

---

APPLIED  
MOLECULAR BIOLOGY

---

UDC 619:578.835.2:616-078

## Use of the Recombinant Nonstructural 3A, 3B, and 3AB Proteins of Foot-and-Mouth Disease Virus in Indirect ELISA for Differentiation of Vaccinated and Infected Cattle

A. S. Yakovleva, A. V. Shcherbakov, A. V. Kan'shina,  
N. S. Mudrak, and T. A. Fomina<sup>†</sup>

*Federal Center for Animal Health, Ministry of Agriculture of the Russian Federation, Vladimir, 600901 Russia*  
*e-mail: kovalishin@arriah.ru*

Received June 29, 2005

**Abstract**—Recombinant foot-and-mouth disease virus (FMDV) proteins 3A, 3B, and 3AB were produced by expressing the corresponding genes in *Escherichia coli* and purified by metal–chelate affinity chromatography. The recombinant proteins were used as antigens in indirect enzyme-linked immunosorbent assay (ELISA) to differentiate between vaccinated and FMD-infected animals. The following parameters were determined: working concentrations of antigens and peroxidase conjugate of cattle anti-IgG, the optimum composition of blocking buffer, and the positive–negative threshold of the reaction. Tests performed with approximately 200 serum samples taken from animals of different immunity states showed that the protocol with protein 3A as the antigen (3A-ELISA) provided the most reliable differentiation. All the newly developed systems proved to outperform the commercial Chekit FMD-3ABC kit in sensitivity, and 3A-ELISA was no less specific.

**DOI:** 10.1134/S0026893306010195

**Key words:** foot-and-mouth disease virus, nonstructural proteins, expression in *Escherichia coli*, metal–chelate affinity chromatography, indirect ELISA, diagnostic sensitivity and specificity

### INTRODUCTION

Foot-and-mouth disease (FMD), which affects artiodactyls, is highly contagious. It ranks first among the most dangerous animal diseases, being able to cause epizootics and great economic loss. The outbreak of 1997 in Taiwan caused a loss of 378 million US dollars [1]. The direct and indirect loss caused by the 2001 FMD epizootic in Great Britain reached 10 billion pounds sterling [2].

Russia is constantly threatened by the penetration of this disease from bordering Asian countries. Examples are FMD outbreaks of 1995, 2000, 2004, and 2005 caused by virus penetration from China to Russia [3, 4]. For this reason, the development and improvement of methods for FMD diagnosis are directly related to the zoosanitary and economic security of Russia.

Foot-and-mouth disease is caused by a nonenveloped RNA virus of the Picornaviridae family, genus *Aphthovirus*. The viral genome codes for four structural and eight nonstructural proteins. Both protein groups induce the production of antibodies in infected animals. However, vaccinated animals generate mainly antibodies against structural proteins. This is related to the fact that vaccine production includes partial purification of the virus, with

most of the nonstructural proteins being eliminated together with cell debris.

Detection of antibodies against nonstructural foot-and-mouth disease virus (FMDV) proteins is an important tool for monitoring the disease because it allows differentiation of vaccinated and infected animals and detection of asymptomatic carriers among vaccinated stock. The anti-FMD vaccination program performed along the southern border of Russia demands diagnostic tools allowing the detection of infected animals in vaccinated herds. At present, several foreign test kits for detecting antibodies against nonstructural FMDV proteins are commercially available. They are based on synthetic peptides or recombinant FMDV proteins produced in *E. coli* or in baculoviral systems. No domestic test kits have as yet been developed in Russia.

Numerous studies show that proteins 3A and 3B are the best candidate antigens for the differentiating test [5–12].

The objectives of the present study were to prepare recombinant FMDV proteins 3A, 3B, and 3AB and to test them for applicability to the differentiation of infected and vaccinated animals.

## EXPERIMENTAL

**Isolation of viral RNA.** Viral RNA was isolated from virus suspension with the use of 6 M guanidine isothiocyanate and GF/F glass fiber filters [13].

