SEL'SKOKHOZYAISTVENNAYA BIOLOGIYA
[AGRICULTURAL BIOLOGY], 2014, № 6, pp. 73-80 ISSN 313-4836 (Online)

Serodiagnosis, immunology

UDC 619:616.98:578.835.2:616-079.4

doi: 10.15389/agrobiology.2014.6.73rus
doi: 10.15389/agrobiology.2014.6.73eng

DIAGNOSTIC VALUE OF A TEST FOR DETECTION OF SECRETORY IgA FOR CONFIRMATION OF ABSENCE OF FMD VIRUS CIRCULATION

D.N. AFONINA, N.YE. KAMALOVA, A.V. KAN'HINA, A.M. TIMINA

Federal Centre for Animal Health, FGBU «VNIIZZh», mkr. Yur'evets, Vadimir, 600901 Russia, e-mail afonina@arriah.ru, kamalova@arriah.ru, kanshina@arriah.ru, timina@arriah.ru
Received March 31, 2014

Abstract

In Russia the preventive vaccination dominates over other protective and preventive measures against foot and mouth disease (FMD). However, the development of the subclinical infection in immune animals should not be ruled out as these animals become virus carriers and pose a potential threat for susceptible animal population. Therefore, the design and reduction to practice of tests for identification of virus-carrier animals continues to be relevant. Secretory immunoglobulins A (sIgA) provide the first line of protection against many infectious agents, they are capable of inhibiting virus intracellular replication and serve as a transmitter of virus neutralization. As reported by foreign authors there is a possibility to identify virus-carrier animals using a rapid ELISA-based test detecting secretory IgA (sIgA-ELISA). Previously we determined sIgA-ELISA optimal conditions for detection virus-specific sIgA in saliva samples in one dilution. In the given paper results of validation and evaluation of the sIgA-ELISA test-system («sandwich» ELISA) are shown as compared with other laboratory methods. The paper presents data on testing bovine biological samples (96 animals with body weight of 200-300 kg at the age of 18-24 months) from FMD-free agricultural enterprises (Vladimir Oblast) collected before and after vaccination from animals, immunized with different batches of FMD vaccine and subject to experimental infection. Samples of saliva, blood sera and esopharyngeal fluid collected with an interval of 3-4 days and up to 94 days after infection were tested using sIgA-ELISA, NP-ELISA (detection of antibodies to virus nonstructural proteins), microneutralization test and Real-Time PCR. Besides, relative sensitivity, specificity and accuracy of the test was evaluated using formulae recommended by the World Organisation for Animal Health (OIE) and compared with results of a reference test conducted by Satya Parida (World Reference Laboratory for Foot and Mouth Disease — WRL, Pirbright Institute, Great Britain) according to a validated method. Out of five preliminary immunized and then infected animals without clinical signs one animal (№ 8895) demonstrated the presence of sIgA in saliva in sIgA-ELISA on day 18 after infection at PP (positivity percent) = 53.00±0.05 % and at other dates (from 21 to 94 days) — at PP from 41.00±0.11 to 115.00±0.41 %. The obtained results were confirmed using the microneutralization test (sIgA titer on day 18 was 1.20±0.05 lg, during a period from day 21 to day 46 the titer was 1.58±0.08-1.90±0.13 lg). At that, the microneutralization test showed significant variations in values and it is undesirable for an identification test. Results of detecting antibibodies to FMD virus nonstructural proteins on day 18-94 were positive. FMD virus RNA was detected using PCR in esopharyngeal fluid samples during a period from day 28 to day 60 after experimental infection, but sIgA-ELISA showed more unambiguous results as compared with PCR. A latent infection was also confirmed in the other animal (№ 8898). Thus, it was demonstrated that the suggested test-system on the basis of indirect «sandwich» ELISA for detection of sIgA to FMD virus in bovine saliva samples made it possible to identify virus-carrier animals according to diagnostic characteristics. After considering results of testing 91 saliva samples the sensitivity of the developed test-system as compared with the reference one (WRL) was 100 %, relative specificity — 97.7 %, accuracy — 99.0 %. The high reproducibility of two test-systems was confirmed on the basis of κ-statistics (κ = 0.99).

