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Testing of diagnostic test-systems for detection of antibodies to foot-and-mouth disease virus structural proteins with enzyme-linked immunosorbent assay for their serotype specificity

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ABSTRACT
A total of 138 serum samples from pigs and cattle vaccinated against foot-and-mouth disease virus (FMDV) of one or two serotypes or infected with FMDV were used for testing of 24 enzyme-linked immunosorbent assay (ELISA) diagnostic tests-systems for detection of antibodies against FMDV structural proteins produced by 6 manufacturers (Federal Centre for Animal Health, Prionics, IZSLER, Innovative Diagnostics, BIONOTE and MEDIAN Diagnostics) for their serotype-specificity. All used test-systems detected apparent serotype-specific activity (homologous reaction) as well as cross-reacting virus-specific antibodies that was accounted for some reasons related to conservative epitopes in amino acid sequence of FMDV virion capsid VP1–VP3 polypeptides, accessibility of internal conservative epitopes of VP4 polypeptide for the animal's immune system during virus replication or vaccine antigen (virus) destruction in the animal's body in the process of immunity development, as well as the pilot anti-FMD vaccine composition, etc. Nevertheless, the analysis of a large data set (about 3,500 tests) showed that the homologous serotype-specific reaction in general was significantly higher and predominant, the proportion of virus-specific non-protective antibodies, including cross-reacting ones, was not significant and did not distort the results of ELISA tests of anti-FMD vaccine for its immunogenicity. Inconclusive test results require confirmation with other serological tests. Complex tests for FMDV using different diagnostic methods such as ELISA with standard and reference test-systems and/or virus neutralization test in cell culture are to be considered as the best option.

Keywords: foot-and-mouth disease, enzyme-linked immunosorbent assay (ELISA), serotype specificity

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Изучение серотипоспецифичности диагностических тест-систем для выявления антител к структурным белкам вируса ящура иммуноферментным анализом

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РЕЗЮМЕ
При изучении серотипоспецифичности 24 диагностических иммуноферментных тест-систем для определения антител к структурным белкам вируса ящура 6 производителей (ФГБУ «ВНИИЗЖ», Prionics, IZSLER, Innovative Diagnostics, BIONOTE и MEDIAN Diagnostics) было исследовано 138 образцов сыворотки крови свиней и крупного рогатого скота, вакцинированных против ящура одного или двух серотипов либо инфицированных вирусом ящура.

Все использованные тест-системы наряду с ярко выраженной серотипоспецифической активностью (гомологичная реакция) выявляли также некоторое количество перекрестно-реагирующих вирусоспецифических антител, что было обусловлено рядом причин, связанных с консервативными эпитопами в аминокислотной последовательности капсидных полипептидов VP1–VP3 вириона возбудителя ящура, с доступностью внутренних консервативных эпитопов полипептида VP4 для иммунной системы животного при репликации вируса или деструкции вакцинного антигена (вируса) в организме животного в ходе иммунного процесса, а также с компонентным составом экспериментальной противоящурной вакцины и др. Тем не менее анализ большого массива данных (около 3500 исследований) показал, что гомологичная серотипоспецифическая реакция в целом была значительно выше и являлась доминирующей, доля вирусоспецифических незащитных антител, в том числе и перекрестно-реагирующих, была не столь значительной и не искажала результаты оценки иммуногенности противоящурной вакцины в иммуноферментном анализе. В сомнительных случаях требуется подтверждение результатов в других серологических реакциях. Оптимальным вариантом следует считать комплексные исследования на ящур с привлечением разных методов диагностики, таких как иммуноферментный анализ с использованием стандартных и референтных тест-систем и/или реакция вирусной нейтрализации в культуре клеток.

Ключевые слова: ящур, иммуноферментный анализ, серотипоспецифичность

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INTRODUCTION

Foot-and-mouth disease (FMD) is one of the most highly contagious viral diseases of domestic and wild cloven-hoofed animals that is well-known since ancient times and can have drastic economic, social, and environmental impact. The disease is caused by aphthovirus of *Picornaviridae* family and is characterized by fever, mucous and epithelial aphthous lesions and other symptoms. There are 7 immunologically distinct serotypes of FMDV: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1 [1, 2].

Specific vaccination is one of important tools used together with other measures to combat the infection. Since the virus of different serotypes practically does not induce any cross-immunity and vaccines based on the virus strains that differ from the field strains of the same virus serotype may confer incomplete protection, thorough selection of the virus strains to be incorporated in the developed vaccines is required to achieve maximum possible protection of animals in the particular region [1, 2].

Two major methods for testing serum samples for antibodies to FMDV structural proteins: virus neutralization test (VNT) and enzyme-linked immunosorbent assay (ELISA), are used for laboratory diagnostics for the purpose of assessment of vaccine effectiveness, immune status and immunity level in susceptible animals [1, 2].

Virus neutralization tests is considered a gold standard for FMDV since its allows direct detection of the virus neutralizing antibodies in serum samples from vaccinated or convalescent animals, thereby determination of the level of protection against FMD caused by the virus of particular serotype.

Enzyme-linked immunosorbent assay based on the inactivated whole virus or recombinant virus antigen covers the whole range of virus-specific antibodies, primarily virus neutralizing ones, owing to the virion structure. This allows ELISA to be used for laboratory FMD diagnosis as an alternative or confirmatory method like VNT.

However, we repeatedly detected cross-reactive antibodies with test-systems for detection of different virus serotypes in serum samples from FMD vaccinated or convalescent animals. Some researchers reported the same phenomenon during performed serological tests [3, 4, 5]. The nature of this phenomenon is unclear and requires further research.

The virion is non-enveloped and is a capsid surrounding RNA-genome. The capsid forms an icosahedral structure with a sedimentation coefficient of 146S and comprises 60 copies of protomer unit consisting of four structural proteins, VP1 to VP4. The virus forms intermediates during its replication: empty 75S capsid lacking nucleic acid, 12S pentamers and 5S protomers. During degradation the capsid appears to dissociate into separate units. The surface proteins VP1–VP3 carry epitopes responsible for FMDV serotype specificity and virus-neutralizing antibody development, while the internal VP4 protein is more conservative in different virus serotypes and antibodies against VP4 epitopes do not confer protection against the infection [3, 6].

Accessibility of internal conservative epitopes for the animal's immune system in the presence of a certain number of 12S and 5S subunits along with an intact antigen (capsid antigen with unchanged structure) contained in the vaccine or during FMDV replication at the time of infection may be one of probable reasons for the

cross-reactivity of antibodies between serotypes. Presence of conservative epitopes in the amino acid sequence of capsid surface proteins (VP1–VP3) may be the other reason. The latter is supported by detection of cross-reactive virus neutralizing antibodies with VNT, which may also be indicative of some cross-serotype protection [4, 5].

In this paper, an attempt was made to explain the nature of the cross-reactions in ELISA, as well as to assess the effect of this phenomenon on the possibility of determination of serotype specificity of post-vaccination and post-infectious antibodies in serum samples from pigs and cattle. For this purpose, mono-specific panel including experimental serum samples collected from infected pigs and cattle or from pigs and cattle single-vaccinated with FMD vaccine based on one FMDV strain. The serum samples were concurrently tested with commercial ELISA test-kits for detection of antibodies against FMDV structural proteins of different manufacturers: Federal Centre for Animal Health (FGBI "ARRIAH"), Russia; Prionics, Netherlands/Switzerland; IZSLER & The Pirbright Institute, Italy/Great Britain; Innovative Diagnostics, France; BIONOTE, South Korea; MEDIAN Diagnostics, South Korea. The used FGBI "ARRIAH" test-kits were strain-specific polyclonal test-systems based on liquid-phase blocking ELISA, used foreign test-kits were serotype-specific monoclonal competitive ELISA test-systems.