**Primer design and synthesis.** Primers were designed on the basis of the FMDV sequence A<sub>22</sub> 550 retrieved from the GenBank bioinformatics system (accession no. X74812). The following primers were chosen for amplification and cloning of the 3A, 3B, and 3AB genes of FMDV:

3AF: 5'-GGATCCATTCCTTCCCAAAGTC-3';

3AR: 5'-AAGCTTTTCAGCTTGTGGTTG-3';

3BF: 5'-GGATCCTACACCGGGCCACT-3';

3BR: 5'-AAGCTTCTCAGTGACAATCAA-3'.

The primers were synthesized by Sintol (Moscow).

**Reverse transcription and polymerase chain reaction (RT-PCR)** were performed in a single test tube. The reaction mixture (50 µl) contained 5 µl of 10× *Taq* polymerase buffer (Promega, USA), 3 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 2.0 U of avian myeloblastosis virus reverse transcriptase (Promega), 2.0 U of *Taq* polymerase (Promega), 20 pmol of each primer, 5 µl of aqueous solution of viral RNA, and water to adjust the volume. The reaction was performed in a Minicycler PTC-100 thermocycler (MJ Research, USA). The program was conducted as follows: reverse transcription at 42°C for 15 min, denaturation of the RNA-DNA complex at 94°C for 2 min, and 35 PCR cycles: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 40 s. The reaction products were analyzed by electrophoresis in 2% agarose gel with 0.001% ethidium bromide at 50 mA.

**Cloning of PCR products** was performed in the pQE expression plasmid and *E. coli* strain M15 (Qiagen, Germany) by conventional methods [14]

**Expression of recombinant protein genes.** *Escherichia coli* cells were cultured on an orbital shaker at 37°C and 150 rpm until the culture reached an optical density of A<sub>550</sub> = 0.6. After that, IPTG (Promega) was added. Protein levels and sizes were determined by vertical electrophoresis in 12% polyacrylamide gel.

**Purification of recombinant proteins.** Recombinant proteins were purified by metal-chelate affinity chromatography. Sedimented *E. coli* cells were treated with lysis buffer (0.01 M Tris-HCl, pH 8.0; 6 M guanidine chloride, and 0.1 M NaH<sub>2</sub>PO<sub>4</sub>) and centrifuged at 12,000 rpm for 5 min. Ni-NTA agarose (Qiagen) was added to the supernatant fluid, and the mixture was incubated at room temperature for 15 min, with shaking. The sorbent with the protein was washed with two portions of the lysis buffer. The proteins adsorbed on agarose were solubilized with

elution buffer (0.01 M Tris-HCl, pH 8.0; 0.4 M imidazole, and 0.1 M NaH<sub>2</sub>PO<sub>4</sub>).

**Indirect ELISA** was performed according to the conventional protocol [15]. The commercial conjugate of bovine anti-IgG with horseradish peroxidase was purchased from the Gamaleya Institute of Epidemiology and Microbiology (Moscow). The reaction was visualized by addition of the substrate 2,2'-azine-bis(3-ethyl)benzothiazoline-6-sulfonic acid (ABTS). Quantitative assessment of the staining was performed with a Uniplan spectrometer (Russia) at 405 nm. We examined 30 sera from clinically healthy animals not vaccinated against FMD, 20 sera from cattle once or repeatedly vaccinated against FMD, and sera from animals experimentally infected with FMDV of serotypes A, O, and Asia 1, which were taken on days 5, 7, and 21 after infection (postinfection days, PID): 34 sera 5PID, 74 sera 7PID, and 17 sera 21PID.

**Statistical evaluation of the results.** The diagnostic sensitivity (D<sub>sens</sub>) and specificity (D<sub>spec</sub>) were calculated as recommended by the Office International des Epizooties [16]: D<sub>sens</sub> = [TP/(TP + FN)] × 100%; D<sub>spec</sub> = [TN/(TN + FP)] × 100%, where TP, FN, TN, and FP are the numbers of true positive, false negative, true negative, and false positive results, respectively.

