CHAPTER «BIOLOGICAL SCIENCES»

PROPERTIES OF STRAINS TESCHOVIRUS A OF NEW SEROTYPES ISOLATED IN UKRAINE

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Abstract. In the period from 1996 to 2018 we have examined 1607 samples of materials, which have been taken from clinically healthy pigs and those that exhibited symptoms of encephalomyelitis, gastroenteritis, pneumonia and pneumoenterytis; pigs that have recovered from the mentioned sicknesses; tools that have been used in working with animals; as well as synanthropic animals and birds in Ukraine. 410 isolates of porcine teschovirus (25,5% of the total samples taken) were identified. 3 viral strains T 3, Ch 863 and Ch 878 new serotypes of Teschovirus A were identified as a result of studying the properties of the virions, genome organization, antigenic and biological properties of the strains. Strains of virusesT 3, Ch 863 and Ch 878 had been isolated from rectal and nasal washout samples, which have been taken from 3–4 months old pigs 2–4 passages. Viruses strains T 3, Ch 863, Ch 878 with morphological, physicochemical and biological properties inherent *Teschovirus* A. The titer of virus strain T 3 was 7,0 lg TCD_{50}/cm^3, Ch 863 – 5,0 lg TCD_{50}/cm^3, Ch 878 – 7,5 lg TCD_{50}/cm^3. Type cytopathic effect was typical for TV-A. Study of pathogenic properties of viral strains T 3, Ch 863, Ch 878. Found that these strains of viruses are not pathogenic for 2-month-old piglets found

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that reproduction of virus in the body of pigs does not cause clinical and pathological complex inherent in Teschen disease. As a result of electron microscopy of viruses which were prepared by purification in sucrose density gradient, it was found that the virions of strains virus T 3, Ch 863, Ch 878 have spherical shape with a diameter of 28–30 nm. In the reaction of neutralization of viruses found that strains of virus T 3, Ch 863, Ch 878 is antigenic ally distinct from the reference strains of viruses of TV-A, SV-A and EV-G. Neutralized only homologous serum antigenic ally differ, therefore, create new serotypes. We consider these strains to belong to new serotypes that were previously unknown. In PCR amplification products form only with primers derived to TV-A. In the reaction neutralization of the virus antigenically not related with any reference strain of TV-A, EV-G and SV-A have no antigenic relationship between them. Thus, strains of T 3, Ch 863, Ch 878 belonging to the family *Picornaviridae*, genus to *Teschovirus*, species *Teschovirus A* and can be attributed to new serotypes and recommended by the International Committee on Taxonomy of viruses as reference strains of new serotypes.

1. Introduction

Teschovirus A (TV-A) is distributed worldwide except Antarctica [8, p. 145]. The current name of the species has been proposed by the International Committee on Taxonomy of Viruses [1, p. 2505; 40, p. 2421]. The *Teschovirus* A had been previously named Porcine teschovirus (PTV) until 2017. The name the species is derived from the name of the disease with symptoms of swine encephalomyelitis, which was first observed by Trefny L. in the Czech town of Teschen [35, p. 235; 18, p. 85]. PTV had been named Porcine enterovirus (PEV) until 1999 [29, p. 1667; 17, p. 657].

A pathogen that casus Teschen disease of pigs was first isolated by Moscovici in 1956 [26, p. 417]. The basic morphological, biological, physical, chemical and antigenic properties of Porcine enterovirus were characterized in 1960s. Serological classification of different isolates of Porcine enterovirus was performed in different countries: Great Britain [4, p. 752; 2, p. 330], Germany [24, p. 183], Japan [26, p. 59], Hungary [35, p. 125], Sweden [33, p. 332], United States [16, p. 1142], Ukraine [31, p. 61].

A reaction of virus neutralization was considered to be a standard test to perform serological classification of PEV. As a result of conducted studies, PEV was divided into first 9 serological groups [4, p. 572] and further, in 1967, 10 serotypes of PEV have been identified by means of cross-neutralization test [2, p. 330].

In 1971, as a result of several studies of the antigenic properties of 72 European, American and Japanese strains of Porcine enterovirus, they have been merged into 8 serotypes [13, p. 619]. This classification was approved by the International Committee on taxonomy of viruses in 1979 [23, p. 132].