MATERIALS AND METHODS

The following samples were used for the test: retained serum samples from pigs and cattle infected with serotype SAT 2 and O FMDV or vaccinated with monovalent and bivalent anti-FMD vaccines. Before testing the samples were kept at minus 20 °C. Monospecific panel contained 81 serum samples including 8 serum samples from convalescent animals and 73 serum samples from animals vaccinated against serotype A (25 samples), serotype O (31 samples), serotype Asia 1 (17 samples) as well as 57 serum samples collected from pigs immunized with pilot bivalent emulsion vaccine against serotype A and O FMD before and after challenge with serotype O FMDV. Serum samples were tested with ELISA and some serum samples were tested with VNT.

Two specimens of pilot emulsion vaccine containing FMDV O 2212/Primorsky/2014 and A 2155/Zabaikalsky/2013 strain antigens at different concentrations were tested in piglets. Challenge tests were carried out in accordance with recommendations of the World Organization for Animal Health (WOAH) [2]. Two groups of animals, 15 pigs per group, were formed for the experiment. The animals were immunized subcutaneously with the vaccine containing different antigen amounts (undiluted, 1:5 and 1:25 diluted antigen, 5 animals per antigen dilution in each group) in a volume of 2.0 cm^3 (1 dose). Another two non-vaccinated animals served as controls. Blood samples were collected from all animals on day 21 after immunization and subjected to serological testing, then the animals were challenged with aphthous FMDV O 2212/Primorsky/2014 strain at a dose of 10^4 ID_{50}/0.2 cm^3. Challenge test results were recorded and blood samples were collected on day 8 after challenge.

All experiments in animals were carried out in strict accordance with GOST 33215-2014, Interstate standard for the laboratory animal keeping and handling, adopted by the Interstate Council for Standardization, Metrology and Certification, as well as in accordance with the requirements of Directive 2010/63/EU of the European Parliament and Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes.

The test-kits based on liquid-phase blocking ELISA for detection of antibodies against structural proteins of different strains of serotype A, O, Asia 1 FMDV (Table 1) developed by the FGBI "ARRIAH" in accordance with the instructions on their use as well as the following commercial test-kits for detection of antibodies against serotype A, O, Asia 1 FMDV produced by leading European and Asian manufacturers in according to their manufacturers' instructions were used:

– PrioCHECK® FMDV Type A (Prionics, Netherlands/Switzerland);

– PrioCHECK® FMDV Type O (Prionics, Netherlands/Switzerland);

– PrioCHECK® FMDV Type Asia 1 (Prionics, Netherlands/Switzerland);

– SPCE for antibodies specific to FMDV Serotype A (IZSLER & The Pirbright Institute, Italy/Great Britain);

– SPCE for antibodies specific to FMDV Serotype O (IZSLER & The Pirbright Institute, Italy/Great Britain);

– ID Screen® FMD Type A Competition (Innovative Diagnostics, France);

– ID Screen® FMD Type O Competition (Innovative Diagnostics, France);

– ID Screen® FMD Type Asia1 Competition (Innovative Diagnostics, France);

– FMD Type O Ab ELISA (BIONOTE, South Korea);

– VDPro® FMDV Type O Ab b-ELISA (MEDIAN Diagnostics, South Korea).

Test results were interpreted based on percentage of inhibition (PI) calculated for each tested sample according to the formula and interpretation criteria indicated by the test-kit manufacturer. To easily perceive large data sets, the results obtained with the test-kits as optical density (OD) values to be expressed as S/N ratio (Innovative Diagnostics – IDVet, MEDIAN), were also recalculated to PI values.

Foot-and-mouth disease virus antigen was serotyped with indirect sandwich ELISA using hyperimmune rabbit serum and guinea pig serum prepared against intact capsid antigen of one of FMDV strains (146S-Ag) as described above with some modifications as capture antibody and detection antibody, respectively [2, 7]. For this purpose, 96-well ELISA plates (NUNC, Denmark) were coated with the solution of strain-specific rabbit serum (serotypes A, O, Asia 1 or SAT 2 FMDV) in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Then, successive steps of adding tested or control antigen samples, detection antibodies (strain-specific guinea pig serum for relevant serotype) and anti-species immunoperoxidase conjugate diluted with 0.01 M tris-buffered saline solution supplemented with 0.01% of Tween 20 detergent containing 10% of bovine fetal serum to the plate wells were carried out. All test steps were carried out at 37 °C for 1 hour and the plates were washed after each step to remove non-bound components. The reaction was visualized with ABTS (2,2'-azino-di(3-ethylbenzothiazoline)-6-sulfonic acid) substrate for 20 min at room temperature; the reaction was stopped with 1% sodium dodecyl sulfate.

The intact antigens of FMDV were also serotyped with direct ELISA when the antigen adsorbed in the plate wells

was detected with commercial peroxidase-conjugated serotype-specific monoclonal antibodies produced by Prionics, IZSLER, Innovative Diagnostics, BIONOTE, MEDIAN Diagnostics companies. The reaction conditions were similar to that ones for sandwich ELISA.

Optical density values of tested samples that were 2.5 times greater than conjugate control OD value (background control) in both assays were considered positive.

Foot-and-mouth disease VNT was carried in continuous IB-RS-2 porcine kidney cells in accordance with the WOAH recommendations [2]. The VNT was carried out using 96-flat-bottom well culture microplates. The virus, 10^2 $TCID_{50}$/0.05 cm^3 (accepted range: $10^{1.5}$–$10^{2.5}$ $TCID_{50}$/0.05 cm^3), was added to two-fold dilutions of tested and control serum samples prepared with the nutrient medium (Eagle's MEM). Then, the plates were incubated at 37 °C in 0.5% CO_2 environment for one hour and IB-RS-2 culture cell suspension at concentration of 10^6 cells/cm^3 was added to all wells, 0.05 cm^3 per well, and the plates were incubated under the same conditions for 48–78 hours. The reaction results were read based on cytopathic effect (CPE) developed in the control wells containing infected cell culture without sera from tested animals. Virus-neutralizing antibody (VNA) titre was defined as the last serum dilution showing 50% inhibition of the virus CPE [2].

VP1 amino acid sequences of FMDV of different serotypes were aligned in accordance with the data obtained from the NCBI and publicly accessible sources [6, 8, 9, 10, 11, 12, 13, 14, 15]:

– A (A/TAN/26/2013); GenBank: AXI68858.1;
– A (A_{22}/IRQ/24/64); GenBank: ARO74643.1;
– A (A/TUR/2/2014); GenBank: QWL55674.1;
– A (A/TUR/2006); GenBank: ACC63168.1;
– A (A/PAK/2013); GenBank: APZ88528.1;
– O (wild type of type O FMDV); PDB: 7ENP_1;
– O (O/TUR/33/2011); GenBank: QWL56951.1;
– O (O/PAK/2019); GenBank: UFI08025;
– Asia1 (Asia1/BAN/DH/Sa-319/2018); GenBank: QED10746.1;
– Asia1 (Asia1/284-3/4_ISB/Pak_2012); GenBank: APZ88631.1;
– Asia1 (Asia1/Shamir/89); GenBank: ASV50713.1;
– C1 (C1/Loupogne BEL/53); GenBank: AAA91488.1;
– SAT2 (SAT2/LIB/2012); GenBank: AFU55197.1;
– SAT2 (SAT2/EGY/Ismailia/2018); GenBank: QZE50286.1.

Electrophoretic separation of the proteins in polyacrylamide gel was carried out as described earlier [16, 17].

