Вирусы и клетка

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COMPARISON OF SOLID-PHASE A-PROTEIN IMMUNE ELECTRON MICROSCOPY, DIRECT ELECTRON MICROSCOPY AND DOT-ELISA FOR DETECTION OF TRANSMISSIBLE GASTROENTERITIS VIRUS IN FECES

The authors have shown the solid-phase A-protein immune electron microscopy permits to detect the fecal coronavirus with the highest sensitivity (54.9 % of 171 fecal samples studied) comparing to direct electron microscopic examination (39.1 %). It is possible to identifity transmissible gastroenteritis virus and to differ it from other virus particles. Dot-ELISA method has shown to demonstrate viral antigen in 51.4 % of the 171 samples investigated. So the solid-phase immune electron microscopy and dot-ELISA are convenient for the veterinary diagnostics.

Introduction. Transmissible gastroenteritis (TGE) is a highly contagious enteric disease of swine characterized by vomiting, severe diarrhea, and high mortality in piglets under 5 weeks of age [1]. Although swine of all ages are susceptible to this viral infection the mortality in swine over 5 weeks is very low. TGE virus belongs to the genus *Coronavirus* of the family *Coronaviridae*.

Diagnosis of TGE virus (TGEV) is usually made on the basis of viral antigen detection, microscopic detection of virus, its isolation and identification [1]. Virus isolation from feces in cell culture is cumbersome to use for routine diagnosis, especially because wild strains of TGEV are difficult to cultivate *in vitro*. Immunofluorescent diagnostic technique is performed on cryostat sections of the small intestine, but its use is limited to dead pigs or to those that can be killed for diagnostic purpose [2, 3]. For these reasons, alternative techniques permitting to detect viral antigen in feces that do not require necropsy specimens, i. e. electron microscopy (EM) or ELISA, would be useful. The sensitivity of EM for detecting coronavirus in feces may be increased by immune electron microscopy having several modifications [4]. Conventional immune electron microscopy (IEM) is based on the observation in the electron microscope of virions clumps specifically formed with homologous antibodies [5]. However, IEM of TGEV does not always produce a clear immunologic reaction that can be readily recognized by EM. Some problems associated with IEM of coronavirus are the following: 1) the virions in a field may not react with the antibodies; 2) virions may not be agglutinated by antibodies and 3) antibodies may coat the virion unevenly. Derrick [6] described a new immune electron technique in which grids were coated with antibodies and used for specific trapping of plant viruses. Shukla and Gough [7] introduced modification in which grids were precoated with protein *A* before coating with specific antiserum to increase the efficiency of trapping by the absorbed antibodies, the immunglobulin molecules being attached to the grid by their *Fc*-fragments. This technique called solid-phase immune electron microscopy (SPIEM) has been applied successfully in the detection of rotavirus [8, 9] as well as for hepatitis A virus [10] in human stools.

Previous attemps to develop ELISA for TGEV have been unsuccessful. This failure has been attributed to 1) low antigen absorbtion on polysterene plates; 2) proteolytic activity in feces which is capable to desorbe immunoreactants; 3) hemoglobin presence in feces causing false-positive results; 4) the appearence of new coronaviruses reported in last years and making the diagnostics rather difficult [11, 12].

The aim of the present study was to develop a SPIEM and dot-ELISA for the detection of TGEV antigen in feces and to compare the results with those obtained using the direct electron microscopy and fecal samples collected at the farms of the Ukraine.

Materials and mothods. Virus strains. We used in this study the porcine rotavirus strain «K» received from Dr F. S. Bobitchevich; the enterovirus strain F7 belonging to the serotype 6 was a gift of Dr E. Rezunenko; Purdue 115 TGEV strain (P115) was taken from Dr V. I. Polulakh. All the other TGEV strains were the gift of Dr E. A. Krasnobayev (Ukrainian Veterinary Research Institute, Kiev).

P115 strain was propagated in the swine embryo renal cell line and harvested 24 h post-infection by three cycles of freezing and thawing, the virus-containing suspension was clarified by low speed centrifugation at 10 000 g for 30 min, stored in aliquots at —20 °C, and used as a standard for optimization of test conditions in SPIEM and ELISA.

Partly purified P115 virus was concentrated and purified by centrifugation through the 70 % glycerol solution (13 ml of the 70 % glycerol (v/v) had been put into the centrifuge tube of PKC-24 rotor); after centrifugation (90 000 *g*, 2 h, 4 °C) the virus pellet was resuspended in Hanks solution. This preparation with the titer about 10^6 TCD_{50}/ml was used for the immunization of rabbits.

