglutination test and immunoperoxidase monolayer assay available in this country. The use of LAT and IPMA can be a reproducible and safe alternative to the conventional methods of ASF serological laboratory diagnosis.

The development and application of rapid, simple and accessible techniques for detection of specific antibodies to ASF virus can accelerate infection diagnosis in the context of limited resources. They will allow the identification of high risk regions, the development of adequate recommendations on ASF occurrence and spread prevention, and may also facilitate the rapid identification and eradication of ASF sources in the infected areas.

MATERIALS AND METHODS

Latex agglutination test. To prepare a specific latex diagnosticum, monodisperse polystyrene latex particles with a mean diameter of 0.9–1.1 μm (DiaM, Russia) were used as carriers.

Prokaryotically expressed recombinant protein p30 of ASF virus was used as a specific antigen to coat polysterene latex particles.

The test was performed according to the common procedure using U-bottom immunoassay plates (semiquantitative analysis) or slides (qualitative assay). Coated latex particles were mixed with phosphate buffered saline (PBS), ASFV-negative and ASFV-positive sera; then the preparations were examined for spontaneous and specific agglutination.

The qualitative assay was carried out using non-diluted porcine serum samples. The results were recorded either as positive or negative. In the semiquantitative analysis, sera were tested in triplicate serial two-fold dilutions (from 1:20 to 1:5120). The agglutination was scored as follows: "++++" – large-flake agglutination, clear background; "+++" – small-flake agglutination, semi-clear background, "++" – small-flake agglutination, turbid background; "+" – hardly visible agglutination, turbid background; "–" – no agglutination, smooth and turbid background. The result was considered positive when agglutination was scored as "++"-"++++", inconclusive – when agglutination was scored as "+", negative – when agglutination was absent.

Immunoperoxidase monolayer assay. Tests were performed using 96-well culture plates with formed monolayer of CV-1 cells infected with ASF/ARRIAH/CV-1 strain of ASF virus adapted for reproduction in CV-1 cell culture.

Peroxidase conjugated goat anti-porcine IgG antibodies were used as secondary (anti-species) antibodies; the working dilution of the conjugate was prepared using a blocking buffer (pH 7.2-7.4).

Substrate buffer was prepared using 20 mg of 3-Amino-9-ethylcarbazole diluted in 2.5 cm^3 of dymethylformamide (storage period at 4 ± 3°C in the dark place is 1 month) and 0.05% acetate buffer with addition of H_2O_2.

The plates were examined under an inverted light microscope for the reaction presence and intensity: red and brown staining of specific ASFV antigen-antibody reaction sites where stained infected cells were counted in 10 randomly selected fields of vision. The serum was considered positive when specific staining was observed in at least 20% of infected cells, provided that no such staining was observed in the control wells. Control wells were those containing porcine sera and intact CV-1 cell culture.

Porcine sera. ASF convalescent pig serum prepared in the process of biological testing carried out in animals according to methodological recommendations was used as a positive control [3]. Field porcine serum containing no anti-ASFV antibodies was used as a negative control. All control sera were pre-tested using ELISA.

RESULTS AND DISCUSSION

To prepare the latex diagnosticum for anti-ASFV antibody detection based on p30 recombinant protein of ASF virus, 5-10% suspension of monomeric polystyrene latex (microspheres with a diameter of 0.9–1.1μm without functional groups) was used.

The bioligand was attached to the microsphere surface via a spacer in order to preserve its native conformation and immune activity. In this test, glutaric aldehyde played a key role of a "bridge" for covalent binding of latex particles and the target protein.

Optimal conditions for LAT were determined through chessboard titration of different p30 recombinant protein concentrations in the range of 500 to 10 μg/ml for coating 2.5% of latex particles and two-fold dilutions of reference ASFV-positive serum and negative control.