RESULTS AND DISCUSSION

Twenty-four ELISA test-kits for detection of antibodies against FMDV structural proteins produced by various manufacturers were tested for their serotype-specificity using the panel of monospecific porcine and cattle sera. The test-kits for FMD diagnosis manufactured by leading foreign companies such as Prionics, IZSLER, Innovative Diagnostics, BIONOTE and MEDIAN Diagnostics, are based on direct competitive ELISA. Serum immunoglobulins and horseradish peroxidase-conjugated monoclonal antibodies compete for specific binding to the virus or recombinant antigen. The FGBI "ARRIAH" tests-kits contain specific and nonspecific components for indirect liquid-phase blocking ELISA. This ELISA variant for detection of antibodies against FMDV structural proteins is a competitive ELISA variant and is characterized by preliminary step of FMDV antigen-tested serum interaction ("liquid phase") during which the immunodominant epitopes in the FMDV antigen amino acid sequence are blocked by specific immunoglobulins. The other steps of the assay are generally similar to other ELISA variants. Competing antibodies are homologous strain-specific polyclonal antibodies in guinea pig blood serum (detection antibodies) that bind to free antigenic determinants [2, 18, 19, 20].

Table 1
FMDV strains used in the test-kits produced by the FGBI "ARRIAH" for virus neutralization tests and/or for monospecific serum preparation

FMD virus serotype	Name of production FMDV strain	Short strain designation	Topotype	Genetic lineage
A	A 2155/Zabaikalsky/2013	A/Zab/13	ASIA	Sea-97
	A 2029/Turkey/2006	A/TUR/06	ASIA	Iran-05
	A 2269/ARRIAH/2015	A/ARRIAH/15	ASIA	G-VII
	A_{22}/Iraq/24/64	A_{22}/IRQ/64	ASIA	Iraq-64
	A/Tanzania/2013	A/TAN/13	AFRICA	G-1
O	O 2047/Saudi Arabia/2008	O/SAU/08	ME-SA	PanAsia2
	O 2356/Pakistan/2018	O/PAK/18	ME-SA	PanAsia2
	O 2212/Primorsky/2014	O/Prim/14	SEA	Mya-98
	O_1/Manisa/Turkey/1993	O_1/Manisa/93	SEA	Mya-98
	O_1/Campos/1994	O_1/Campos/94	EURO-SA	–
	O 2344/Mongolia/2017	O/MOG/17	ME-SA	Ind 2001
	O 2311/Zabaikalsky/2016	O/Zab/16	ME-SA	Ind 2001
	O 2620/Orenburg/2021	O/Orenburg/21	ME-SA	Ind 2001
	O/Kenya/2017	O/KEN/17	EA-2	–
Asia 1	Asia-1 1946/Shamir 3/89	Asia1/Shamir/89	ASIA	Shamir
	Asia-1 2145/Tajikistan/2011	Asia1/TAJ/11	ASIA	Singh-08
	Asia-1 2356/14/Pakistan/2018	Asia1/PAK/18	ASIA	Singh-08
SAT 2	SAT2/LIB/39/2012	SAT2/LIB/12	VII	Lib-12
	SAT2/ERI/98	SAT2/ERI/98	VII	–

Figure 1 shows that none of above test-kits used for testing sera collected from animals on day 21–28 after their vaccination with anti-FMD monovalent vaccines based on the virus strains indicated in Table 1 demonstrated 100% serotype-specificity. The reaction to homologous sera was predominant, however, the number of cross-reacting samples turned out to be significant.

The cross-reactivity was also measured during the tests of 6 serum samples from SAT 2 FMD convalescent pigs and cattle (Table 2).

Analysis of the obtained results showed that the majority of used test-systems detected cross-reactive antibodies in sera collected from animals on day 21–31 after their infection with FMDV SAT2/ERI/98 strain. Therewith, the detected cross-reactivity was significantly lower than that one detected by SAT2/LIB/12-ARRIAH test-system: all 6 samples were tested positive with PI_{mean} = 97.6%. Four monoclonal test-systems: A-PrioCHECK, O-PrioCHECK,

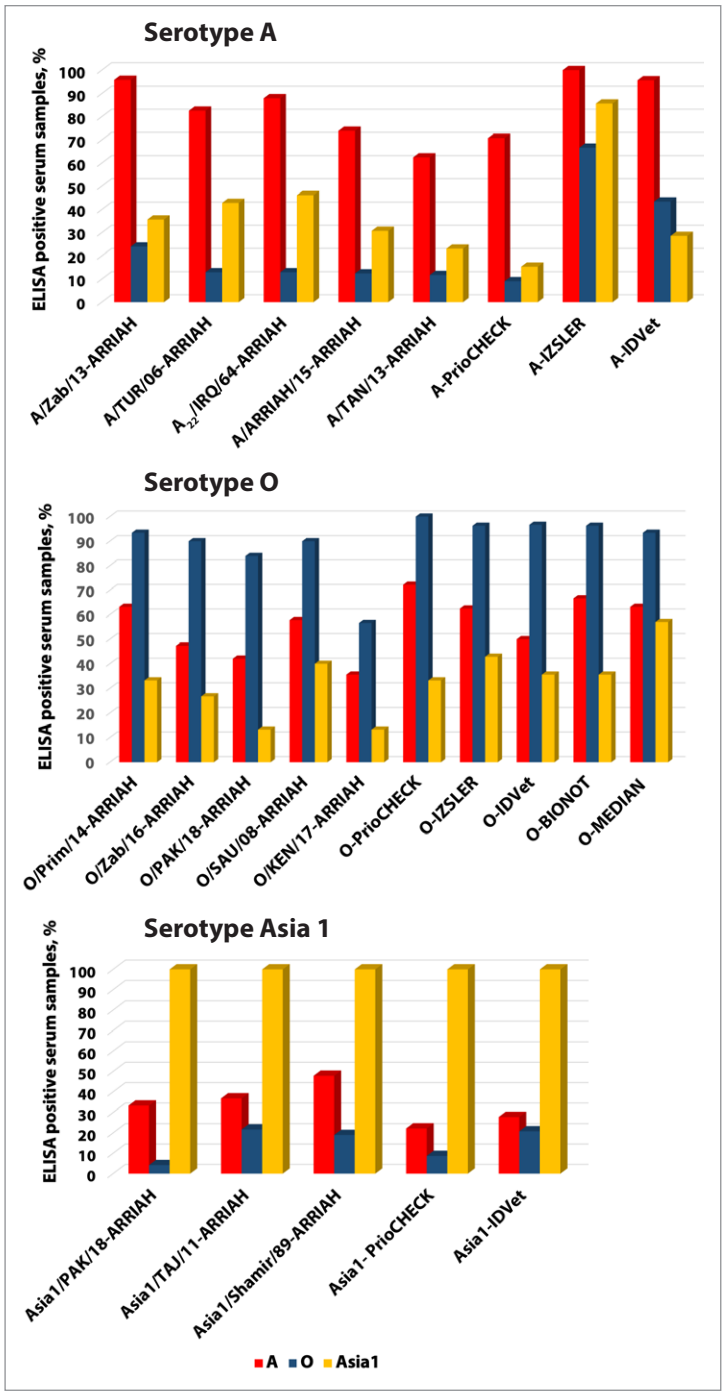

Fig. 1. Results of ELISA tests of serum samples from animals immunized with monovalent anti-FMD vaccine (n = 73)

Table 2
Results of ELISA tests of serum samples from animals infected with SAT 2 FMDV

Diagnostic test-system	Serum samples from cattle and pigs, 21–31 dpi SAT2/ERI/98 (*n* = 6)	
	PI_{mean}, %	pos/n
A/Zab/13-ARRIAH	***64.9***	*5/6*
A/TUR/06-ARRIAH	***66.7***	*5/6*
A_{22}/IRQ/64-ARRIAH	***62.8***	*4/6*
A/ARRIAH/15-ARRIAH	***54.5***	*5/6*
A/TAN/13-ARRIAH	***62.6***	*3/6*
A-PrioCHECK	*36.3*	*0/6*
A-IZSLER	***74.8***	*4/6*
A-IDVet	*45.0*	*3/6*
O/Prim/14-ARRIAH	***60.2***	*5/6*
O/Zab/16-ARRIAH	***51.8***	*4/6*
O/PAK/18-ARRIAH	***52.8***	*4/6*
O/SAU/08-ARRIAH	***64.8***	*5/6*
O/KEN/17-ARRIAH	***61.9***	*3/6*
O-PrioCHECK	*34.8*	*0/6*
O-IZSLER	*45.6*	*0/6*
O-IDVet	*35.8*	*3/6*
O-BIONOT	*15.5*	*1/6*
O-MEDIAN	*37.0*	*3/6*
Asia1/PAK/18-ARRIAH	*37.4*	*2/6*
Asia1/TAJ/11-ARRIAH	***57.8***	*4/6*
Asia1/Shamir/89-ARRIAH	***71.9***	*5/6*
Asia1-PrioCHECK	***52.8***	*4/6*
Asia1-IDVet	*31.4*	*0/6*
SAT2/LIB/12-ARRIAH	**97.6**	6/6

dpi – days post infection; pos/n – number of ELISA positive serum samples to total number of tested serum samples; results of tests with heterologous ELISA test-systems are given in italics, positive PI values are given in bold.