Keywords: foot and mouth disease, secretory immunoglobulin A, solid phase immunosorbent assay, virus carrier state, post-vaccination control.

A secretory IgA (sIgA) is essential for the first line of defense against many pathogens and play a critical role in mucosal immunity (1). IgAs can bind and aggregate bacteria and viruses in secretions preventing their adhesion on mucosal surfaces, and at high concentrations IgAs block viruses from attaching to cell membrane. At low concentration sIgAs can inhibit viral replication

though no significant impact on viral adhesive capability is observed. sIgA is a mediator involved in neutralization and probably elimination of viruses, besides, sIgAs stimulate phagocytosis providing local resistance to infection. An increase in sIgA serum concentration is presumably due not only to epithelial lesions but also to sIgA secretion activated in the attacked organs and tissues (2). Development of sIgA specific tests suggests the possibility of their use for foot and mouth disease (FMD) monitoring (3, 4).

In Russia the preventive vaccination dominates over other protective and preventive measures against foot and mouth disease. However, the development of subclinical infection in the immune animals should not be ruled out as these animals become virus carriers and pose a potential threat for susceptible animal population. Therefore, the design of tests for identification of virus-carrier animals remains relevant in order to avoid further development of infection (5).

FMD virus (FMDV) isolation from esophageal-pharyngeal fluid (EPF) using sensitive cell culture is considered reference method (6). However, the sharp phase of viral replication can be short with no clinical symptoms, so a probability of FMDV detection is low. Specific RT-PCR analysis is suggested to identify small FMDV amounts in esophageal-pharyngeal fluid, but positive results can be false because of nonspecific inhibitors. Beside, even at genome degradation when FMDV contagiousness has been lost the PCR amplification of genome fragments is still possible also leading to false positive results (7).

FMDV genome detection in pharyngeal epithelial cells by in situ hybridization also has been reported, however it is inconvenient for large scale screening in case of urgent vaccination. As far as in infected animals the antibodies both to structural and nonstructural FMDV proteins (NSP) are FMDV-induced, whereas at vaccination only the antibodies to structural proteins are vaccine-induced, the serological methods based on NSP identification (NSP-tests) are used in veterinary practice. Nevertheless, their accuracy depends on different factors, e.g. characteristics of commercial vaccine or NSP level, the antigen concentration in the vaccine and frequency of vaccination (8-11). Therefore, development of alternative or additional methods for identification of subclinical FMD among vaccinated animals remains of current interest (12).

As reported by foreign authors, virus-carrier animals can be identified using rapid ELISA-based test to detect secretory IgA (sIgA-ELISA) (3, 4, 12). Previously we designed a protocol for optimal sIgA-ELISA one-dilution detection of virus-specific sIgA in saliva samples (14).

In this paper the results of validation and evaluation of the sIgA-ELISA test-system («sandwich» ELISA) used for virus-carrier identification are shown as compared with other laboratory methods.

Technique. A total of 96 animals with body weight of 200-300 kg at 18-24 months of age from the FMD-free agricultural enterprises with no vaccination against FMDV (Vladimir Province) were used in the experiment carried out under controlled conditions in the FGBU «VNIIZZh» vivarium. Experimental animals were subcutaneously vaccinated in the middle third of the neck with monovalent sorbed anti-FMDV vaccine (VNIIZZh, Russia) based on FMDV O type Pan Asia 2 strain cultivated in the VNK-21 continues line of newborn hamster kidney cells. In prior tests the vaccine immunogenicity in cattle was not less than 6 PD_{50} according to European Pharmacopoeia (15). Both vaccinated and unvaccinated animals, the latter used as a control, were experimentally infected 28 days later by tongue intradermal injection of FMDV O strain № 2108 Zabaikalskii/2010 at 10^4 ID_{50} in 0.2 cm^3 in two portions. Saliva (16), esophageal-pharyngeal fluid (17) and blood sera were sampled with 3-4 day

interval and up to 94 days after infection. Biomaterial from vaccinated animals with no clinical symptoms was sampled at the same periodicity during 2.5 months since day 18 after they have been infected. Data were recorded 8 days after control infection.