The sensitivity of LAT was the highest (1:2560-1:5120) when p30 recombinant protein was spontaneously adsorbed onto the surface of polystyrene microspheres either at antigen concentration of 150 μg/ml in 0.01 M PBS, pH 7.2–7.4 (1:4 dilution), or at antigen concentration of 30 μg/ml provided that latex particles had been treated with 0.05% glutaric aldehyde solution prepared using 0.01 M PBS, pH 7.2–7.4 (1:16 dilution). The recombinant protein p30 was diluted to make a concentration that was suitable for latex particle coating without impeding the reaction.

To perform latex agglutination test, 0.025 cm^3 of test serum and 0.025 cm^3 of loaded latex particles were added to the wells of the U-bottom immunoassay plate. The reaction can be observed in 5-15 minutes, the final result is obtained in 30-60 minutes. The latex particle agglutination is clearly visible to a naked eye against a dark background or at low magnification under the microscope (Fig. 1).

The tests performed showed that binding via a spacer (glutaric aldehyde) was stronger and more stable than conventional spontaneous adsorption of latex particles by the bioligand; the use of glutaric aldehyde enabled a better antigen adsorption onto latex particles without increasing the non-specific binding of antibodies and the recombinant antigen adsorbed onto latex.

To identify optimal conditions for immobilization of ASFV recombinant protein p30 on the latex particles, various temperature regimes and incubation times were tried out. It was found that a temperature of +37 °C and a 6-hour incubation period were optimal for adsorption of p30 recombinant protein at a concentration of 30 ng/μl onto polysterene latex particles treated with 0.05% glutaric aldehyde solution prepared using 0.01 M PBS, pH 7.2–7.4. Test results are presented in Table 1.

Data presented in Table 1 suggest that 6-hour exposure at a temperature of +37 °C can be considered optimal, since further extension of immobilization period did not significantly increase the sensitivity of LAT.

As can be seen from the above, optimal conditions for coating latex particles with p30 recombinant protein were determined as a result of the work.

Using latex agglutination technique, 30 positive and 20 negative (based on ELISA results) porcine sera were tested. The results of LAT and those obtained when the said sera were tested using ELISA were 100% identical. The developed LAT technique, therefore, allows effective and rapid detection of anti-ASFV antibodies in porcine sera.

For further development and improvement of diagnostic tests, fixed test specimen for IPMA were prepared. Before adding porcine sera, test specimens were washed one time with PBS (pH 7.2). Serial two-fold dilutions of sera (from 1:10 to 1:640) were transferred to the plates (2 wells for each dilution); the plates were incubated on a shaker at a temperature of 37 °C for 60 minutes. Then the plates were washed three times with PBS, and 100 µl of peroxidase-conjugated goat anti-porcine IgG or protein A were added to each well. The plates were incubated at 37 °C for 45 minutes. The plates were washed three times; then 100 µl of substrate buffer were added to each well. The colour reaction was allowed to proceed for 20 minutes; after that, the plates were washed one time with PBS and examined under the inverted light microscope.

Specific antibodies to ASF virus were detected in 1:20-1:160 diluted sera using IPMA; no non-specific background reaction was observed.

The detection of specific reaction of anti-ASFV antibodies contained in porcine sera with ASFV antigens in CV-1 cell culture is shown in Figure 2.

As the figure shows, specific peroxidase staining is only observed when infected CV-1 cell culture interacts with sera of ASFV-positive pigs and is not observed with sera containing no anti-ASFV antibodies.

In addition to ASFV-negative porcine sera, various dilutions of sera positive to heterologous viral infections (CSF, porcine influenza) were tested using the prepared test specimens. No specific interaction with antibodies was observed when the said sera were tested using immunoperoxidase assay. The results of testing different sera using IPMA are presented in Table 2.

