Part 1. Veterinary medicine

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EXPERIMENTAL-CLINICAL ANALYSIS OF SOME ASPECTS OF THE CORONAVIRUSES EMERGENCE IN PIGGERY DURING 1987–2020

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Summary. The paper presents an analysis of own clinical and experimental data on the participation of ducks in the possible emergence of swine alpha-coronavirus — a virus of endemic diarrhea (PED-CoV), as well as of the porcine beta-coronavirus threats (hemagglutinating encephalomyelitis virus, PHE-CoV) in the COVID pandemia context. The coronavirus of duck enteritis (DE-CoV) was studied in the nineties of the twentieth century: biodiversity of its morphology includes the morphological variants identical to the morphology of PED-CoV and PHE-CoV. Moreover, hemagglutinins of all these viruses have a bilateral affinity among them on the level 24–42%. Obtained data suggest the real risk of ducks' participation in the emergence of at least alpha-coronavirus infections in pigs. There are also threats of the emergence of porcine beta-coronavirus infection under the influence of COVID-19 in industrial pig herds

Keywords: duck enteritis, porcine epidemic diarrhea, porcine hemagglutinating encephalomyelitis, electron microscopy

Introduction. The modern world practice of pig breeding in the last 40–30 years has made significant progress in regulating the epidemic process of coronavirus etiology. Experience in this area has crystallized in the collision with four coronavirus infections. It is summarized and widely highlighted in the specialized periodicals on pig breeding as are presented in Hancox (2020):

Disease	Known as	Clinical signs	Notes
Porcine epidemic diarrhea	PED	• Watery diarrhea and vomiting • Rapid spread • 100% mortality in pigs < 7 days old	If you see these clinical signs contact your vet immediately
Transmissible gastroenteritis	TGE		
Hemagg-lutinating encephalo-myelitis virus	Vomiting and wasting disease	• Often no symptoms when infected • Suckling pigs may show vomiting, anorexia, and wasting	Clinical disease is rarely seen
Porcine respiratory coronavirus	PRC	• Often no symptoms	Virus is widespread and antibodies to it protects against TGE

The COVID-2019 pandemic attracted special attention to swine coronaviruses. On the one hand, it posed a direct threat to the pig business through quarantine measures that affected pork purchase (Conklin, 2020).

On the other hand, the pandemic was alarming given the possibility of human coronavirus survival in animal's populations and contra versa (Pennisi, 2020; Komisarenko, 2020).

The Italians were the first to feel this alarm (including the threats from swine) after the COVID-2019 outbreak in northern Italy (Leopardi, Terregino and Paola, 2020).

This is quite justified because the biological host range' expansion is strictly correlated with the emerging potential of viruses (Zhao et al., 2019).

Besides, the new (for 1980) duck coronavirus of Chinese origin, as it was known to a narrow circle of former Soviet experts (Knyazev, 2011), in 1990 became the main cause of the collapse of the duck industry in some republics of the former USSR (see Discussion). Below we will try to analyze some of our own experimental and clinical data on duck and porcine coronaviruses, obtained in various scientific teams in 1989–2020 in the aspect of current threats for piggery.

Materials and methods. Coronaviruses (CoV) of duck enteritis (DE-CoV) and porcine endemic diarrhea (PED-CoV) were isolated by bioassay on 3–5-day-old ducklings, followed by their adaptation to the permanent cell culture of the Vero line. Hemagglutinating encephalomyelitis virus (PHE-CoV) was isolated by bioassay on albino mice weighing 10–12 g, followed by an adaptation of the isolated virus to the permanent cell culture of the BHK-21 line. Virus-containing 10–30%

suspensions of the intestinal mucosa (DE-CoV and PED-CoV) or brain (PHE-CoV) were prepared on sodium phosphate buffer (pH 7.2–7.4). To get rid of the host fatty compounds they were shaken for up to 5 min with chloroform (final concentration 10–15%) at room temperature (RT, 18–24°C), followed by low-speed centrifugation (300–400 xg, 30 min, RT). Degreased tissue suspensions were passed through membrane filters in syringe cartridges with a pore diameter of 0.22 μm (Sartorius PVDF, Maharashtra, and analogs) before bioassay or cell culture inoculation.