The existing classification of Dunne H. et al. was extended by adding three new serotypes of Porcine enterovirus that have been isolated in United Kingdom [19, 201] and two new serotypes that have been isolated in Germany [3, p. 1]. Additionally, 4 new serotypes of PEV have been identified in Japan [15, p. 49], and in Ukraine in on the basis of the study of the antigenic properties of viruses, isolated of pigs on the territory of Ukraine, Russia and Uzbekistan have established 14 PEV new serotypes [32, p. 94] that are not included in the international classification.

The results of the studies of nucleotide sequences, organization and structure of the PEV genome have led to significant changes in the taxonomy of the virus [29, p. 1667; 17, p.657]. According to a decision of 11 International Congress of Virology in 1999, serotypes PEV 1–7 and 11–13 have been classified as a separate genus *Teschovirus* to species *Porcine teschovirus*, which includes 11 serotypes. Serotype PEV 8 has been reclassified as Porcine enterovirus A, serotypes PEV 9 and 10 – as Porcine enterovirus B, which belongs to the genus *Enterovirus*.

In 2009, the International Committee on Taxonomy of Viruses has reclassified Porcine enterovirus A species as a separate genus *Sapelovirus*, species *Porcine sapelovirus* (PSV) and further, in 2012 species Porcine enterovirus B has been reclassified as species *Enterovirus G* (EV-G) within the genus *Enterovirus*. Later Porcine sapelovirus was reclassified as *Sapelvirus A* [1, p. 2505; 40, p. 2421].

Thus, serological classification of swine *Picornaviridae* have constantly updated with new serotypes, changing their taxonomy constantly, range, and therefore the aim of our work was to study the properties of strains of swine viruses isolated by us in Ukraine and establish their taxonomic position.

2. Materials and methods

Virus strains. The paper used 20 reference strains and 14 serotypes of *Teschovirus A*, *Enteroviruses G* and *Sapelovirus* A from collections of Friedrich-Loeffler-Institute, Federal Research Institute for Animal Health, Germany, have been used in the study (Table 1).

Cell culture. Used transplantable line cultures cells pigs' embryonic kidney (PEKV), pigs' kidney (PK-15), pigs' testicles (PTP) and kidney newborn Syrian hamster (BHK-21).

The growth medium. Growth medium 199 (LTD "Bio-Test Laboratory", Ukraine), Eagle's medium, 0,5% solution of hydrolyzed lactate-albumin (HLA) (Institute of poliomyelitis and virus encephalomyelitis RAMS, Russia), 5% solution of hydrolyzed blood (Scientific-Research Institute of Experimental Medicine, Republic of Belarus), bovine serum (JSC "Konotopmyaso", Ukraine) and fetal bovine serum (Sigma-Aldrich, USU) have been used for the growth of cell cultures.

Growth of cell cultures has been performed in flat flasks and test tubes at the temperature of 37˚.

Table 1

Reference strains *Teschovirus A, Sapelovirus A* and *Enterovirus G*

Genus	Genus	Serotype	Reference strains of viruses
Teschovirus	*Teschovirus A*	1	Talfan, Teschen 199, Tirol, DS1520/93
		2	T 80, O 3b
		3	O 2b
		4	PS 36
		5	F 26
		6	PS 37
		7	F 43
		8	UKG 173/74, DS 805/92
		9	VIR 2899/84
		10	VIR 460/88
		11	Dresden
Sapelovirus	*Sapelovirus A*	8	V13, Potsdam 5116
Enterovirus	*Enterovirus G*	9	UKG 410/73
		10	LP 54

Isolation of viruses from selected samples has been performed by successive passages of cell cultures PEKV and BHK 21 clone 13 according to the methodology developed by Bogel K. and Mayr A. [5, p. 908].

Studying the biological activity of the viruses. Prepared tenfold dilution of virus in physiological solution. Viruses were incubated at 37°C. An assessment of the cytopathic effect (CPE) has been performed on days from 3 to 7. Virus titer was calculated by the method of Reed L.J. and Muench H. [30, p. 495].

Pathogenic properties of viruses have been determined in studies on intact 2-month-old piglets which have been injected intracerebrally with a suspension of virus-containing culture at a dose of $0{,}4x10^6$ TCD_{50}/cm^3. The piglets have been clinically observed for 60 days.

Physicochemical properties of virions. Resistance to non-polar solvents (diethyl ether and chloroform), proteolytic enzymes (trypsin), water-based environments with a range of pH values from 2,2 to 11, thermal resistance in the presence of 1 M solution of $MgCl_2$, the effect of an inhibitor of synthesis of deoxyribonucleic acid (DNA) – 5-bromo-2-deoxyuridine, have been studied according to the commonly used virological methods [20, p. 463; 21, p. 137; 22, p. 141; 38, p. 504].