Antisera and antibodies. Two rabbits were immunized six times with purified P115 virus suspension (5 ml) following the method of immunization often used in our institute. Intervals among immunizations were 10 days, the blood was obtained 7 days post the last antigen injection. The neutralizing antibody titers of the antisera were 1 : 256 with P115 strain.

Rabbit immunoglobulin G preparations were isolated using McKinney and Parkinson protocol [13]. Their titers were 1 : 1024 (neutralization test).

Monoclonal anticoronaviral antibodies (MAbs) 13-19 (against spike protein from D52 strain) were received from Dr O. Morenkov (Institute of Biological Physics, Academy of Sciences of the Russian Federation, Pushtchino-on the Oka, Moscow district). Their neutralizating titers were 1 : 16 000.

Antimouse antibodies and mouse PAP-complex (peroxidase-antiperoxidase) preparations were received from Bio-Test-Laboratory (Kiev) and used in a dilution 1 : 1 000 for all the ELISA tests.

Direct electron microscopy-EM. For conventional electron microscopic negative staining fecal extracts were clarified by low speed centrifugation; E-aminocaproic acid was added to virus suspensions (up to 3 %), to prevent proteolytic action; grids with a formvar support were incubated for 1 min with a drop of suspension, stained with 2 % phosphotungstic acid, pH 6.8, and examined in electron microscope EMV 100 at an accelerating voltage of 75 kV and an instrument magnification of 40 000.

Solid-phase immune electron microscopy — SPIEM. All incubations were performed at room temperature using 300 mesh copper grids; freshly prepared grids supported with a formvar film were floated for 30 min on a drop of protein *A* solution (Sankt-Peterburg Pasteur Research Institute of Epidemiology and Microbiology) 250 μg/ml in phosphate buffer (PBS), 0.01 M, containing 0.15 M NaCl, pH 7.2—7.4. The grids were then washed with three drops of PBS and drained on filter paper. Protein *A*-coated grids were incubated (30 min) on a drop of antiTGEV rabbit immunoglobulin suspension diluted 1 : 80 in

PBS, the grids were washed again with three drops of 0.1 % solution of bovine serum albumin (BSA) and incubated overnight on a drop of a viral or fecal suspension; they were finally washed by placing them on a series of six drops of PBS and drained on filter paper. The grids were stained with 2 % phosphotungstic acid, pH 6.8, and examined in the electron microscope. Mean numbers of virus particles per grid square were estimated from at least five randomly chosen squares.

Dot-ELISA. Dot immunoassay test for the TGEV detection were made according to our previous protocol [14]. The specimens (2 μl) to be studied were put on the nitrocellulose membrane filters («Millipore», 0,22 μm) and treated by acetate buffer pH 4.8 containing 3 % H_2O_2 and 3 % BSA to eliminate non-specific reactions. The filters were then incubated (30 min) in 3 % BSA solution to saturate the free links and in the solution of antiviral MAbs 13-19 (diluted 1 : 500) in PBS) overnight 4 °C. The membranes were carefully washed with PBS and incubated with antimouse antibody suspension (1 h, 37 °C), again washed with PBS, later with mouse PAP solution (1 h, 37 °C). They were finally washed with PBS and the enzyme reaction was developed using diaminobenzidine tetrahydrochloride as chromogene (1 mg/ml in PBS). Virus positive dots became brown stained.

Results and discussion. Sensitivity and specifity of different methods of virus detection. It is evident from our data (table 1) that is possible to differenciate between several virus species. SPIEM as well as dot-ELISA permit to differ porcine epidemic diarrhea virus, porcine rotavirus and porcine enterovirus from TGEV strains in the suspensions of low virus concentration. It is interesting to compare some results from the table 1; the particles of B-950 TGEV strain were not found in nondiluted suspension using EM although they were detected in diluted suspensions of same specimens with SPIEM and dot-ELISA.

It may due to low concentration of viral particles which does not permit their observation in EM [4]; SPIEM is a method capable to trap the virus particles from the virus suspension of low particle concentration (10^{-4}/ml) [4, 18]; the fecal suspension of B-950 strain may contain the proteolytic enzymes degradating virus, so the viral proteins may be trapped by dot-ELISA, but the virus particles in such samples are not seen. TOK and P115 strains were readily detected with SPIEM and dot-ELISA using diluted virus suspension where EM study had found no particles.