## RESULTS

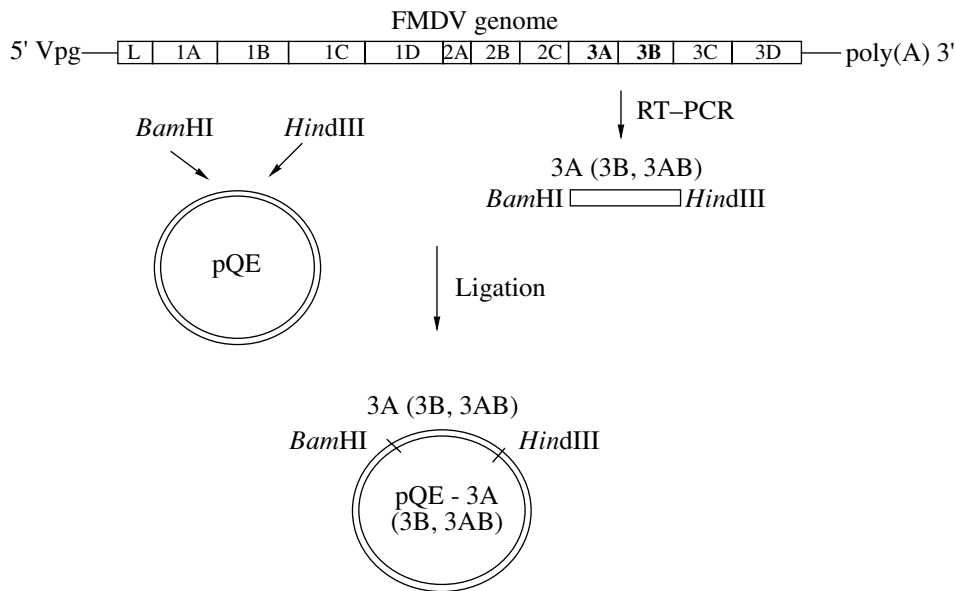
Recombinant nonstructural proteins were constructed on the basis of FMDV strain A<sub>22</sub> 550. We obtained cDNAs of the 3A, 3B, and 3AB genes of the virus by RT-PCR with primers including *Bam*HI and *Hind*III restriction sites: primers 3AF and 3AR for 3A, 3BF and 3BR for 3B, and 3AF and 3BR for 3AB. After digestion with corresponding restriction endonucleases, the PCR products were cloned into plasmid pQE (Fig. 1). Transformation of *E. coli* strain M15 with the recombinant plasmids yielded clones producing the 3A, 3B, and 3AB FMDV proteins with six histidine residues at their N termini.

The results of PAGE analysis of the recombinant proteins are shown in Fig. 2. The molecular weights of the proteins corresponded to predicted values: 3A, 22 kDa; 3B, 11 kDa; and 3AB, 33 kDa.

Experiments on optimization of gene expression were performed in order to increase production of the proteins by *E. coli* cells.

The effect of IPTG concentration on expression was studied in the concentration range of 0.01 to 2 mM. The levels of recombinant FMDV proteins reached their maximum values at 0.2 mM IPTG. Further elevation of IPTG concentration did not increase them.

To study the time course of protein accumulation, culture samples were taken 0.25, 0.5, 1, 2, 4, and 18 h after induction and analyzed by PAGE. The maximum amount of recombinant proteins was obtained 4 h after



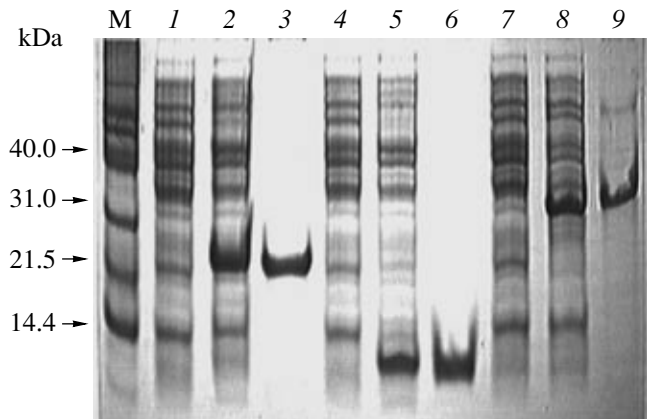
**Fig. 1.** Cloning of the 3A, 3B, and 3AB FMDV genes into the pQE plasmid.

induction, without further increase. All the three proteins were stable, as no proteolysis was observed even 18 h after induction.