O-IZSLER and Asia1-IDVet did not detect anti-FMDV antibodies in convalescent animals. However, two serum samples from control pigs infected with aphthous serotype O FMD virus-containing suspension during vaccine tests were tested positive with heterologous A-PrioCHECK test-system (Table 3).

The cross reactions were observed in samples from pigs after their challenge with FMDV O 2212/Primorsky/2014 strain during tests of bivalent emulsion anti-FMD vaccine based on A 2155/Zabaikalsky/2013 and O 2212/Primorsky/2014 strains (Table 3). Six heterologous test-systems for serotypes Asia 1 и SAT 2 detected low cross reactivity to serotypes O and A or did not detect any cross reactivity to serotypes O and A, however, only two-test kits (Asia1-PrioCHECK and Asia1-IDVet) did not detect any anti-FMDV antibodies in sera from both vaccinated and control animals after challenge. The following interesting dependence was observed when sera were tested with test-systems for serotype A FMDV: the number of seropositive animals increased and the antibody level increased significantly 8 days after infection with FMD serotype O compared to that ones before infection, however, mean PI value in the test group was lower than that one in control (unvaccinated) animals. This could be the evidence of partial neutralization of serotype O FMDV with antibodies against serotype A FMDV, i.e. the evidence

of cross-protection. This was confirmed by results of VNT of FMDV A 2155/Zabaikalsky/2013 strain. Tables 3 and 4 show that number of animals demonstrating positive VNT reaction to serotype A FMD virus before and after infection remained unchanged but it was not possible to determine the proportion of cross-reactive VNAs since the animals were vaccinated against serotype A FMDV. But virus-specific antibodies of this category were detected in sera from control animals and protective VNA titre was 1:45 or 1.65 lg, similar to VNA titre detected when FMDV Asia-1 2356/14/Pakistan/2018 strain was used.

One animal in each of two groups of pigs vaccinated with the vaccine diluted 1:25 demonstrated systemic disease after challenge with aphthous virus. Therewith, no ELISA antibodies to serotype A FMDV were detected and threshold antibody levels to serotype O FMDV were detected using tested ELISA test-kits for serotype O, except for the FGBI "ARRIAH" test-kits for O 2356/Pakistan/2018, O 2311/Zabaikalsky/2016, O 2047/Saudi Arabia/2008, O/Kenya/2017 strains, in these two pigs before challenge. Virus neutralizing antibody titres against FMDV A 2155/Zabaikalsky/2013 and O 2212/Primorsky/2014 strains in

Table 3
ELISA and VNT results for serum samples from pigs vaccinated with bivalent vaccines against type A and O FMDV before and after challenge test using type O FMDV

Diagnostic test-system	Serum samples collected from pigs on					
	21 dpv (vaccination against serotype A and O FMDV)		29 dpv (vaccination against serotype A and O FMDV), 8 dpi (infection with serotype O FMDV)		8 dpi (infection with serotype O FMDV) non-vaccinated controls	
	PI_{mean}, %	pos/n	PI_{mean}, %	pos/n	PI_{mean}, %	pos/n
A/Zab/13-VNT	n/d	12/29	n/d	12/28	n/d	2/2
A/Zab/13-ARRIAH	39.6	13/28	**59.2**	18/28	***76.0***	*2/2*
A/TUR/06-ARRIAH	36.9	10/28	**57.9**	18/28	***75.1***	*2/2*
A_{22}/IRQ/64-ARRIAH	48.0	15/28	**67.6**	22/28	***80.7***	*2/2*
A/ARRIAH/15-ARRIAH	28.7	4/28	**54.1**	17/28	***73.2***	*2/2*
A/TAN/13-ARRIAH	38.2	9/28	**58.7**	17/28	***65.5***	*2/2*
A-PrioCHECK	36.0	3/28	41.8	9/28	***52.4***	*1/2*
A-IZSLER	**77.3**	23/29	**76.1**	21/28	***84.5***	*2/2*
A-IDVet	**51.54**	15/29	**58.52**	19/28	***78.6***	*2/2*
O/Prim/14-VNT	n/d	10/29	n/d	28/28	n/d	2/2
O/Prim/14-ARRIAH	**74.3**	26/28	**87.8**	28/28	**91.3**	2/2
O/Zab/16-ARRIAH	**53.1**	18/28	**76.8**	24/28	**90.0**	2/2
O/PAK/18-ARRIAH	**50.9**	16/28	**75.6**	23/28	**94.2**	2/2
O/SAU/08-ARRIAH	**51.9**	16/28	**73.4**	23/28	**89.8**	2/2
O/KEN/17-ARRIAH	45.3	12/28	**73.1**	21/28	**93.9**	2/2
O-PrioCHECK	**73.5**	25/28	**82.0**	27/28	**89.9**	2/2
O-IZSLER	58.6	5/29	**71.2**	16/28	**71.4**	2/2
O-IDVet	**63.4**	13/29	**77.9**	21/28	**77.7**	2/2
O-BIONOT	49.4	18/29	**73.1**	25/28	**80.1**	2/2
O-MEDIAN	**52.0**	23/29	**68.0**	27/28	**60.0**	2/2
Asia1/PAK/18-ARRIAH	*23.0*	*0/29*	*39.9*	*6/28*	***78.5***	*2/2*
Asia1/TAJ/11-ARRIAH	*28.7*	*3/29*	*50.3*	*13/28*	***80.3***	*2/2*
Asia1/Shamir/89-ARRIAH	*29.4*	*1/29*	*47.9*	*13/28*	***76.2***	*2/2*
Asia1-PrioCHECK	*21.5*	*0/29*	*24.8*	*0/28*	*35.9*	*0/2*
Asia1-IDVet	*6.6*	*0/29*	*5.8*	*0/28*	*2.0*	*0/2*
SAT2/LIB/12-ARRIAH	*6.8*	*0/29*	*19.0*	*4/28*	***61.6***	*2/2*

dpv – days post vaccination; dpi – days post infection; pos/n – number of ELISA positive serum samples to total number of tested serum samples; n/d – not detected; results of tests with heterologous ELISA test-systems are given in italics, positive PI values are given in bold.

the serum samples were 1.08 and 0.78 lg, respectively, that was lower than protective level (data are not provided). At the same time, serum samples from challenged control pigs were tested positive by 22 out of 24 ELISA test-kits and 7 out of 14 VNT both serotype O-homologous and serotype O-heterologous ones (Tables 3 and 4).

Our studies have detected and repeatedly confirmed the presence of cross-reactive antibodies in sera tested with ELISA and, to a lesser extent, with VNT. In our opinion, this could be accounted for as follows:

– presence of conservative epitopes in VP1–VP3 capsid polypeptide amino acid sequences;

– accessibility of internal conservative epitopes of VP4 polypeptide for the animal's immune system due to presence of 12S and 5S subunits in the vaccine, during FMDV replication or vaccine antigen (virus) degradation in the animal body during immunity development.