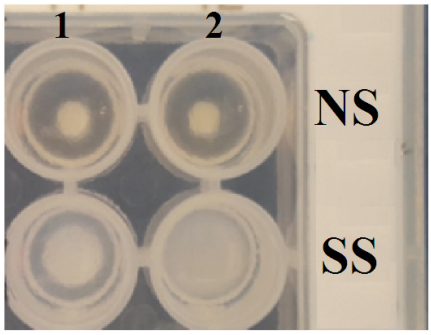

Fig. 1. Detection of specific antibodies to ASF virus using LAT

Column 1 – latex diagnosticum prepared by passive adsorption;
column 2 – latex diagnosticum prepared using covalent bonds (glutaric aldehyde);
NS (top row) – normal porcine serum;
SS (bottom row) – specific control serum positive to ASF virus.

The results given in the table demonstrate that specific staining was observed in the wells containing infected cell culture when ASFV-specific porcine sera at dilutions up to 1:160 were added. No staining was detected in the wells containing uninfected cell culture, normal pig and boar sera, as well as sera positive to heterologous viral infections (KSF, swine influenza).

CONCLUSION

The developed p30 recombinant protein based LAT technique presented in this paper is a specific, sensitive, rapid, easy to perform and cost-effective method that does not require sophisticated equipment and high qualification of personnel. It is therefore the most acceptable technique for field serological monitoring of ASF in the veterinary practice. Latex agglutination test based on p30 recombinant protein is effective in diagnostic testing and eliminates the need for working with infectious virus material.

Immunoperoxidase monolayer assay also has a number of advantages. These include the following: plates with

Table 1
Latex agglutination test results in relation to exposure period

Serum dilution	Exposure period (hours)						
	2	3	4	5	6	7	8
1:10	+	++	+++	++++	++++	++++	++++
1:20	+	+	++	+++	++++	++++	++++
1:40	–	+	++	+++	+++	++++	+++
1:80	–	+	++	++	+++	+++	+++
1:160	–	–	+	++	+++	++	+++
1:320	–	–	–	+	++	++	++
1:640	–	–	–	–	–	+	–
1:1280	–	–	–	–	–	–	–

Exposure temperature was +37 °C, p30 recombinant protein concentration for LAT – 30 µg/ml.

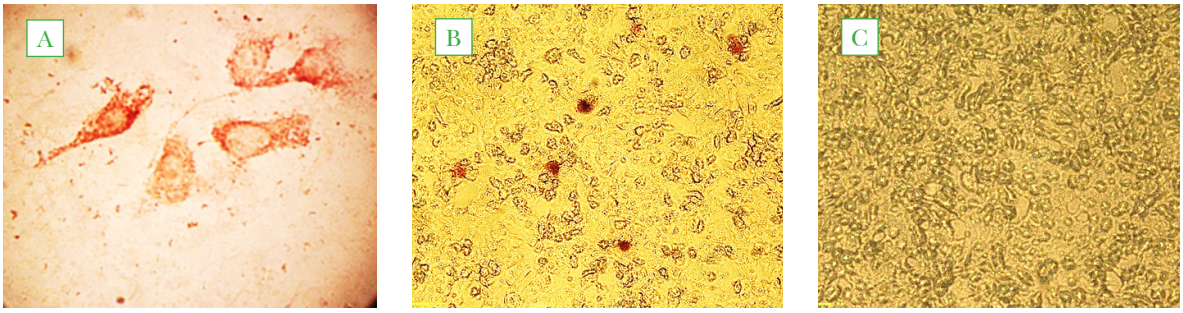

Fig. 2. Detection of specific antibodies to ASF virus in porcine sera using IPMA

A – positive immunoperoxidase staining of infected cells, 400-fold magnification;
B – positive reaction with 1:40 diluted specific serum, 200-fold magnification;
C – negative reaction with 1:40 diluted normal porcine sera, 200-fold magnification.