Bioassay on ducklings was performed by oral injection to them (4–5 ducklings per isolate) of the above-mentioned suspensions the DE-CoV or PED-CoV in a dose of 1 ml. Suspensions of PHE-CoV virus were injected intraperitoneally in adult albino mice (2–3 mice per isolate) or to suckling mice (nests from 5–8 mice per isolate) — intracerebral: in doses of 0.5–0.7 ml and 0.05 ml, respectively. Control animals (mock) were injected with similar tissue extracts made according to the scheme described above from the same organs of healthy pigs or ducks from farms which free from coronavirus infections. Animals were kept under biosafety conditions that preclude their mix-infection and following the euthanasia requirements according to the Good Laboratory Practice principles (WHO, 2009). Clinical observations of infected and control animals were performed until the moment of their death or maximum the disease manifestation with each bioassay duration up to 10 days.

Adaptation of DE-CoV and PED-CoV isolates to Vero cell culture were performed using the standard procedure of trypsin treatment of inoculums at a final concentration of 1.5–4.0 mg/ml at an exposure of 45–60 min on 37°C (Hofmann and Wyler, 1988; Menachery et al., 2020).

Cell cultures of Vero and BHK-21 were grown and inoculated by the contact method using full monolayers of tissue culture in 25–50 ml flask or biological tubes as described elsewhere (Cutts et al., 2019).

As a control (mock) used tissue extracts from healthy pigs or ducks, which were manufactured according to the above-mentioned scheme. The cytopathic effect was recorded by microscopy and infectious activity of isolates was counted by the Reed-Mench method (Grimes, 2002).

Cell culture isolates of CoV were identified by hemaglutination (HA) and its inhibition (HI) tests using the following sera against coronaviruses:

(a) duck enteritis, manufactured by the former All-Union Research Institute of Veterinary Virology and Microbiology, Pokrov, Vladimir Region, Russia ($S^{DE\text{-}CoV\text{-}1989}$, VNIIVViM) and by the Kharkiv State Zooveterinary Academy, Kharkiv, Ukraine ($S^{DE\text{-}CoV\text{-}2002}$, KhSZVA);

(b) porcine endemic diarrhea, manufactured by the former VNIIVViM ($S^{PED\text{-}CoV\text{-}1999}$), as well as by the National Veterinary Research Institute, Puławy, Poland ($S^{PED\text{-}CoV\text{-}2005}$, PIWet) and by the National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine', Kharkiv, Ukraine ($S^{PED\text{-}CoV\text{-}2012}$, NSC 'IECVM');

(c) transmissible gastroenteritis of swine, produced by VNIIVViM ($S^{TGE\text{-}CoV\text{-}1993}$), as well as by PIWet ($S^{TGE\text{-}CoV\text{-}2003}$);

(d) bovine enteritis, produced by the former All-Union Institute of Experimental Veterinary Medicine, Moscow, Russia ($S^{BD\text{-}CoV\text{-}1990}$, VIEV) and NSC 'IECVM' ($S^{BD\text{-}CoV\text{-}2010}$).

HA-HI tests were performed in polystyrene plates with U-like wells using 0.8–1.0% suspension of mouse erythrocytes, viral hemagglutinins of DE-CoV, PED-CoV, and PHE-CoV, mentioned virus-specific sera — all according to the instructions for diagnosis 'Kit for diagnosis of bovine coronavirus by hemagglutination method'. IHA cross tests were conducted according to the titration scheme of the above sera with 8 HAU of each of the viruses, as we described previously (Semenikhin et al., 1994).