The diagnostic kits used in our study had their own distinctive features. First, they differed in competitive antibody origin. Monoclonal antibodies are used as competitive ones in foreign test-systems (Prionics, IZSLER, Innovative Diagnostics, BIONOTE and MEDIAN Diagnostics), the FGBI "ARRIAH" produces the test-kits based on polyclonal antibodies. Serotype-specificity is the main goal of test system developers for any test variant, the serotype specificity is determined by surface capsid epitopes. For this purpose, laboratory animals being antibody donors are immunized with FMDV intact antigen that mainly consists of 146S subunits. In the FGBI "ARRIAH" highly purified FMDV antigen subjected to sucrose density gradient fractionation in the form of the intact capsids not divided into smaller subunits is used for preparation of hyperimmune polyclonal rabbit and guinea pig serum for generating capture and detection antibodies, respectively. Thus, the probability of generating antibodies to internal highly conserved epitopes is low. However, according to the publications of some researchers, surface polypeptides also have conservative antigenic determinants inducing cross-reactive VNAs [4, 5].

Table 4
Results of testing of serum samples from control animals ($n = 2$) infected with FMDV 02122/Primorsky/2014 strain with VNT (8 dpi)

FMDV strain	Virus dose, $TCID_{50}/0.05\ cm^3$	T_{mean} VNA, lg
A 2029/Turkey/2006	$10^{1.58}$	*1.08*
A 2155/Zabaikalsky/2013	$10^{1.46}$	***1.65***
A 2269/ARRIAH/2015	$10^{1.76}$	*0.98*
A_{22}/Iraq/24/64	$10^{1.69}$	*1.34*
A/Tanzania/2013	$10^{1.5}$	*1.34*
O 2356/Pakistan/2018	$10^{1.69}$	**2.56**
O 2311/Zabaikalsky/2016	$10^{1.58}$	**2.03**
O 2212/Primorsky/2014	$10^{1.76}$	**2.26**
O 2047/Saudi Arabia/2008	$10^{2.06}$	**2.10**
O/Kenya/2017	$10^{1.8}$	**2.40**
Asia-1 1946/Shamir/Israel/3/89	$10^{1.5}$	*1.20*
Asia-1 2145/Tajikistan/2011	$10^{1.69}$	*1.08*
Asia-1 2356/14/Pakistan/2018	$10^{1.69}$	***1.65***
SAT2/LIB/39/2012	$10^{1.76}$	*1.08*

T_{mean} VNA – mean titre of virus neutralizing antibodies; results of tests with heterologous VNT are given in italics, protective level of virus neutralizing antibodies is given in bold.

He Y. et al. [4] detected and identified serotype O/A cross-reactive neutralizing antibodies (R50) in cattle with single B cell antibody isolation technique as well as determined virus-NAb complex structures with cryo-electron microscopy. R50 were shown to engage FMDV-O capsids as well as FMDV-A capsids and to bind to BC/EF/GH-loop of VP1 polypeptide and to GH-loop of VP3 polypeptide, revealing a previously unknown antigenic site. The cross-serotype neutralizing epitope recognized by R50 is highly conserved among serotypes O/A.

To illustrate this, 14 amino acid sequences up to 214 a. a. in length, of VP1, one of surface proteins, of FMDV of different serotypes were analyzed in order to determine the probability of conservative epitope presence [9, 10, 11, 12, 13, 14, 15, 16, 17]. The sequences were aligned with FMDV A/TAN/26/2013 strain (GenBank: AXI68858.1). Figure 2 shows that this probability exists, since conservative sites with a minimum number of substitutions are found.

Putative epitopes of surface polypeptides were assessed for their immunogenicity during serotyping the intact antigen of different FMDV strains with ELISA variants: sandwich ELISA with capture and detection polyclonal antibodies, direct ELISA with horseradish peroxidase-conjugated monoclonal antibodies (Tables 5 and 6).

Analysis of obtained data shows that FMDV intact antigen at concentration of 0.025 mg/mL was distinctly serotyped by most of the ELISAs using both polyclonal and monoclonal antibodies. Two direct ELISAs with conjugates detected cross-reaction: A-IZSLER (serotype SAT2/LIB/12 antigen) and A-IDVet (serotype Asia 1 antigen), these findings together with cross-reaction detected with VNT confirmed, in our opinion, the presence of conservative epitopes in surface polypeptides. These epitopes appeared to be less immunogenic as compared to loop-regions (Figure 2) due to their location on capsid surface. Protruding loop regions of amino acid sequence induce primary immune response. The cross-reactive antibody response detected with both ELISA as well as with VNT in convalescent animals was lower than homologous (serotype-specific response) response (Tables 2, 3 and 4) the similar results were obtained when sera from vaccinated animals were tested with the ELISAs (Figure 1).

The performed tests allow us to conclude that parallel tests with the test-systems designed for different FMDV serotypes are required for determination of serotype-specificity of anti-FMDV antibodies in sera from infected animals. The serotype should be determined based on predominant results. Cross-reactive antibody level is not important for post-vaccination immunity level, since this category of antibodies, regardless of the cause of their appearance, is virus-specific and indicates the animal's immune system state. The level of the animal protection from infection is directly proportional to the total number of virus-specific antibodies that are predominantly VNAs due to the structural features of the virion.

Table 7 shows responses of two cattle (animal No. 1 and animal No. 2) to administration of the pilot