At the next stage, we developed a protocol for purification of recombinant FMDV proteins. All the three recombinant proteins were present in *E. coli* cells in the insoluble state. Therefore, we purified them under denaturing conditions. The proteins were dissolved only partially when 8 M urea was added to the lysis buffer. The proteins could be completely solubilized only with 6 M guanidine hydrochloride. After solubi-

lization, the proteins were purified by metal–chelate affinity chromatography on Ni-NTA agarose (Qiagen). However, the elution conditions recommended by the manufacturer were not applicable, because none of the three proteins could be eluted under standard conditions (at low pH or in the presence of 0.2 M imidazole). The bulk of each protein could be eluted only at imidazole concentrations in the elution buffer elevated to 0.4 M (pH 8.0). The yields of the purified proteins from 100 ml of the *E. coli* cell culture were 5, 1, and 1.5 mg for 3A, 3A, and 3AB, respectively.

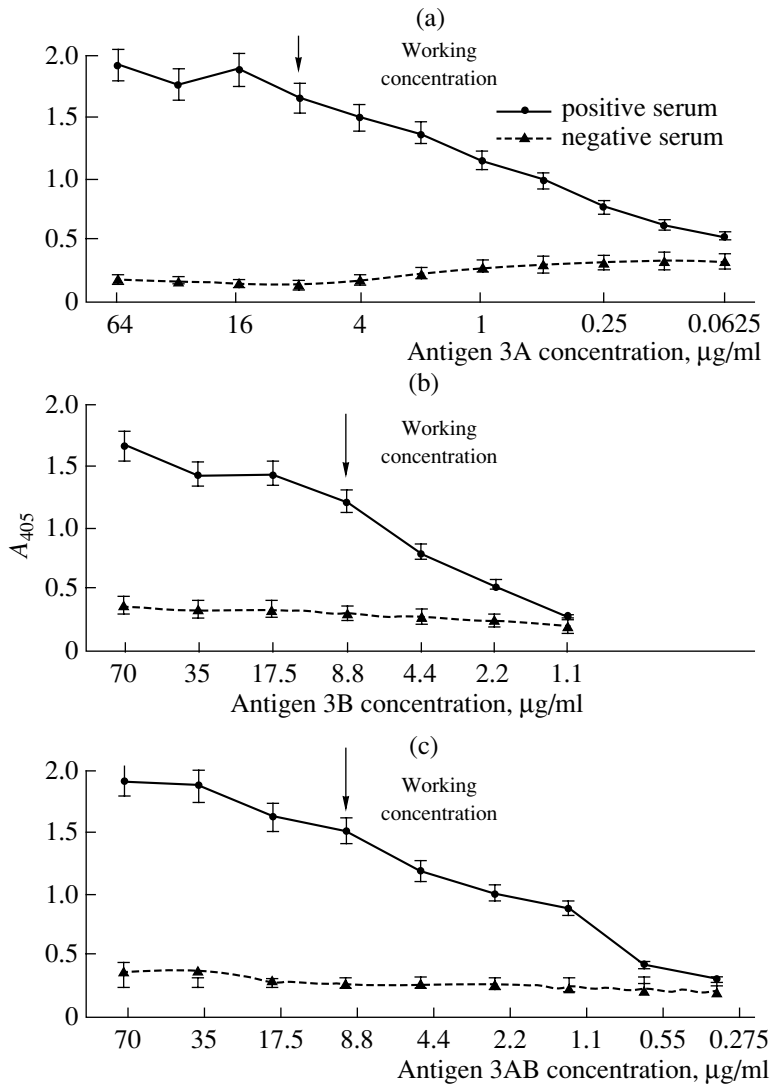
The antigenic activity and specificity of the purified preparations of recombinant proteins were studied.



**Fig. 2.** Electrophoresis of recombinant proteins in 12% polyacrylamide gel. Expression of the genes for: (1–3) the recombinant 3A protein, (4–6) the recombinant 3B protein, and (7–9) the recombinant 3AB protein. Lanes: (1, 4, 7) lysate of *E. coli* cells before induction, (2, 5, 8) 4 h after induction; (3, 6, 9) purified recombinant 3A, 3B, and 3AB proteins, respectively; (M) protein molecular weight marker, kDa.