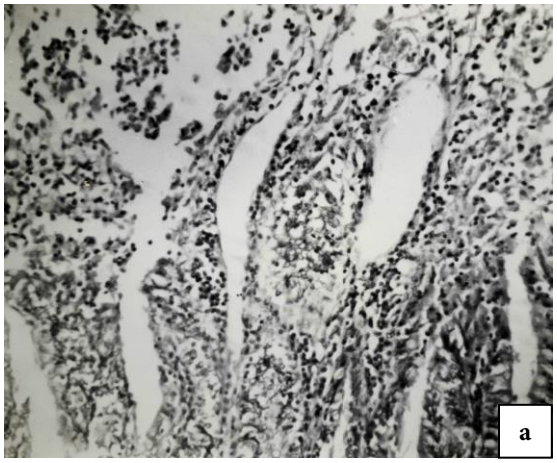
In addition, individual isolates of DE-CoV, PED-CoV, and PHE-CoV were identified by the classical negative stain method of electron microscopy (EM) using transmission electron microscope PEM-125K (OJSC SELMI, Ukraine), EM-grids with the phosphoric-tungsten acid films at the subject magnification of up to 60–80 thousand times (X).

Results. ***Trials on coronaviruses isolation.*** As we received the experience, the isolation of porcine and duck CoVs was the most successful when we began to use a bioassay with degreased tissue samples. In our trials, chloroform was the best choice for degreasing 10–30% tissue suspensions. Fig. 1 presents the illustrations of the results of the bioassay.

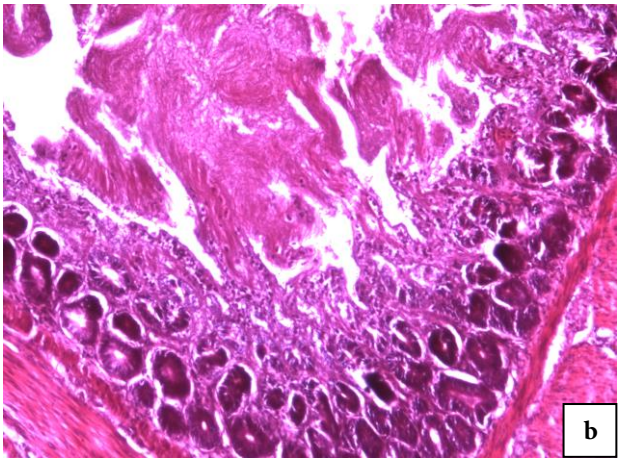
As shown in Fig. 1a–b, the manifestation of clinical signs in ducklings (drastic diarrhea, 'crawling by hocks': $n \approx 100$ for DE-CoV and $n = 18$ for PED-CoV) was escorted by the destruction of upper parts of the duodenum villi on 4–5th days for DE-CoV and PED-CoV isolates after administration *per os*. The PHE-CoV isolates which are known since 2008 ($n = 7$), evoke the porcine (Fig. 1f) and mouse (Fig. 1g) brain damage which is typical for encephalomyelitis. As a consequence of this brain damage, the diseased suckling piglets in enzootic holdings ($n = 3$) had a clinical sign of 'vomiting disease' and the disease in post-weaning piglets was manifested by 'wasting disease' (Fig. 1c) and convulsions/tremor signs in the terminal phase of the disease (Fig. 1d). The convulsions/tremor signs in the terminal phase of disease we observed also in mice (Fig. 1e) infected intraperitoneal ($n = 12$) and orally ($n = 4$).

The porcine and duck CoVs isolation in cell cultures was optimal when we began to use the specimens' trypsinization and application of trypsin (2–3 μg/ml) in maintenance cultural mediums at the first 3–5th passages of isolates.

Fig. 2 presents the illustrations of the PED-CoV (2 isolates) and PHE-CoV (1 isolate) cytopathology in Vero and BHK-21 cell lines, respectively.