A/TAN/26/2013	1	ttatgesadp	vtttvenygg	etqvqrrhht	svefimdrfv	klgvsspthv	idlmqthqhg
A_{22}/IRQ/24/64	1	--t-------	----------	----------	d-t-t-----	-i-nln----	----------
A/TUR/2/2014	1	--ta------	----------	---a------	d-g-------	-inpv-----	---------a
A/TUR/2006	1	--ta------	----------	---a------	d-g------a	-ispv-----	---------a
A/PAK/2013	1	--ta------	----------	---a------	d-g-v-----	-inpv-----	---------a
O, wild type of FMDV	1	-s--------	--a-------	----------	d-s--l----	-vtpkdsin-	l-----ps-t
O/TUR/33/2011	1	--s-------	--a-------	v---------	d-s--l----	-vtpkdqin-	l-----pa-t
O/PAK/2019	1	--s-------	----------	----------	d-s--l----	-vtpkdqin-	l-----pa-t
Asia1/BAN/DH/2018	1	----------	----------	---ta--l--	d-a-vl----	--nepks-q-	l----ipa-t
Asia1/ISB/Pak_2012	1	--tv------	----------	---aa--l--	d-g-vl----	--tnpka-qt	l----ipp-t
Asia1/Shamir/89	1	--t-------	----------	---ta--l--	d-a--l----	--tapkniqt	l----ips-t
C1/Loupogne BEL/53	1	----------	----------	----------	d-a-vl----	-vt--gnq-t	l-v--a-kdn
SAT2/LIB/39/2012	1	--sa--g--v	---dpsth--	nvqeg--k--	e-a-ll--st	hvhtgktsf-	v---n-kka
SAT2/EGY/Ismailia/2018	1	--sa--g--v	---dpsth--	nvqeg--k--	e-a-ll--st	hvhtgktsf-	v---n-keka
A/TAN/26/2013	61	lvgallraat	yyfsdlevvv	rhegnltwvp	ngapeaalan	tsnptayhke	pftrlalpyt
A_{22}/IRQ/24/64	61	----------	-------i--	--d-------	--------s-	-g-----l-a	----------
A/TUR/2/2014	61	----------	-------i--	----------	-----g--v-	----------	----------
A/TUR/2006	61	----------	-------i--	--d-------	----ve----	---------q	----------
A/PAK/2013	61	----------	-------i--	--d-------	----vg----	---------q	----------
O, wild type of FMDV	61	-------t--	---a-----	k-k-d-----	----v---d-	-t-------a	-l--------
O/TUR/33/2011	61	-------t--	---a-----	k---------	--------d-	-t-------a	-l--------
O/PAK/2019	61	-------t--	---a-----	k---------	--------d-	-t-------a	-l--------
Asia1/BAN/DH/2018	61	-------s--	--------al	v-t-pa----	--s-kt--d-	qt-----q-q	-i--------
Asia1/ISB/Pak_2012	61	-------s--	--------al	v-t-pv----	----kt--dc	qt-----q-q	-i--------
Asia1/Shamir/89	61	-------s--	--------al	v-a-pv----	----kd--n-	qt-----q-k	-i--------
C1/Loupogne BEL/53	61	i---------	-------ia-	t-t-k-----	----vs--d-	-t-------g	-l--------
SAT2/LIB/39/2012	61	----i---s-	---c---iac	vgdhtrvfwq	pngaprttqp	gd--mvfa-g	gv--f-i-f-
SAT2/EGY/Ismailia/2018	61	----i---s-	---c---iac	vg-htrvfwq	pngapr-tql	gd--mvfa-g	gv--f-i-f-
A/TAN/26/2013	121	aphrvlatvy	ngtskysaat	sgrrgdlgsl	aarvaaqlpa	sfnygalrat	tihellvrmk
A_{22}/IRQ/24/64	121	----------	--------gg	t-------p-	-----------	---f--iq--	----------
A/TUR/2/2014	121	----------	--v----ttg	g---------	---------s	---f--i---	n---------
A/TUR/2006	121	----------	--v----ttg	n-------p-	---------s	---f--i---	----------
A/PAK/2013	121	----------	--v----tts	g---------	---------g	---f--ik--	----------
O, wild type of FMDV	121	----------	---c--aegs	lpnvrgdlqv	l-qk--rplp	tsfnygaika	-rvte-lyrm
O/TUR/33/2011	121	----------	--nc--gesh	tanvrgdlqv	l-qk--rtlp	tsfnygaika	-rvse-lyrm
O/PAK/2019	121	----------	--nc--geg-	vtnvrgdlqv	l-qk--rtlp	tsfnygaika	-rvte-lyrm
Asia1/BAN/DH/2018	121	----------	--ktt-get-	-r-gdlaala	qrvsrqlpts	fnygavkaen	itel-irmkr
Asia1/ISB/Pak/2012	121	----------	--kta-gqe-	pr-gdlaaia	qrvstslpts	fnygavkadn	itel-irmkr
Asia1/Shamir/89	121	----------	--kta-get-	sr-gdmaala	qrls-rlpts	fnygavkad-	itel-irmkr
C1/Loupogne BEL/53	121	--------a-	t--tt-t-s-	r-dlah-aat	h--hlptsfn	fgavk-eti-	ellvrmk-ae
SAT2/LIB/39/2012	121	----l-s---	--ecd-nktv	tai---raa-	--ky-dntht	lpstfnfgfv	-vdkpvdvyy
SAT2/EGY/Ismailia/2018	121	----l-s---	--ecv-kkpv	asi---raa-	--ky-dstht	lpptfnfgfv	-vdkpvdvyy
A/TAN/26/2013	181	raelycprpl	latevsager	ykqkiiapak	qll		
A_{22}/IRQ/24/64	181	----------	------sqd-	h---------	---		
A/TUR/2/2014	181	----------	------sqd-	h---------	---		
A/TUR/2006	181	----------	-----lsqd-	h---------	---		
A/PAK/2013	181	----------	------sqd-	h--q------	-		
O, wild type of FMDV	181	kraetycprp	-lavhpsaa-	h----v----			
O/TUR/33/2011	181	kraetycprp	-laihpnea-	h----v----	--		
O/PAK/2019	181	kraetycprp	-laihpe-a-	h----v----			
Asia1/BAN/DH/2018	181	aetycprpl-	aldttqdrrk	qeiiapekqv	l		
Asia1/ISB/Pak_2012	181	aetycprpl-	aldttqdrrk	qeiiapekq			
Asia1/Shamir/89	181	aetycprpl-	aldttqdrrk	q-iiapekqv	lnf		
C1/Loupogne BEL/53	181	lycprpil-i	qp-gdrhkqp	lvapakqll			
SAT2/LIB/39/2012	181	rmkraelycp	rpllptydha	grdrfd--ig	verq		
SAT2/EGY/Ismailia/2018	181	rmkraelycp	rpllpaydha	grdrfd--ig	verq		

Fig. 2. Amino acid sequence of surface VP1 polypeptide of FMDV of different serotypes, 214 a. a. (according to the GenBank NCBI data; GH-loop region is indicated in red)

monovalent anti-FMD vaccine based on Asia-1 1946/Shamir/Israel/3/89 strain. Clinically healthy same-aged young bulls kept under similar conditions demonstrated different patterns of immune response development. If sera from both animals tested with ELISA on day 14 after their vaccination reacted only to serotype Asia 1, then on day 23 after vaccination antibodies were detected in serum from animal No. 1 with 7 out of 8 ELISA test-kits for serotype A and with 3 out of 10 ELISA test-kits for serotype O whereas the serum from animal No. 2 remained serotype-specific. Therewith, the level of specific antibodies against serotype Asia 1 FMDV was the same in both animals. This could be accounted for by different rate of the immunity development in the bulls capable of provoking destruction of the injected vaccine antigen. According to our observations, the level of humoral immunity, as a rule, reaches a peak on the 21st day after vaccination and achieves a plateau, over time (observation period: 14–45 days after vaccination), the number of serotype-specific antibodies practically remains unchanged or the changes are insignificant, and cross-reactive antibodies show stable dynamics (data are not provided).

When the antigen with the structure disrupted for various reasons is used for immunization, a large-scale cross-reaction of virus-specific antibodies can occur which makes serotyping with ELISA difficult.

Thus, cattle sera collected on day 21 after their vaccination against FMD with the vaccine based on FMDV O 2620/Orenburg/2021 strain demonstrated high cross

Table 5
Results of serotyping of FMDV intact antigen with sandwich ELISA

146S-FMDV Ag specimens (C ≈ 0.025 mg/mL)	Sandwich ELISA (FGBI "ARRIAH") for detection of FMDV Ag of the virus serotype:							
	A		O				Asia 1	SAT 2
	A/Zab/13	A/TUR/06	O/Zab/16	O/PAK/18	O/SAU/08	O/Prim/14	Asia1/Shamir/89	SAT2/LIB/12
A/Zab/13	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
A/TUR/06	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
A/ARRIAH/15	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
A_{22}/IRQ/64	**pos.**	**pos.**	neg.	neg.	neg.	neg.	neg.	neg.
O/Zab/16	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/PAK/18	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/SAU/08	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/Prim/14	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
O/KEN/17	neg.	neg.	**pos.**	**pos.**	**pos.**	**pos.**	neg.	neg.
Asia1/Shamir/89	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**	neg.
Asia1/TAJ/11	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**	neg.
Asia1/PAK/18	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**	neg.
SAT2/LIB/12	neg.	neg.	neg.	neg.	neg.	neg.	neg.	**pos.**

FMDV Ag – antigen of FMDV.

Table 6
Results of FMDV intact antigen serotyping with direct ELISA using commercial monoclonal antibody-horse radish peroxidase conjugates

146S-FMDV Ag specimens (C ≈ 0.025 mg/mL)	Direct ELISA with commercial conjugate for detection of FMDV Ag										
	PrioCHECK		IZSLER				IDVet			BIONOTE	MEDIAN
	A	O	A	O	Asia 1	SAT 2	A	O	Asia 1	O	O
A/Zab/13	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
A/TUR/06	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
A/ARRIAH/15	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
A_{22}/IRQ/64	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.
O/Zab/16	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	**pos.**
O/PAK/18	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.
O/SAU/08	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	neg.	neg.	**pos.**	**pos.**
O/Prim/14	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.
O/KEN/17	neg.	**pos.**	neg.	**pos.**	neg.	neg.	neg.	**pos.**	neg.	**pos.**	**pos.**
Asia1/Shamir/89	neg.	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.	**pos.**	neg.	neg.
Asia1/TAJ/11	neg.	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.	**pos.**	neg.	neg.
Asia1/PAK/18	neg.	neg.	neg.	neg.	**pos.**	neg.	**pos.**	neg.	**pos.**	neg.	neg.
SAT2/LIB/12	neg.	neg.	**pos.**	neg.	neg.	**pos.**	neg.	neg.	neg.	neg.	neg.