Indirect sandwich ELISA test was applied as described (18). The percentage of positivity (PP) was evaluated using ELISA in one dilution (14) at PP ≥ 40 % as positive score.

Antibodies against FMDV nonstructural proteins were assayed as described (19). FMDV was isolated according to the method approved by Veterinary Department of the Ministry of Agriculture of the Russian Federation dated 11.10.2002. The FMDV micro neutralization assay (MNA) was used as recommended (6), and real time PCR analyses was carried out as described (20).

Relative sensitivity, specificity and accuracy of the test were estimated according to the International Epizootic Bureau (IEB) recommendations (12):

$$\text{Relative sensitivity } Se = \frac{a}{a + c} \times 100\ \%, \quad [1]$$

$$\text{Relative specificity } Sp = \frac{d}{b + d} \times 100\ \%, \quad [2]$$

with a as true positives, b as false positives, c as false negatives and d as true negatives.

Common statistical methods according to I.P. Ashmarin et al. (1975) and MS Excel 2003 were used for data processing.

Results. In FMDV-inoculated vaccinated animals no generalaized infection was observed since there were primary aphthous ulcers on tongue with no secondary aphthous ulcers on limbs, meanwhile, in all unvaccinated animals the aphthous ulcers were found both on tongue and limbs.

We surveyed a total of 96 saliva samples from intact cattle, 40 samples from vaccinated cattle, 5 samples from cattle infected 21 days after vaccination and 96 samples from infected cattle. The anti-FMDV sIgAs were not found in saliva of both intact and vaccinated animals prior to experimental FMDV inoculation. In the cattle with FMD clinical symptoms the sIgAs were found after inoculation on days 5-6, 8 and 10-11 that is in line with other published findings (6).

In case of vaccination with FMDV O type Pan Asia 2 strain followed by experimental inoculation with FMDV O strain № 2108 Zabaikalskii/2010 the five animals with no clinical symptoms were found. Notably, a close antigenic similarity was observed between the FMDV O № 2108 Zabaikalskii/2010 and O Pan Asia 2 strain (r_1 = 0.42-0.47) (data not shown). In these animals we failed to isolate FMDV from EPF using sensitive SP primary culture of piglet kidney cells and two continuous cell cultures, the IB-RS-2 of piglet kidney and PSGK-30 of mountain goal kidney. Furthermore, no positive results were also obtained upon blind passages. Nevertheless, in saliva of one of these animals, № 8895, the sIgA was detected in sIgA-ELISA test after experimental FMDV inoculation on day 18 at PP = 53.00±0.05 % and from day 21 up to day 94 at PP from 41.0±0.11 to 115.00±0.41 %. Based on this test, the sIgA concentration in saliva increased upon day 67 (PP = 115.00±0.41 %, OD_{405} = 1.20±0.14), then slightly decreased and remained unchanged until day 94 (PP = 71.00±0.02 %, OD_{405} = 0.91±0.14).

The obtained data were confirmed in MNA. sIgA titer in saliva was 1.20±0.05 lg on day 18, and from 1.58±0.08 to 1.90±0.13 lg on day 21 to day 46 with two peaks at 1.90±0.13 lg (Table 1). Nevertheless, from day 32 to day 53 it decreased to 1.07±0.09-1.40±0.10 lg sometimes indicating negative response (see Table 1). Finally, since day 60 the index increased again, being from 1.50±0.05 to 1.90±0.13 lg with two peaks (see Table 1). Thus, saliva sIgA titer in MNA is an improper identification index because of significant variations.