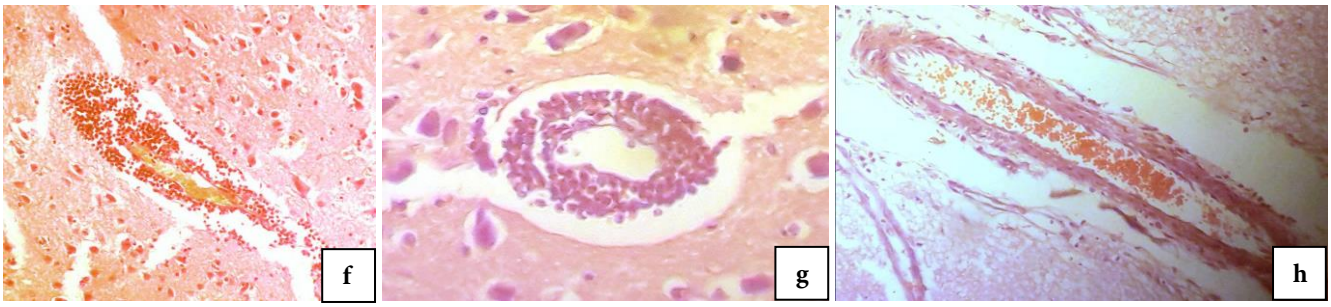


The DE-CoV isolate 'Taranivka-1991' (a): total destruction of upper parts of the ducklings' duodenum villi on the 4th day after oral administration (together with V. P. Knyazev, VNIIVViM, 1993)



The PED-CoV isolate 'Globino-2010' (b): total destruction of upper parts of the ducklings' duodenum villi on the 5th day after oral administration (together with M. M. Surkova, KhSZVA, 2015)

The PHE-CoV isolate 'Ryasne-2015': piglets under one month old with 'vomiting disease' and 'waste disease' in an age of 2.5–3.0 months (c); convulsions and tremor signs in terminal phase of disease in swine (d) and mouse (e) (together with O. V. Prokhoryatova, NSC 'IECVM', 2008)



The PHE-CoV isolate 'Lipczy-2008': the porcine (f) and mouse (g) brains with the typical vasculitis signs ('clotting' of leukocytes around blood vessels). In comparison with normal vessels (g, h) on the 7th day after intraperitoneal inoculation of mouse (together with P. O. Shutchenko, NSC 'IECVM', 2008)

Figure 1. Main results of bioassays on DE-CoV (a, b) and PHE-CoV (c–h) isolations. For more details, see the text

In Vero cells the 'Globino-2010' and 'Dnipro-2013' PED-CoV isolates reached titers 3.0–3.5 lg $TCID_{50}$/ml on the 3rd passage; the 'Sahnovsky-2013' PED-CoV isolate reached this titer only on the 7th passage. The first signs of the mentioned virus cytopathic effect were observed in 40–48 hrs p.i. (Fig. 2a–c). Full monolayer destruction by this PED-CoV dose occurred on the 5–7th day p.i.

The appearance of cytopathology in the case of PHE-CoV in BHK-21 cells monolayer occurred some faster than with PED-CoV in Vero cells.

Fig. 2d–e shows the typical cytopathic effect of the PHE-CoV isolate 'Lipczy-2008' already in 18 hrs p.i., while its infectious activity was 3.75 lg $TCID_{50}$/ml on the 2nd passage of 'mouse brain' virus.

Study of the coronavirus immunological relationships. Data on cross-reactions of CoVs hemagglutinins (8 HA-units) in HA-HI tests with the use of corresponding antiserums and mouse erythrocytes are summarized in Table 1.