FMDV Ag – antigen of FMDV.

reactivity when tested with 11 out of 13 ELISA test-kits for detection of antibodies to structural proteins of serotype A, Asia 1 and SAT 2 FMDV comparable to homologous reaction and low response when tested with other two test-kits (A-PrioCHECK, Asia1-PrioCHECK), Table 7. In this case, the cross-reactivity was caused by disruption of capsid antigen integrity that resulted in development of large numbers of antibodies against internal conservative epitopes of VP4 polypeptide.

Electrophoretic analysis of the antigen-containing material used for the pilot vaccine preparation showed the cleavage of VP1–VP3 structural polypeptide molecules.

The non-disrupted 146S-Ag of FMDV Asia-1 2145/Tajikistan/2011 strain (intact antigen, band 1) is given for comparison (Figure 3).

However, the VNAs at the amount of 2.70 lg (VNA titre – 1:512), determined by VNT for FMDV O 2620/Orenburg/2021 strain was sufficient to protect from the infection (Table 8). When the same serum sample was tested with VNT for cross virus neutralizing activity, serum neutralization of FMDV A 2155/Zabaikalsky/2013 strain was observed up to final serum dilution of 1:128 (2.10 lg). At the same time, VN testing of the serum against O 2356/Pakistan/2018 showed significant cross-serotype neutralization of FMDV SAT2/LIB/39/2012 strain (1.65 lg).

Thus, the altered vaccine antigen structure did not interfere with the immune response development, however, it could hamper ELISA testing of the vaccine for their protective properties.

Nevertheless, this must be avoided during large-scale vaccine production through strict control of the vaccine components. The above example was used for better understanding of possible causes of cross-reactivity in ELISA.

Our study results are consistent with the data published by the Pirbright Institute (Great Britain) [3]. Testing of 294 monovalent mainly bovine sera collected following infection, vaccination, or vaccination and infection with one of five FMDV serotypes showed that over half of the samples, representing all three immunization categories, were positive to at least one heterologous serotype and some were positive to all serotypes tested. Preliminary studies with stabilized recombinant capsid antigens of serotypes O and A that did not detect internal epitopes showed reduced cross-reactivity of serum samples, supporting the hypothesis that capsid integrity can affect the serotype-specificity of the SP-ELISAs. The residual

Table 7
Results of ELISA tests of serum samples from cattle immunized with monovalent vaccine against type O and Asia 1 FMDV

Diagnostic test-system	Sera from cattle, PI_{mean} (%)				
	21 dpv O/Orenburg/21	animal No. 1 Asia1/Shamir/89		animal No. 2 Asia1/Shamir/89	
		14 dpv	23 dpv	14 dpv	23 dpv
A/Zab/13-ARRIAH	***97.1***	*35.4*	***82.2***	*21.7*	*17.3*
A/TUR/06-ARRIAH	***93.8***	*6.5*	***63.0***	*1.1*	*22.3*
A_{22}/IRQ/64-ARRIAH	***89.2***	*36.4*	***68.8***	*3.8*	*6.56*
A/ARRIAH/15-ARRIAH	***90.1***	*4.4*	*40.5*	*23.3*	*25.9*
A/TAN/13-ARRIAH	n/t	*3.1*	***81.5***	*8.7*	*37.3*
A-PrioCHECK	***58.3***	*23.3*	***63.8***	*17.9*	*35.1*
A-IZSLER	***95.8***	***71.0***	***81.6***	*67.4*	*70.3*
A-IDVet	***97.7***	*35.6*	***81.4***	*36.6*	*34.3*
O/Prim/14-ARRIAH	**96.6**	*48.3*	***58.4***	*31.2*	*39.5*
O/Zab/16-ARRIAH	**95.9**	*16.7*	*38.3*	*6.4*	*14.7*
O/PAK/18-ARRIAH	**96.1**	*35.2*	*33.7*	*12.4*	*18.6*
O/SAU/08-ARRIAH	**96.0**	*34.5*	***68.2***	*22.8*	*25.2*
O/KEN/17-ARRIAH	n/t	*2.1*	*3.3*	*5.3*	*7.1*
O-PrioCHECK	**97.3**	*40.5*	*48.4*	*23.9*	*25.5*
O-IZSLER	**95.8**	*56.1*	*41.5*	*38.6*	*55.6*
O-IDVet	**85.9**	*4.2*	*1.0*	*–26.6*	*–43.6*
O-BIONOT	**98.9**	*32.0*	*39.7*	*21.0*	*22.9*
O-MEDIAN	**90.0**	*40.0*	***54.0***	*30.0*	*20.0*
Asia1/PAK/18-ARRIAH	***92.2***	**67.3**	**84.6**	**63.7**	**84.5**
Asia1/TAJ/11-ARRIAH	***88.7***	**62.3**	**72.1**	**61.6**	**71.5**
Asia1/Shamir/89-ARRIAH	***90.6***	**72.8**	**90.8**	**71.9**	**89.9**
Asia1-PrioCHECK	*47.1*	**74.8**	**80.4**	**74.2**	**81.7**
Asia1-IDVet	***81.9***	**85.2**	**88.9**	**78.9**	**75.9**
SAT2/LIB/12-ARRIAH	***81.5***	n/t	n/t	n/t	n/t

dpv – days post vaccination; n/t – not tested; results of tests with heterologous ELISA test-systems are given in italics, positive PI values are given in bold.

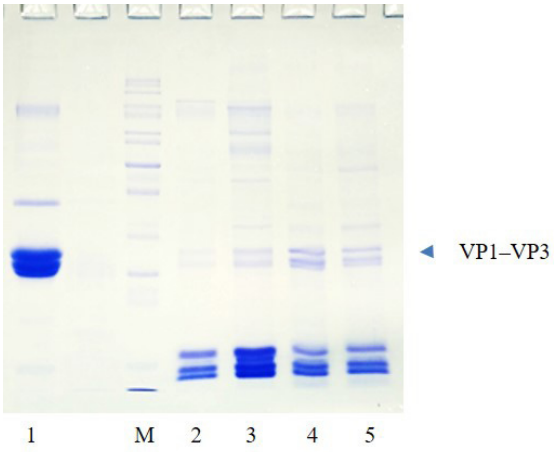

Fig. 3. Polyacrylamide gel (12%) electrophoresis of FMDV antigen specimens:
M – protein marker, 10–200 kDa;
1 – 146S-Ag Asia1/TAJ/11 (intact antigen);
2 – Ag_{UCF35} O/Orenburg/21;
3 – Ag_{PRECIP} O/Orenburg/21;
4 – Ag_{UCF35} Asia1/PAK/18;
5 – Ag_{PRECIP} Asia1/PAK/18.
Ag_{UCF35} – antigen concentrated with 35% sucrose gradient ultracentrifugation (UCF);
Ag_{PRECIP} – antigen precipitated with 8% polyethylene glycol 6,000

Table 8
Results of tests of serum samples from cattle vaccinated with anti-FMD vaccine based on O 2620/Orenburgsky/2021 or O 2356/Pakistan/2018 strain with neutralization test using homologous and heterologous FMDV serotypes

FMDV strain	Virus dose, $TCID_{50}$/0.05 cm^3	T_{mean} VNA, lg	
		O/Orenburg/21	O/PAK/18
A 2155/Zabaikalsky/2013	$10^{1.46}$	***2.10***	*1.38*
O 2356/Pakistan/2018	$10^{1.35}$	**≥ 2.86**	**3.00**
O 2620/Orenburg/2021	$10^{1.88}$	**2.70**	**1.70**
Asia-1 1946/Shamir/Israel/3/89	$10^{2.29}$	*≤ 0.78*	*1.08*
SAT2/LIB/39/2012	$10^{1.16}$	*0.90*	***1.65***

T_{mean} VNA – mean titre of virus neutralizing antibodies; results of tests with heterologous VNT are given in italics, protective level of virus-neutralizing antibodies is given in bold.

cross-reactivity associated with capsid surface epitopes was consistent with the evidence of cross-serotype virus neutralization.