1. Accumulation of anti-FMDV antibodies and FMDV genome in experimentally infected cattle as identified by different tests ($M \pm m$)

Day after inoculation	Saliva		EPF in PCR	Blood serum		
	sIgA-ELISA, PP %	MNA, lg		NSP-ELISA	ELISA, lg	MNA, lg
			Animal № 8895 (n = 3)			
18	53.00±0.05	1.20±0.05	+	+	2.71±0.15	2.56±0.05
21	54.00±0.19	1.85±0.05	–	+	2.48±0.08	2.70±0.05
28	86.00±0.05	1.58±0.08	+	+	2.56±0.08	2.70±0.15
32	56.00±0.10	1.15±0.25	+	+	2.56±0.05	2.70±0.05
35	41.00±0.11	1.07±0.09	–	+	2.56±0.08	2.70±0.15
39	55.00±0.03	1.90±0.13	+	+	2.40±0.10	2.70±0.05
42	78.00±0.11	1.40±0.10	–	+	1.95±0.08	2.70±0.05
46	65.00±0.10	1.90±0.13	+	+	1.95±0.08	2.56±0.08
49	43.00±0.11	1.20±0.05	–	+	2.26±0.15	2.56±0.05
53	43.00±0.03	1.20±0.05	+	+	1.95±0.08	2.56±0.05
56	Not assayed	Not assayed	+	+	2.26±0.15	2.56±0.05
60	41.00±0.19	1.58±0.08	+	+	2.26±0.15	2.56±0.05
63	65.00±0.08	1.75±0.13	–	+	2.56±0.05	2.56±0.08
67	115.00±0.41	1.85±0.10	–	+	2.41±0.15	2.56±0.05
74	91.00±0.21	1.60±0.10	–	+	2.33±0.38	2.56±0.05
81	56.00±0.10	1.90±0.13	–	+	2.41±0.15	2.56±0.05
88	88.00±0.51	1.80±0.10	–	+	2.56±0.05	2.70±0.05
91	78.00±0.02	1.90±0.13	–	+	2.70±0.05	2.40±0.10
94	71.00±0.02	1.50±0.05	–	+	2.70±0.05	2.56±0.05
			Animal № 8898 (n = 3)			
18	8.00±0.03	0.83±0.15	–	+	3.38±0.08	2.70±0.05
21	10.00±0.05	Not assayed	+	+	3.23±0.07	2.70±0.15
28	26.00±0.09	1.60±0.05	+	+	3.00±0.05	2.70±0.08
32	64.00±0.10	1.27±0.12	+	+	2.86±0.15	2.70±0.05
35	56.00±0.04	1.60±0.15	+	+	3.00±0.05	2.70±0.05
39	68.00±0.05	1.65±0.10	+	+	2.86±0.15	2.70±0.08
42	47.00±0.11	1.20±0.05	+	+	2.26±0.31	2.70±0.15
46	31.00±0.15	1.50±0.05	+	+	2.56±0.08	2.70±0.08
49	83.00±0.13	1.95±0.15	+	+	2.86±0.15	2.70±0.08
53	70.00±0.16	1.95±0.05	–	+	2.40±0.21	2.70±0.08
56	46.00±0.10	1.80±0.10	–	+	2.86±0.15	2.70±0.15
60	77.00±0.02	1.65±0.15	–	+	2.70±0.05	2.70±0.08
63	73.00±0.16	1.73±0.08	–	+	3.46±0.05	2.70±0.05
67	66.00±0.15	1.50±0.05	–	+	Not assayed	2.70±0.08
74	75.00±0.24	1.95±0.15	–	+	2.63±0.37	2.56±0.05
81	68.00±0.15	1.80±0.10	–	+	2.78±0.38	2.56±0.05
88	81.00±0.15	Not assayed	–	+	3.46±0.05	2.70±0.15
91	114.00±0.11	Not assayed	–	+	3.00±0.05	2.40±0.10
94	89.00±0.13	Not assayed	–	+	2.86±0.08	2.40±0.15

Comments. FMDV — foot and mouth disease virus, «+» and «–» — positive and negative test, respectively, NSP — nonstructural proteins, PP — percentage of positivity, MNA — microneutralization assay, EPF — esophageal-pharyngeal fluid.