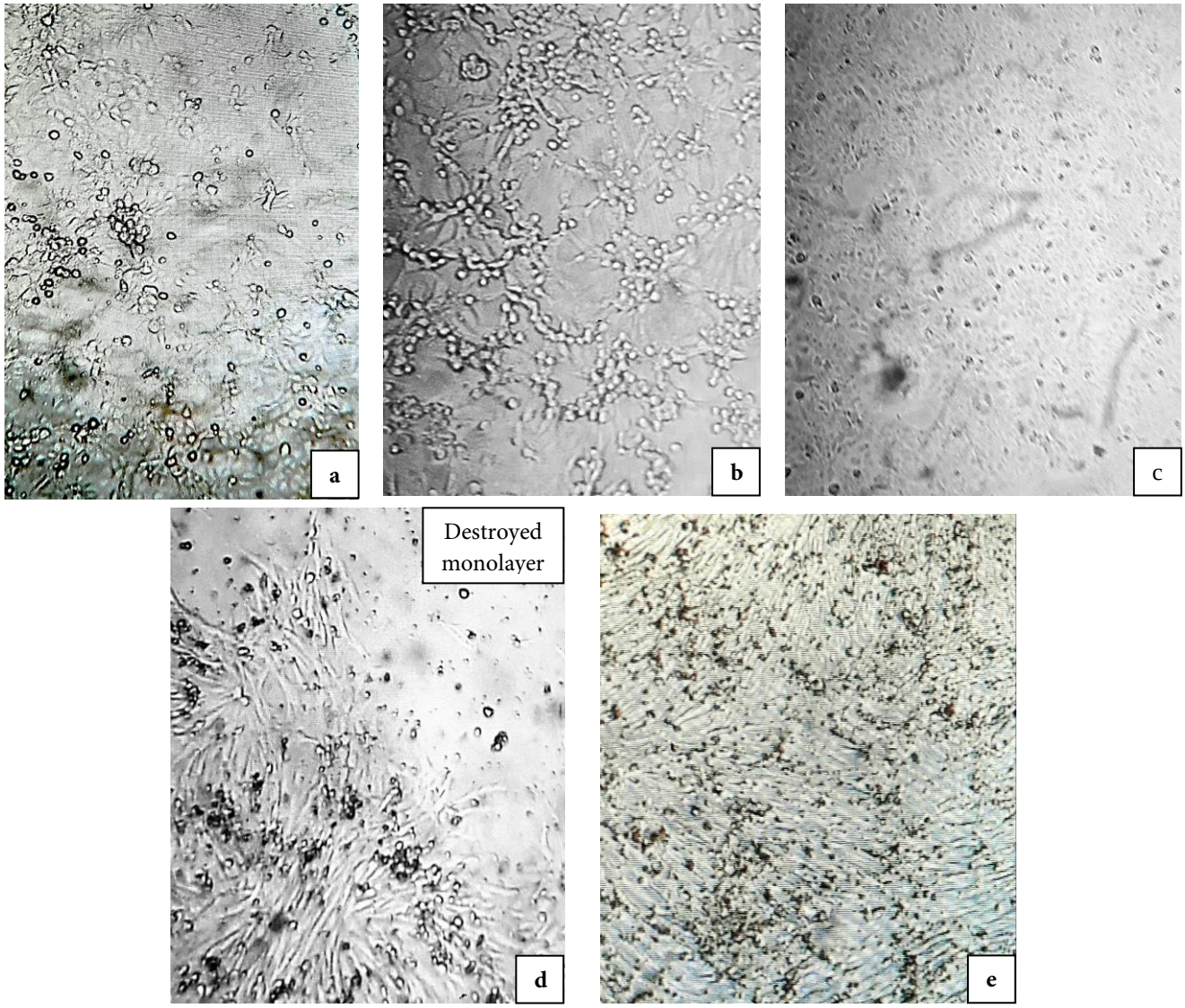


Figure 2. Cytophatology of the PED-CoV in Vero cells monolayer (a — isolate 'Globino-2010', the 3rd passage; b — isolate 'Dnipro-2013', the 2nd passage; c — mock) and PHE-CoV in BHK-21 cells monolayer (d — isolate 'Lipczy-2008', the 2nd passage; e — mock). For more details, see the text

Table 1 — Results of the HA-HI tests for study of CoVs immunological relationships