CONCLUSION

Thus, all ELISA diagnostic test-kits for detection of antibodies to FMDV structural proteins produced by different manufacturers: FBGI "ARRIAH" (Russia), Prionics (Netherland/Switzerland), IZSLER & The Pirbright Institute (Italy/Great Britain), Innovative Diagnostics (France) BIONOTE (South Korea), MEDIAN Diagnostics (South Korea) were shown to detect some cross-FMDV serotype reactive antibodies. Such cross-reactive antibodies could develop due to the following: presence of conservative epitopes in FMDV surface polypeptide amino acid sequence, disruption of the capsid integrity provoking development of antibodies against internal highly conservative polypeptide. Nevertheless, the analysis of a large data set (about 3,500 publications) showed that homologous serotype-specific reaction in general was significantly higher and predominant, the proportion of virus-specific non-protective antibodies, including cross-reactive ones, was not significant and did not distort the results of ELISA tests of anti-FMD vaccine for its immunogenicity. In exceptional cases, the test results require confirmation with other serological tests. Complex tests for FMDV using different diagnostic methods such as enzyme-linked immunosorbent assay (ELISA) with standard and reference test-systems and/or virus neutralization test in cell culture are to be considered as the best option.

REFERENCES

1. Ferrari G., Paton D., Duffy S., Bartels C., Knight-Jones T. Foot and mouth disease vaccination and post-vaccination monitoring: Guidelines. Ed. by S. Metwally, S. Münstermann. Rome: FAO, OIE; 2016. 111 p. https://www.fao.org/documents/card/en/c/56c4f441-1aad-46b5-bc7a-c0ba17c1a11d

2. Foot and mouth disease (infection with foot and mouth disease virus). *In: WOAH. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals.* 2022; Chapter 3.1.8. https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.01.08_FMD.pdf

3. Ludi A. B., Morris A., Gubbins S., Asfor A., Afzal M., Browning C. F., et al. Cross-serotype reactivity of ELISAs used to detect antibodies to the structural proteins of foot-and-mouth disease virus. *Viruses.* 2022; 14 (7):1495. https://doi.org/10.3390/v14071495

4. He Y., Li K., Cao Y., Sun Z., Li P., Bao H., et al. Structures of foot-and-mouth disease virus with neutralizing antibodies derived from recovered natural host reveal a mechanism for cross-serotype neutralization. *PLoS Pathogens.* 2021; 17 (4):e1009507. https://doi.org/10.1371/journal.ppat.1009507

5. Meloen R. H., Briaire J. A study of the cross-reacting antigens on the intact foot-and-mouth disease virus and its 12S Subunits with antisera against the structural proteins. *Journal of General Virology.* 1980; 51 (1): 107–116. https://doi.org/10.1099/0022-1317-51-1-107

6. Dong H., Lu Y., Zhang Y., Mu S., Wang N., Du P., et al. A heat-induced mutation on VP1 of foot-and-mouth disease virus serotype O enhanced capsid stability and immunogenicity. *Journal of Virology.* 2021; 95 (16):e00177-21. https://doi.org/10.1128/JVI.00177-21

7. Roeder P. L., Le Blanc Smith P. M. Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: A sensitive, rapid and reliable technique for primary diagnosis. *Research in Veterinary Science.* 1987; 43 (2): 225–232. https://doi.org/10.1016/S0034-5288(18)30778-1

8. Casey-Bryars M., Reeve R., Bastola U., Knowles N. J., Auty H., Bachanek-Bankowska K., et al. Waves of endemic foot-and-mouth disease in eastern Africa suggest feasibility of proactive vaccination approaches. *Nature Ecology and Evolution.* 2018; 2 (9): 1449–1457. https://doi.org/10.1038/s41559-018-0636-x

9. Dill V., Beer M., Hoffmann B. Simple, quick and cost-efficient: A universal RT-PCR and sequencing strategy for genomic characterization of foot-and-mouth disease viruses. *Journal of Virological Methods.* 2017; 246: 58–64. https://doi.org/10.1016/j.jviromet.2017.04.007

10. Di Nardo A., Ferretti L., Wadsworth J., Mioulet V., Gelman B., Karniely S., et al. Evolutionary and ecological drivers shape the emergence and extinction of foot-and-mouth disease virus lineages. *Molecular Biology and Evolution.* 2021; 38 (10): 4346–4361. https://doi.org/10.1093/molbev/msab172

11. Ali M. R., Alam A. S. M. R. U., Amin M. A., Siddique M. A., Sultana M., Hossain M. A. Emergence of novel lineage of foot-and-mouth disease virus serotype Asia1 BD-18 (G-IX) in Bangladesh. *Transboundary and Emerging Diseases.* 2020; 67 (2): 486–493. https://doi.org/10.1111/tbed.13381

12. Dill V., Hoffmann B., Zimmer A., Beer M., Eschbaumer M. Adaption of FMDV Asia-1 to suspension culture: Cell resistance is overcome by virus capsid alterations. *Viruses.* 2017; 9 (8):231. https://doi.org/10.3390/v9080231

13. Martínez M. A., Dopazo J., Hernández J., Mateu M. G., Sobrino F., Domingo E., Knowles N. J. Evolution of the capsid protein genes of foot-and-mouth disease virus: Antigenic variation without accumulation of amino acid substitutions over six decades. *Journal of Virology.* 1992; 66 (6): 3557–3565. https://doi.org/10.1128/JVI.66.6.3557-3565.1992

14. Hassan A. M., El-Mayet F. S., El-Habbaa A. S., Shahein M. A., El Zowalaty M. E., Hagag N. M., Sharawi S. S. A. Molecular characterization of newly emerging foot-and-mouth disease virus serotype SAT 2 of Lib-12 lineage isolated from Egypt. *Virus Research.* 2022; 311:198651. https://doi.org/10.1016/j.virusres.2021.198651

15. Ahmed H. A., Salem S. A. H., Habashi A. R., Arafa A. A., Aggour M. G. A., Salem G. H., et al. Emergence of foot-and-mouth disease virus SAT 2 in Egypt during 2012. *Transboundary and Emerging Diseases.* 2012; 59 (6): 476–481. https://doi.org/10.1111/tbed.12015

16. Laemmli U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227 (5259): 680–685. https://doi.org/10.1038/227680a0

17. Lugovskaya N. N., Scherbakov A. V., Yakovleva A. S., Tsyvanyuk M. A., Mudrak N. S., Drygin V. V., Borisov A. V. Detection of antibodies to avian infectious bronchitis virus by a recombinant nucleocapsid protein-based enzyme-linked immunosorbent assay. *Journal of Virological Methods.* 2006; 135 (2): 292–296. https://doi.org/10.1016/j.jviromet.2006.03.019

18. Hamblin C., Barnett I. T. R., Hedger R. S. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus I. Development and method of ELISA. *Journal of Immunological Methods*. 1986; 93 (1): 115–121. https://doi.org/10.1016/0022-1759(86)90441-2

19. Hamblin C., Barnett I. T. R., Crowther J. R. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus: II. Application. *Journal of Immunological Methods*. 1986; 93 (1): 123–129. https://doi.org/10.1016/0022-1759(86)90442-4

20. Hamblin C., Kitching R. P., Donaldson A. I., Crowther J. R., Barnett I. T. R. Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus: III. Evaluation of antibodies after infection and vaccination. *Epidemiology and Infection.* 1987; 99 (3): 733–744. https://doi.org/10.1017/s0950268800066590

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