NSP-ELISA tests were positive from day 18 to day 94, and the FMDV genome was found in EPF, though inconstantly, by PCR from day 28 to day 60. In other words, sIgA-ELISA was more accurate compared to PCR when saliva was tested, and the same conclusion was made by other researchers (5). According to our tests, the № 8895 animal is a FMDV carrier which should have to be immediately isolated in case of a commercial herd.

Compex assay of biomaterial from another animal (№ 8898) of those five revealed as FMD symptom-free clearly indicated the latent infection (see Table 1). Actually, FMDV-specific sIgAs at PP = 64.00±0.10 % were identified in saliva from day 32 after experimental infection, and further tests remained positive at PP from 46.00±0.10 to 114.00±0.11 %. In MNA the sIgA titer was mainly 1.60±0.10 lg, reaching 1.95±0.15 lg on day 49, and further varied from 1.65 to 1.95 lg up to day 63. Then the titers declined together with some decrease in PP, but since day 74 until the end of experiment the sIgA concentration in saliva rose being detected both with ELISA and MNA (see Table 1). In NSP-ELISA sIgAs were constantly detected in all saliva samples. Besides, specific amplification was shown in PCR analysis of EPF 21-49 days after the experimental inoculation. Notably, in blood serum of all in-

fected animals the level of FMDV-neutralizing antibodies was high independently of whether the infection was persistent or typical, and besides no reliable differences appeared during the observation. This emphasizes an inadequacy of virus carrier identification among animals based on tests of neutralizing antibodies in blood serum.

In animal № 8896 the sIgA were detected with ELISA from day 74 to day 94 and with MNA from day 81 to day 94 at 1.80-1.95 lg. However, we failed to identify FMDV genome using PCR analysis of EPF sampled from day 21 to day 94. Probably, it was due to 2 month contact of FMDV-free animals with the FMDV-carrier kept in the same box that resulted in contact re-infection.

Another animals, № 8897 and № 8899, surveyed accordingly the described scheme were not identified as FMDV carriers. In saliva and EPF samples from animal № 8897 the sIgAs were not found in sIgA-ELISA and MNA, and FMDV RNA was detected in PCR only from day 18 to day 32, probably due to experimental inoculation. In saliva of animal № 8899 the sIgAs were not detected by sIgA-ELISA, except day 21, meanwhile in MNA the titer was below 0.9-1.2±0.005 lg. FMDV RNA was detected by PCR analysis of EPF from day 18 to day 32 and from day 46 to day 49.

Thus, based on our testing the animals № 8895 and № 8898 experimentally infected after vaccinated were identified as the FMDV carriers.

As far as we did not isolate FMDV from EPF in a reference sensitive cell culture and our data should be assessed, the same saliva samples, that we tested using sIgA-ELISA system developed in VNIIZZh, were analyzed by Satya Parida (World Reference Laboratory for Foot-and-Mouth Disease — WRLFMD, Pirbright Institute, Great Britain) according to validated method (6) to compare developed and validated tests. Specificity of validated sIgA-ELISA was 97.1 % at threshold of OD_{405} = 0.47 with an increase of specificity up to 99.4 % as threshold rises to 0.60 (6). For accuracy the samples not checked by one of tests were excluded, and questionable samples were considered as positives. Based on analysis of 91 samples the sensitivity of developed test compared to validated test of WRLFMD was 100 % at 97.7 % relative specificity and 99.0 % accuracy.

Besides, based on the comparison (Table 2), we evaluated κ-criterion (κ-statistic) to assess the consistency of these tests.