Antiserums against CoV*:	Haemagglutinins of CoV isolates (8 HA-units)					
	DE-CoV	PED-CoV			PHE-CoV	
	'Taranivka-1991'	'Globino-2010'	'Sahnovsky-2013'	'Dnipro-2013'	'Lipczy-2008'	'Ryasne-2015'
Reciprocal titers of duck antiserums against DE-CoV						
$S^{DE\text{-}CoV\text{-}1989}$	112.5 ± 29.12	28.1 ± 18.34	31.1 ± 10.23	57.3 ± 22.04	14.7 ± 1.54	n.d.
$S^{DE\text{-}CoV\text{-}2002}$	168.4 ± 27.91	42.1 ± 8.41	47.5 ± 17.77	64.4 ± 31.14	15.3 ± 0.33	n.d.
Reciprocal titers of porcine antiserums against PED-CoV						
$S^{PED\text{-}CoV\text{-}1999}$	22.3 ± 0.75	111.2 ± 9.12	100.9 ± 8.49	n.d.	n.d.	7.7 ± 3.91
$S^{PED\text{-}CoV\text{-}2005}$	29.5 ± 1.53	134.4 ± 7.73	88.7 ± 8.81	n.d.	n.d.	7.3 ± 4.86
$S^{PED\text{-}CoV\text{-}2012}$	23.2 ± 0.82	87.6 ± 9.71	79.5 ± 9.19	135.1 ± 7.55	n.d.	11.8 ± 1.29
Reciprocal titers of bovine antiserums against BovE-CoV						
$S^{BD\text{-}CoV\text{-}1990}$	97.2 ± 17.33**	n.d.	n.d.	n.d.	n.d.	n.d.
$S^{BD\text{-}CoV\text{-}2010}$	107.4 ± 31.12	76.3 ± 19.77	68.3 ± 19.88	53.8 ± 24.64	7.5 ± 2.05	n.d.
Reciprocal titer of mouse antiserum against PHE-CoV						
$S^{PHE\text{-}CoV}$	48.9 ± 2.13	12.4 ± 2.33	13.2 ± 3.24	13.4 ± 2.47	107.6 ± 2.88	122.8 ± 5.78

Notes: * — see descriptions in Materials and Methods; ** — together with N. L. Sokolova, 1994; n.d. — not done.

As we have no certified reference reagents for CoVs identification, our results should be considered preliminary. However, our data indicate a high probability of the definite immunological affinity of all the hemagglutinins learned PED-CoV and PHE-CoV isolates with hemagglutinins of DE-CoV. If we take the titers of homologous antiserum as 100%, then PED-CoV hemagglutinins are related to DE-CoV haemagglutinins (by bilateral affinity) by 24% vv.32% ($n = 8$, $P \leq 0.01$), and to PHE-CoV hemagglutinins — by 35% vv. 42% ($n = 8$, $P \leq 0.01$). At the same time, according to our results (together with N. L. Sokolova), hemagglutinins DE-CoV are related to bovine enteritis virus hemagglutinins by 73% ($n = 4$, $P \leq 0.02$). bovine enteritis virus hemagglutinins are related to PED-CoV haemagglutinins by 54% ($n = 4$, $P \leq 0.02$), but to PHE-CoV haemagglutinins — by 6% ($n = 5$, $P \leq 0.01$) only.

Results of coronaviruses microscopy. Unexpected patterns of comparative CoVs morphology were revealed by the traditional negative contrast method of electron microscopy (Fig. 3). Fig. 3a–c presents the evidence of a high level of the DE-CoV polymorphism in environmental samples ($n = 25$): up to 70% of elongated and up to 15% pleomorphic and up to 15% rounded from all registered DE-CoV virions. At the same time, we can see the relatively homogenous morphology of PED-CoV virions (Fig. 3d–e) in its cultural samples ($n = 5$) — rounded particles up to 90% and pleomorphic virions (not shown — up to 10%). In the case of PHE-CoV (Fig. 3f–g) we can see the viral particles by 30% larger in size than DE-CoV virions; among them up to 90% of them were pleomorphic and up to 10% — rounded virions.

In other words, electron microscopy data show that DE-CoV samples contain virions of specific for DE-CoV morphology (elongated and even 'Ebola-like' particles, Fig. 3c) and with morphology that resemble and PED-CoV (rounded particles), and PHE-CoV morphology (pleomorphic particles).