The activity of recombinant proteins was tested by indirect ELISA with the reference cattle sera. A serum from an animal with a history of FMD was used as a positive control, and a healthy-cattle serum was used as a negative control. The results of the test are shown in Fig. 3. All the three proteins showed specific antigenic activity. The minimum protein concentrations at which the optical density of the positive control was twice as high as that of the negative one were different: 0.25 µg/ml for 3A, 2.2 µg/ml for 3B, and 1.1 µg/ml for 3AB. These concentrations corresponded to the following protein dilutions: 1:32,000, 1:800, and 1:3200 for 3A, 3B, and 3AB, respectively. Thus, 3A has the highest antigenic activity.

The antigenic specificity of the recombinant proteins was tested by indirect ELISA with cattle sera containing antibodies against bovine leukemia virus, parainfluenza virus, infectious bovine rhinotracheitis virus, and bovine coronavirus. The activity of all the proteins with the heterologous sera did not exceed the background level with the nonimmune serum. The



**Fig. 3.** Antigenic activity of recombinant proteins (a) 3A, (b) 3B, and (c) 3AB with control sera. The activity was tested by indirect ELISA. Serum of an animal with a history of FMD was used as a positive control and normal cattle serum was used as a negative control.

absence of the reaction of the proteins with heterologous sera is indicative of their antigenic specificity.

After the test for protein activity and specificity, we performed experiments on optimizing the conditions of ELISA in order to bring the method to completion. In particular, the optimal composition of the blocking buffer, the working concentrations of the antigen and conjugate, and the positive–negative reaction threshold were determined. The working concentrations of all proteins were approximately equal: 8–8.8  $\mu\text{g/ml}$  (Fig. 3).

In the course of development of the blocking solution, we tested 10% yeast extract, 3% and 10% defatted milk, and 10% normal equine and rabbit sera with the TBST buffer (0.02 M Tris-HCl, pH 7.6; 0.14 M sodium chloride, and 0.01% Tween 20). Among them, 10% equine serum proved to be the best additive.

After optimizing all parameters, we accepted the following ELISA protocol. Aliquots (50  $\mu\text{l}$ ) of a recombinant protein at the working concentration were placed into each well of the plate and incubated at 4°C for 16–18 h. Then 50  $\mu\text{l}$  of the blocking buffer (TBST, pH 7.6, with 10% equine serum) was added to each well without washing, and the plate was incubated at 37°C for 1 h. After washing the wells with TBST, they were filled with twofold serial dilutions of the test sera with the blocking buffer (beginning from 1:100, 50  $\mu\text{l}$  per well), and the plate was incubated on a shaker at 37°C for 1 h. Thereafter, the wells were washed from nonbound components, peroxidase-conjugated anti-IgG at the working concentration was added, and the plate was incubated again under the same conditions. After washing, the ABTS substrate was added to the wells, and the results of the

## Results of analysis of cattle sera with various test systems

Animal state	3A-ELISA	3B-ELISA	3AB-ELISA	Chekit-ELISA
Healthy (30 sera)	29 negative, 1 positive	29 negative, 1 ambiguous	28 negative, 2 ambiguous	30 negative
Once or repeatedly vaccinated (20 sera)	18 negative, 2 ambiguous	17 negative, 2 ambiguous, 1 positive	16 negative, 3 ambiguous, 1 positive	20 negative
Experimentally infected with FMDV, 5PID (34 sera)	34 negative	34 negative	34 negative	34 negative
Experimentally infected with FMDV, 7PID (74 sera)	54 positive, 11 ambiguous, 6 negative	53 positive, 18 ambiguous, 3 negative	53 positive, 18 ambiguous, 13 negative	13 positive, 10 ambiguous, 51 negative
Experimentally infected with FMDV, 21PID (17 sera)	17 positive	17 positive	17 positive	17 positive

reaction were measured with a Uniplan spectrometer at 405 nm.

The positive–negative threshold of the differentiating test was determined by studying cattle blood sera with different levels of antibodies against nonstructural FMDV proteins. The sera were tested prior to the study with a commercial Chekit FMD-3ABC kit (Bommeli Diagnostics, Switzerland). The ELISA studies with antigens 3A, 3B, and 3AB showed that the titer of antibodies against nonstructural FMDV proteins were below 1:400 in negative sera, between 1:400 and 1:800 in ambiguous sera, and 1:800 or higher in positive sera.