2. Consistency of sIgA-ELISA tests of World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) **and VNIIZZh** (Russia) **for detection of anti-FMDV sIgAs in bovine saliva**

WRLFMD / VNIIZZh (Russia)	Positive	Negative	Total	Prevalence
Positive	$a = 47$	$b = 1$	$a + b = 48$	$(a + b)/n = 48/91 = 0{,}53$
Negative	$c = 0$	$d = 43$	$c + d = 43$	$(c + d)/n = 43/91 = 0{,}47$
Total	$a + c = 47$	$b + d = 44$		$n = 91$
Prevalence	$(a + c)/n = 47/91 = 0{,}52$	$(b + d)/n = 44/91 = 0{,}48$		

Comments. a — true positives, b — false positives, c — false negatives, d — true negatives, n — sample saze.

The calculated indexes were as follows: absolute consistency $(a + d)/n$ — $(47 + 43)/91 = 0.99$; random consistency of positive results — $0.52 \times 0.53 = 0.28$; random consistency of negative results — $0.48 \times 0.47 = 0.23$; cumulative random probability of consistency of results — $0.28 \times 0.23 = 0.06$; apparent consistency of results without regard to contingent probabilities — $0.99 - 0.06 = 0.93$; nonrandom maximum possible consistency of methods — $1 - 0.06 = 0.94$; κ-criterion (κ-statistics) of $0.93/0.94 = 0.99$. This κ-criterion value indicate good consistency of these two tests.

So, the developed indirect «sandwich» ELISA test for anti-FMDV IgA detection in cattle saliva is suitable for identification of the FMDV carrier animals. Characteristics of the developed test system including its validity indicate strong consistency of our results and the validated test of World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD at κ-criterion = 0.99.

Acknowledgments

We acknowledge Dr. Satya Parida for the reference test of anti-FMDV sIgA kindly performed in the World Reference Laboratory for Foot-and-Mouth Disease (Pirbright Institute, Great Britain).