Discussion and conclusions. Modern studies have shown chickens and Pekin ducks were not susceptible to SARS CoV-2 infection (Schlottau et al., 2020; Shi et al., 2020). In summer 2020 American scientific team had challenged the chickens, turkeys, ducks, quail, and geese with SARS-CoV-2 or MERS13 CoV. No disease was observed, no virus replication was detected, and antibodies were not detected in serum. Neither virus replicated in embryonated chicken eggs. Therefore poultry is unlikely to serve a role in the maintenance of either virus (Suarez et al., 2020). However, our data show that DE-CoV populations are very pleomorphic and contain some virions which morphologically are indistinguishable from viruses PED-CoV and PHE-CoV (Fig. 3) and are closely related to them by immunology (Table 1). Therefore we can suggest a real risk of ducks participating in the emergence of at least alpha-coronavirus infections in pigs. This hypothesis is based also on our preliminary investigations.

Brief, duck coronaviral enteritis was diagnosed in USSR from 1987 to 1995 in the frame of special observations in 9 duck industrial state holdings in Russia (North Caucasus, Southern Volga, and Central Regions, West Siberia), and also in Ukraine and Belarus (Lagutkin et al., 1994).

In 1987, the 'Ivano-Frankivsk virus' (isolate 'IF-87') was isolated from samples of organs of dead ducklings of 5–30 days of age from the 'Gorodenkivska' poultry farm (Ukraine) in the former All-Union Research Institute of Veterinary Virology and Microbiology, Pokrov, Vladimir Region, Russia (Knyazev et al., 2003).

According to electron microscopy data, viral particles of the pathogen were polymorphic: spherical, elongated, and curved; the sizes of spherical virions ranged from 80 to 200 nm, and elongated and curved — from 70 to 300 nm. On the surface of the particle envelope, clavate peplomers with a length of 7 to 12 nm were found, forming a crown. A thickened core was found in the center of all viral particles (Buzun et al., 1995a).

The duck disease by clinic, epizootology, and pathology resembled the porcine transmissive gastroenteritis (Buzun et al., 1995b, 1995c).

According to state statistics in the 1980s, industrial farms, where the breeding stock was staffed with Peking ducks imported from China, gradually lost their profitability due to the growing year by year lethality of ducklings. Particularly catastrophic consequences in state farms were caused by the mass placement of 'Medeo' and 'Temp' crosses, which were bred in Kazakhstan and Belarus, respectively, based on breeding ducks of Peking breed from China (duckling lethality to 70–90%). Subsequent studies have shown the affinity of hemagglutinins of viruses DE-CoV and BE-CoV (Semenikhin et al., 1996).

Much more, the nucleocapsids of viruses DE-CoV and TGE-CoV were revealed as affinity in immunoblotting; under experimental conditions was proved the prophylactic efficacy of intranasal application to 1–2-day-old ducklings ($n = 1{,}300$) of the virus vaccine against TGES from the strain 'VGNKI' in doses of 400–800 $TCID_{50}$ (once, intranasal the 1.5–2.0 ml) (Musukaeva, 1997).

This approach was not introduced in post-soviet practice — only stamping out with the replacement of duck herds. But we can not vouch that TGE-vaccination in duck industry was not conducted off outrange of the former USSR and it not had as consequence the appearance the new porcine CoV — i.e. PED-CoV. In this context, against the background of the COVID pandemic, we expect an increase in the incidence of pigs for beta-coronavirus infections — in particular PHE-CoV.

Acknowledgments. We are grateful to all colleagues who helped us to conduct these investigations and to summarize the results, and will not lose the hope to continue the development of this topic.