The vaccine TGEV strain Rims was shown to react very poorly with rabbit antiP115 antibodies and with antiD52 *S*-protein MAbs. Both P115 and D52 appear to share few common antigens with Rims strain reflect-

Table 1

The virus detection using different diagnostic methods

Virus	Mean number of particles per grid square					Antigen detection Dot-ELISA	
	UD	EM		SPIEM			
		1/10	1/100	1/10	1/100	1/10	1/100
TGEV:							
Purdue 115	17.4	0	0	ND	36.4	+	—
TOK	5.4	0	0	ND	1.2	+	—
B-950	0	0	0	5.0	0.4	+	+
Rims	ND	37.0	1.3	ND	5.3	+	—
Rotavirus	1.8	0	0	0	0	—	—
Enterovirus	30.6	ND	0	0	0	—	—
PEDV/EVD20	9.0	0.6	0	0	0	—	—

UD — Undiluted viral suspension; ND — not done; (—) — no antigen was detected; (+) —viral antigen is present.

ing host-dependent virus modification in the course of enteric virus adaptation. Perhaps it the cause of its low protective value.

Field specimens study from swine farms having ill animals with diarrhea. 171 samples were investigated io our experiments; we used EM, SPIEM and dot-ELISA. The results were evaluated according to the presence or absence of the virions on the grids with EM and SPIEM tests, and the presence of antigen was scored in immunoenzyme reaction. In 59 samples no method applied detected TGEV particles or antigen; 112 were found to be positive. Table 2 demonstrates the possibility of virus or viral antigen detection by three methods used separately and after independent verification with more than one type of assay. EM found coronavirus particles in 67 samples (39.1 %), SPIEM in 94 samples (54.9 %) and dot-ELISA in 88 samples (51.4 %) taken from diarrhea ill piglets.

Six EM-positive samples demonstrating coronalike particles were shown by SPIEM and dot-ELISA negative; it may be due to the presence of other, non-TGEV coronavirus in such preparations i. e. porcine epidemic diarrhea; futher investigations are necessary to confirm such preliminary data. Among 112 samples being positive 76 were found positive both by SPIEM and dot-ELISA tests, but 18 samples were ELISA-negative. It may be due to such factors: 1) in a lot of samples the peplomers were absent when they were examined by EM methods, but antiTGEV MAb 13-19 reacts only with spike protein carried by peplomers; 2) in dot-ELISA we used an antiTGEV MAb that detects only one epitope absent in the particles containing in dot-ELISA-negative samples. To assure more high virus recovery in dot-ELISA, it is necessary to use more than one MAb; it would be useful to have different MAbs against all the four neutralizing spike protein epitopes [15, 16], as well as those against matrix protein.

The binding of virus antigen by dot-ELISA (12 samples) in EM- and SPIEM-negative fecal samples may be caused by several factors: the samples stored during a long time before examination without enzyme inhibitors and the virions were destroyed, although the viral antigen being yet «alive», but having already no virus particles morphology.

So we are capable to evaluate some features of the natural TGEV strains including their antigenic characters. Comparing them it becomes possible to understand the interrelations between epidemic strains detected during diarrhea outbreaks in different farms. Polyclonal antibodies-based SPIEM was shown to be the most sensitive among several techniques when the virions were present in the sample. Protein A using SPIEM technique increases the rate of electron microscopy detection, but it requires special equipment and is rather time consuming. Dot-ELISA was demonstrated to be a reliable trustworthy permitting to detect virus

Table 2

Concordant and non-concordant virus and viral antigen detection by different methods *

Method used	Positive samples	
	Number	Percent (%)
EM	67(6)**	39.1(3.5)**
SPIEM	94(8)**	54.9(4.7)**
Dot-ELISA	88(12)**	51.4(7.0)**
EM+SPIEM	100	58.5
EM+dot-ELISA	104	60.8
SPIEM+dot-ELISA	106	62.0
EM+SPIEM+dot-ELISA	112***	65.5

* 171 samples were analyzed; ** the samples positve only when this technique was used; *** 112 were positive at least by one method.

antigen during express-investigation of a great number of field samples. The limiting factor of ELISA is the possibility of false-positive results due to the microflora contamination and the presence of hemoglobin in some feces samples [11, 17]. The membrane treatment with acetic buffer containing 3 % H_2O_2 and 3 % BSA decreases the percent of false-positive results.

Van Nieuwstadt et al. [18] compared SPIEM and sandwich ELISA techniques using hyperimmune serum in SPIEM and MAbs in ELISA; their experimental fecal samples were obtained from the infected gnotobiotic piglets. Our data and their results are not contradictory although these authors had no field materials.

Our data prove that the TGEV diagnostics is made better when several alternative techniques are used confirming and verifying each other.

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