The three tests, hereafter referred to as 3A-, 3B-, and 3AB-ELISA, were applied to four serum groups: from healthy cattle not vaccinated against FMD, from once or repeatedly vaccinated cattle, and from animals experimentally infected with FMDV. The sera were taken 5, 7, and 21 days after infection (PID). The results of the tests are shown in the table.

The 3A-ELISA test provided the most reliable differentiation of FMD-infected and uninfected animals. In this test, none of the samples from healthy unvaccinated or vaccinated cattle fell within the range of positive results. The 3B- and 3AB-ELISA tests proved to be less specific. They showed false positive results in one animal each, with the animals being different.

In tests of sera from experimentally infected animals, all the three methods revealed antibodies against FMDV starting from 7PID. Sera taken 5 days after infections showed negative results. All sera from the 21PID group were positive in all the three tests.

The table also includes the results of testing sera with the commercial Chekit FMD-3ABC kit. This system, Chekit-ELISA, showed 100% specificity in our studies; however, its sensitivity was considerably lower than those of 3A, 3B, and 3AB-ELISA for 7PID

sera. Only 13 of 74 sera of the 7PID group were positive in Chekit-ELISA, whereas 57 sera were positive in 3A-ELISA and 53 sera were positive in 3B- and 3AB-ELISA. In the 21PID group, all the four tests showed 100% sensitivity and perfect correlation.

The results of tests presented in the table were used for determining the diagnostic sensitivity and specificity of the test systems. The sensitivity of the developed methods was 93.8% for 3A-ELISA, 87.5% for 3AB-ELISA, and 96.8% for 3B-ELISA. The sensitivity of Chekit-ELISA was considerably lower: 65.9%. The specificity was 100% for 3A-ELISA and Chekit-ELISA and 98.04% for 3B- and 3AB-ELISA.

## DISCUSSION

The main objectives of our study were to prepare the 3A, 3B, and 3AB recombinant nonstructural FMDV proteins, to develop enzyme immunoassay methods for differentiating vaccinated and FMDV-infected animals, and to compare the developed methods in order to choose the most specific and sensitive test.

Molecular cloning of the genes for the 3A, 3B, and 3AB FMDV proteins yielded *E. coli* clones producing corresponding recombinant proteins. All the three proteins accumulated in cells in large amounts, visually constituting no less than 10% of the total weight of cell proteins. The recombinant proteins showed similar accumulation properties: the optimum IPTG concentration for their induction was 0.2 mM, and their maximum accumulation in the form of inclusion bodies was recorded 4 h after induction. In spite of significant loss of recombinant proteins during purification, we obtained 5 mg of 3A, 1 mg of 3B, and 1.5 mg of 3AB from 100 ml of the cell culture.

The test for antigenic properties of the recombinant proteins by ELISA with control cattle sera showed that they had high antigenic activities and

specificities. The best indices were recorded for the 3A protein. The titer of this antigen was much higher: 1:32,000, compared to 1:800 and 1:3200 for 3B and 3AB, respectively. Optical densities with the negative control serum were very low (<0.2) even at initial 3A dilutions. These advantages of the 3A protein demonstrated by experiments with control sera were then confirmed by analysis of numerous sera from animals with different immunity states.

An indirect ELISA protocol was developed with the use of the recombinant proteins for differentiation of vaccinated and FMDV-infected cattle. The working concentrations of antigens and horseradish peroxidase-conjugated anti-IgG were determined, and the optimum composition of the blocking buffer was chosen. The positive-negative threshold of the reaction was determined. The method showed well-reproducible results. Proteins adsorbed on ELISA plates retained their antigenic activity without any loss for at least one year (time of observation).

One of the main tasks was to find out which non-structural FMDV protein, 3A, 3B, or 3AB, was most suitable as a component of a test kit for differentiation of vaccinated and FMDV-infected cattle. Tests of about 200 sera from animals with known immunity states showed that all three proteins were suitable, but 3A-ELISA showed the best combination of diagnostic specificity (100%) and sensitivity (93.8%).

Comparison with the commercial Chekit FMD-3ABC kit showed that all the tests developed by us outperformed it in sensitivity in testing sera early after infection.