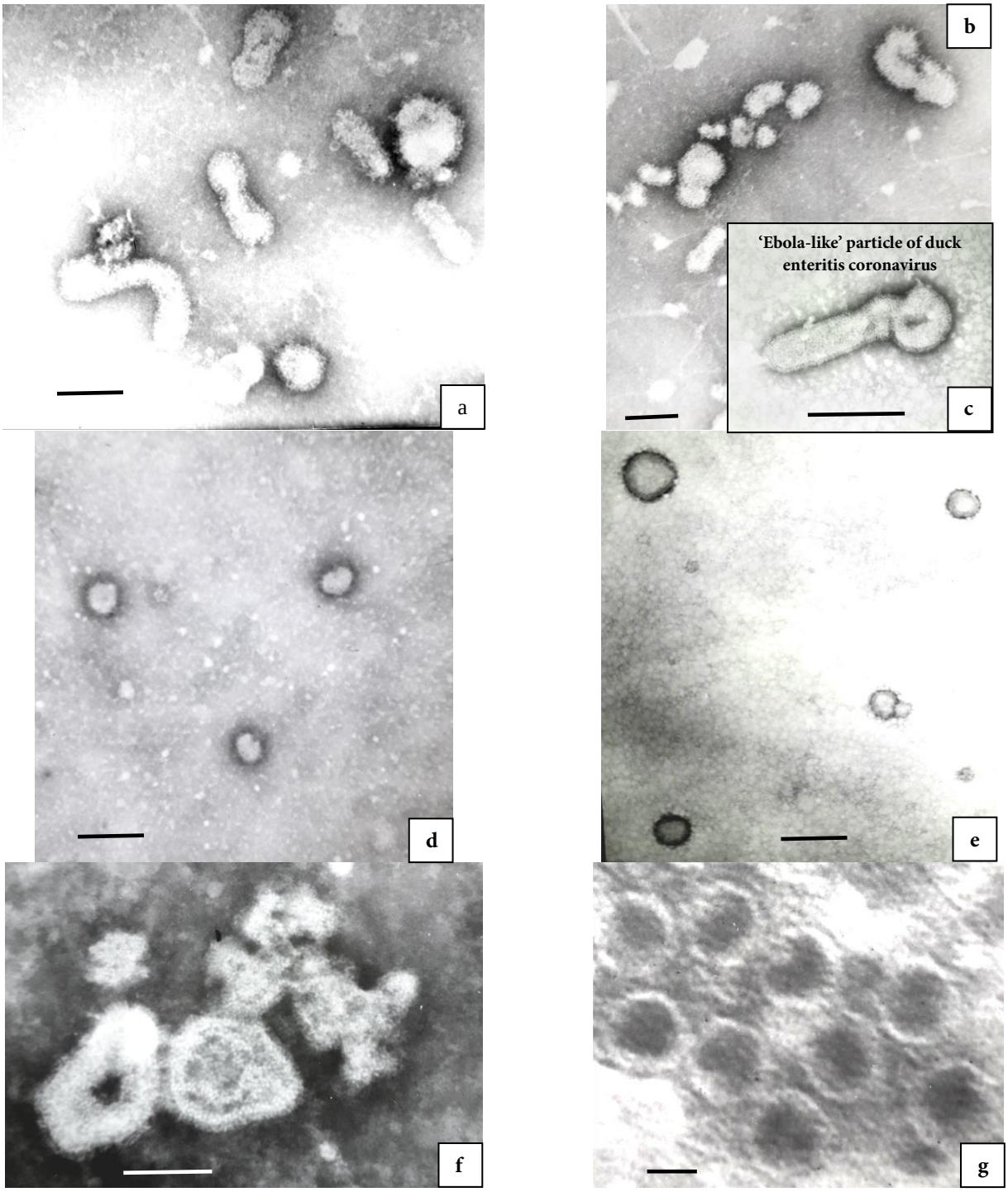


Figure 3. Negative-stain electron microscopy image of: a–c — DE-CoV isolate 'Taranivka-1991', environmental sample (fatty cap of slurry collector, together with M. Malahova, 1993); d–e — PED-CoV isolate 'Globino-2010': samples of porcine intestine (d) and Vero cells (e, the 3rd passage of PED-CoV); f–g — PHE-CoV isolate 'Ryasne-2015': samples of mouse brain (f) and BHK-21 cells (g, the 2nd passage of PHE-CoV). The magnification bars in the pictures represent 100 nm in length. For more details, see the text

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