Table 2
Detection of anti-ASFV antibodies in porcine sera using IPMA

Serum dilution / Column No.	NS (pig)		SS positive to ASF virus No. 1		NS (boar)		SS positive to CSF virus		SS positive to swine influenza virus		SS positive to ASF virus No. 2	
	1	2	3	4	5	6	7	8	9	10	11	12
1:20	–	–	–	+	–	–	–	–	–	–	–	+
1:40	–	–	–	+	–	–	–	–	–	–	–	+
1:80	–	–	–	+	–	–	–	–	–	–	–	+
1:160	–	–	–	+	–	–	–	–	–	–	–	–
1:320	–	–	–	–	–	–	–	–	–	–	–	–
1:640	–	–	–	–	–	–	–	–	–	–	–	–

Odd-numbered columns – uninfected cell culture (control);
even-numbered columns – infected cell culture;
«+» and coloured boxes – specific staining of cells in the wells;
«–» – no staining.

virus infected cells are easy to prepare, and they can be stored for a long time. Besides, the assay allows easy and objective interpretation of results using only an inverted light microscope. Staining results are stable, and stained specimens can be stored for several months.

To sum up, latex agglutination test and immunoperoxidase monolayer assay are simple, rapid and reproducible techniques that are comparable to reference ASF diagnostic methods and suitable for use in the regions where laboratory resources are limited. These tests have potential as inexpensive alternative methods for detection of ASFV-specific antibodies and estimation of their levels (high/low) either in a single test or in screening (monitoring) tests, including those performed in the field.

Conflict of interests. The authors declare that there is no conflict of interests.

REFERENCES

1. Brovkina A.N. Development of Salmonella detection method and test-system using latex agglutination [Razrabotka metoda i test-sistemy vyyavleniya bakterij roda Salmonella na osnove lateks-agglyutinacii]. *Scientific Journal KubSAU*. 2011; 72 (08). URL: http://ej.kubagro.ru/2011/08/pdf/31.pdf (in Russian).

2. Makarov V. V. African swine fever [Afrikanskaya chuma svinej]. M.: RUDN, 2011 (in Russian).

3. Methodical instructions for preparation of type-specific pig blood serum to ASFV for hemadsorbtion inhibition test performance: approved by the Rosselkhoznadzor in 2017 [Metodicheskie ukazaniya po polucheniyu tipospecificheskoj syvorotki krovi svinej k virusu afrikanskoj chumy svinej dlya postanovki reakcii zaderzhki gemadsorbcii: utv. Rossel'hoznadzorom v 2017 g]. MU 07-15; FGBI "ARRIAH". Vladimir, 2017 (in Russian).

4. Stanishevsky Ya. M. Biomedical polymer disperse systems [Polimernye dispersnye sistemy mediko-biologicheskogo naznacheniya]: authors extract. dis. … candidate of science (Biology). M., 2001 (in Russian).

5. Chaika N. A. Latex agglutination test [Reakciya agglyutinacii lateksa]. *Immunodiagnosis of viral infections [Immunologicheskaya diagnostika virusnyh infekcij]*. F. S. Noskov, A. A. Smoroditsev, Yu. M. Brodyansky [et al]; ed. by T. V. Peradze, P. Halonen. M.: Medicine. 1985; 121–143 (in Russian).

6. African swine fever. *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (mammals, birds and bees)*. OIE. 7th ed. Paris, 2012; 2 (2.8.1): 1067–1079.

7. Development of an immunoperoxidase monolayer assay for the detection of antibodies against peste des petits ruminants virus based on BHK-21 cell line stably expressing the goat signaling lymphocyte activation molecule. J. Zhang, W. Liu, W. Chen [et al.]. *PLoS ONE*. 2016; 11 (10):e0165088; DOI: 10.1371/journal.pone.0165088.

8. Hechemy K., Michaelson E. Latex particle assays in laboratory medicine. Part I–II. *Laboratory Management*. 1984; 27 (40): 26–34.

9. Molecular characterization of African swine fever virus isolates originating from outbreaks in the Russian Federation between 2007 and 2011. A. Malogolovkin, A. Yelsukova, C. Gallardo [et al.]. *Vet. Microbiol.* 2012; 158 (3–4): 415–419; DOI: 10.1016/j.vetmic.2012.03.002.

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