Note that the relatively low values of the diagnostic sensitivity of Chekit FMD-3ABC found in our experiments were accounted for by the fact that 74 out of 91 doubtless positive sera tested in the study were taken from the 7PID group of animals experimentally infected with FMDV. In spite of the fact that some scientists reported detection of antibodies against non-structural FMDV proteins as early as 6PID [7], most of the commercial kits are not designed for such early detection of antibodies. As assured by the developers of Chekit FMDV-3ABC, the kit allows reliable detection of antibodies against FMDV on day 10–14 after infection. The FMDV-Ab kit by Svanova (Sweden) is more sensitive; however, it detects antibodies only on day 8. The fact that 3A-, 3B-, and 3AB-ELISA revealed antibodies against FMDV in most sera of the 7PID group is indicative of the high sensitivity of our methods.

Thus, we obtained the recombinant nonstructural 3A, 3B, and 3AB FMDV proteins, determined the optimal conditions of their synthesis and purification, and developed an indirect ELISA protocol based on use of the recombinant proteins as antigens. Their comparison showed that all the three test systems (3A-, 3B-, and 3AB-ELISA) were suitable for the differentiation of vaccinated and FMDV-infected cattle, but

3A-ELISA proved to be the most promising as a differentiating test.

## REFERENCES

1. Sung W.H.T. 2002. In: *FMD: Control Strategies: Proc. Int. Symp.* Lyons, pp. 97–105.
2. Donaldson A.I., Alexandersen S. 2002. In: *FMD: Control Strategies: Proc. Int. Symp.* Lyons, pp 173–181.
3. Shcherbakov A.V., Andreev V.G., Drygin V.V., Gusev A.A. 2002. Molecular epizootiology of foot-and-mouth disease in Russia and CIS countries. *Agrarnaya Rossiya*. **2**, 8–11.
4. Valarcher J.F., Knowles N.J., Ferris N.P., Paton D.J. 2005. Recent spread of FMD virus serotype Asia 1. *Vet. Rec.* **2**, 30.
5. Clavijo A., Wright P., Kitching P. 2004. Developments in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. *Vet. J.* **167**, 9–22.
6. Bergmann I.E., Malirat V., Neitzert E., Beck E., Paniz-zutti N., Sanchez C., Falczuk A. 2000. Improvement of serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: A combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. *Arch. Virol.* **145**, 473–489.
7. Clavijo A., Zhou E.M., Hole K. 2004. Development and use of a biotinylated 3ABC recombinant protein in a solid-phase competitive ELISA for the detection of antibodies against foot-and-mouth disease virus. *J. Virol. Meth.* **120**, 217–227.
8. De Diego M., Brocchi E., Mackay D., De Simone F. 1997. The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Arch. Virol.* **142**, 2021–2033.
9. Mackay D.K.J., Forsyth M.A., Davies P.R., Berlinzani A., Belsham G.J., Flint M., Ryan M.D. 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. *Vaccine*. **16**, 446–459.
10. Rodriguez A., Dopazo J., Saiz J.C., Saiz J.C., Sobrino F. 1994. Immunogenicity of non-structural proteins of foot-and-mouth disease virus: Differences between infected and vaccinated swine. *Arch. Virol.* **136**, 123–131.
11. Marquardt O. 1996. In: *Report of the Third Annual Meeting*. Tubingen, pp.19–20.
12. Sun T., Lu P., Wang X. 2004. Localization of infection-related epitopes on the non-structural protein 3ABC of foot-and-mouth disease virus and the application of tandem epitopes. *J. Virol. Meth.* **119**, 79–86.
13. Gribanov O.G., Shcherbakov A.V., Perevozchikova N.A., Gusev A.A. 1996. Application of aerosil A-300 and GF/F (GF/N) filters to purification of DNA fragments, plasmid DNA, and RNA. *Biokhimiya*. **61**, 1064–1070.
14. Maniatis, T., Fritsch, E.F., Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, N.Y.: Cold Spring Harbor Lab. Press.
15. *Teoriya i praktika immunofermentnogo analiza (ELISA: Theory and Practice)*. Eds. Egorov A.M., Osipov A.P., Dzantiev D.D., Gavrilova E.M. Moscow: Vysshaya Shkola.
16. Jacobson R.H. 1998. Validation of serological assays for diagnosis of infectious diseases. *Rev. S. Techn. (OIE)*. **17**, 469–526.