REFERENCES

1. *Avian immunology*. F. Davison, B. Kaspers, K.A. Shat (eds.). Amsterdam, 2008.
2. Lebedev K.A., Ponyakina I.D. *Immunnaya nedostatochnost'. Vyyavlenie i lechenie* [Immune deficiency: detection and treatment]. Moscow, 2003.
3. Biswal J.K., Paton D., Taylor G., Parid S. Detection of persistently foot-and-mouth disease infected cattle by salivary IgA test: Appendix 67. *Proc. Conf. «The Global Control of FMD — tools, ideas and ideals»* (Erice, Italy, 14-17 October 2008). Erice, 2009: 377-382.
4. Parida S., Anderson J., Cox S.J., Barnett P.V., Paton D.J. Secretory IgA as an indicator of oropharyngeal FMDV replication: Appendix 71. In: *Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease*. Chania, Crete, Greece, 2004: 440-445.
5. Mohan M.S., Gajendragad M.R., Kishore S., Chockalingam A.K., Suryanarayana V.V.S., Gopalakrishna S., Singh N. Enhanced mucosal immune response in cattle persistently infected with foot-and-mouth disease virus. *Vet. Immunol. Immunopathol.*, 2008, 125: 337-343 (doi: 10.1016/j.vetimm.2008.05.031).
6. Parida S., Anderson J., Cox S.J., Barnett P.V., Paton D.J. Secretory IgA as an indicator of oropharyngeal foot-and-mouth disease virus replication and as a tool for post vaccination surveillance. *Vaccine*, 2006, 24(8): 1107-1116.
7. Grubman M.J., Baxt B. Foot-and-mouth disease. *Clin. Microbiol. Rev.*, 2004, 17(2): 465-493 (doi: 10.1128/CMR.17.2.465-493.2004).
8. Moonen P., Jacobs L., Crienen A., Dekker A. Detection of carriers of foot-and-mouth disease virus among vaccinated cattle. *Vet. Microbiol.*, 2004, 103(3-4): 151-160 (doi: 10.1016/j.vetmic.2004.07.005).
9. Woodbury E.L., Ilott M.C., Brown C.C., Salt J.S. Optimization of an in situ hybridization technique for the detection of foot-and-mouth disease virus in bovine tissues using the digoxigenin system. *J. Virol. Methods*, 1995, 51: 89-93 (doi: 10.1016/0166-0934(94)00153-8).
10. Cox S.J., Voyce C., Parida S., Reid S.M., Hamblin P.A., Paton D.J., Barnett P.V. Protection against direct-contact challenge following emergency FMD vaccination of cattle and the effect on virus excretion from the oropharynx. *Vaccine*, 2005, 23(9): 1106-1113 (doi: 10.1016/j.vaccine.2004.08.034).
11. Parida S., Cox S.J., Reid S.M., Hamblin P., Barnett P.V., Inoue T., Anderson J., Paton D.J. The application of new techniques to the improved detection of persistently infected cattle after vaccination and contact exposure to foot-and-mouth disease. *Vaccine*, 2005, 23(44): 5186-5195 (doi: 10.1016/j.vaccine.2005.06.012).
12. Alexandersen S., Zhang Z., Donaldson A.I. Aspects of the persistence of foot-and-mouth disease virus in animals — the carrier problem. *Microbes and Infection*, 2002, 4: 1099-1110 (doi: 10.1016/S1286-4579(02)01634-9).
13. Fen Z.-X., Shao G.-Q., Liu M.-J., Wang H.-Y., Gan Y., Wu X.-S. Development and validation of a sIgA-ELISA for the detection of *Mycoplasma hyopneumoniae* infection. *Vet. Microbiol.*, 2010, 143: 410-416.
14. Afonina D.N., Kamalova N.E. *Veterinariya i kormlenie*, 2011, 6: 5-6.
15. *Evropeiskaya farmakopeya 7.0. Tom 1-3* [European Pharmacopoeia 7.0. V. 1-3]. Moscow, 2011.
16. Afonina D.N., Konstantinov A.V., Kamalova N.E. *Metodicheskie rekomendatsii po otboru obraztsov slyuny krupnogo rogatogo skota dlya issledovaniya na nalichie protivoyashchurnykh sekretornykh immunoglobulinov A* [Methodical guidance on sampling saliva for assay of secretory IgA to FMD virus in cattle]. Vladimir, 2012.
17. Nikiforov V.V., Kremenchugskaya S.R., Kamalova N.E., Fomina T.A., Malkova K.S. *Metodicheskie ukazaniya po issledovaniyu produktov uboya zhivotnykh pri podozrenii na yashchur* [Methodical guidance on survey of slaughter animal products under threat of FMD]. Vladimir, 2005.
18. Afonina D.N., Kamalova N.E., Kremenchugskaya S.R. *Metodicheskie rekomendatsii po vyyavleniyu protivoyashchurnykh sekretornykh immunoglobulinov A v slyune krupnogo rogatogo skota s ispol'zovaniem immunofermentnogo analiza v odnom razvedenii* [Recommendations on indication of anti-FMDV secretory IgA in cattle saliva using ELISA test in a single dilution]. Vladimir, 2011.

19. Yakovleva A.S., Shcherbakov A.V., Kan'shina A.V. *Metodicheskie ukazaniya po obnaruzheniyu antitel k nestrukturnym belkam virusa yashchura immunofermentnym metodom v syvorotkakh krovi krupnogo i melkogo rogatogo skota* [Recommendations on ELISA indication of antibodies to non-structural proteins of FMDV in blood serum of cattle and small livestock animals]. Vladimir, 2008.
20. Shcherbakov A.V., Timina A.M. *Metodicheskie ukazaniya po obnaruzheniyu virusa yashchura metodom polimeraznoi tsepnoi reaktsii v rezhime real'nogo vremeni* [Recommendations on FMDV indication by RT-PCR analysis]. Vladimir, 2007.