Purification of viruses have been performed in stepwise sucrose density gradient using preparative ultracentrifuge UCP–35 (Ukraine) and VAC–601 (Gemany) according to the method of P. Minor [25, p. 44–50].

Morphology of viruses has been studied by negative staining with 2% solution of phosphotungstic acid at pH 7.0 using transmission electron microscopes EM-1 (Ukraine), Tesla DS-540 (Slovakia) and GEM-1400 (Japan) with instrumental zoom 20000–22000 and accelerating voltage 60–75 kV.

Hyperimmune rabbit serum to virus strains has been obtained according to our modified method, which implies alternative introduction of antigens intradermally without adjuvant and subcutaneously with an addition of adjuvant Montanide ISA 25 (SEPPIC, France) [37, p. 1].

Determination of the serotype. The serotypes have been determined by means of the reaction of neutralization in cell culture with 100 TCD_{50} of the virus and 10 neutralizing doses of hyperimmune rabbit serums against etalon strains of *Teschovirus A*, *Sapelovirus A* and *Enterovirus G* [7, p. 11].

Primers. The primers of our own original design have been used in the study [14, pp. 158–163] The primers have been designed according to the results of

the analysis of the genomes of TV-A, SV-A and EV-G by means of "Align X (Vector NTI Suite)" software and databases such as GenBank, EMBL, DDBJ.

Primers for identification TV-A had the following sequences:

Sense Primer: **TeschoF51 5'– CCAGCAGCCTCTGTTCAGAAAG**

AntisensePrimer:**TeschoR515'–GC(A/G)TACTTGTATGAGGCCCATC**

They have flanked the portion of the RNA molecule with the length of 650 nucleotide bases, with the onset at the nucleotide number 5271 and the end at the nucleotide number 5920 (AF296096, Gene Bank) [10, p. 1].

The primers with the length of 458 nucleotide bases, which flank the area of the genome of the virus from the position at the nucleotide 3141 to the position at the nucleotide 3598 have been designed in order to identify SV-A (AF406813, Gene Bank) [11, p. 1]:

Sense Primer: **Pev8F6 5'-TGCCAAACTAAGAACGCCACTG**

Antisense Primer**: Pev8R6 5'-TCACCTTCTGCCATCCACAATC**

Species-specific primers for EV-G had the following sequences:

Sense Primer: **Pev9F1 5'– GGATTGCGGTCAAGCACTTCTGTT**

Antisense Primer: **Pev9R1 5'-CGTGGTTAGGATTAGCCGCATTC**

The primers have been blueprinted in order to cover the area of the viral genome between nucleotides 187–513 (AF363453, Gene Bank). The length of the amplicon was 327 nucleotide bases [12, p. 1].

Polymerase chain reaction (PCR). Ribonucleic acids of TV-A, EV-G and SV-A were isolated from the samples using "AmpliSens® RIBO-sorb" kit (InterLabService, Moscow, Russian Federation). cDNA has been obtained from previously isolated RNA using «Amplisens® Reverta-L» kit (InterLabService, Moscow, Russian Federation) according to the manufacturer's manual. RT-PCR have been performed on thermal cycler with 4-well capacity "Tertsyk" (SPC "DNA Technology", Russia). 5 µl of the cDNA were used for the PCR with the employment of different primer sets. 25 µl of the PCR mixture had contained: 67 mM Tris-HCl (pH 8,8), 16,6 mM $(NH4)_2SO_4$, 2,0 mM $MgCl_2$, 0,01% Tween-20, 0.2 mM of each dNTPs, 0.2 µM of each primer, 5 µl of sample cDNA, 2.0 units TaqF DNA-polymerase («InterLabService-Ukraine», Kyiv, Ukraine). Thin layers containing 30 µl of mineral oil have been added over the surfaces of each sample in order to prevent evaporation.

The amplification of the specific areas of the viral cDNA have been carried out with the use of the parameters, which are given in Table 2.

Table 2

Temperature and timing of specific areas cDNA amplification

Number of cycles	Amplification temperature, °C	Time. min.	Number of cycles
1	95	5	1
2	94	1	5
	58	1	
	74	1	
3	94	0,5	35
	58	0,5	
	73	0,5	
4	72	5	1
5		Storage	

Detection of PCR products have been performed by means of electrophoresis through 1.5% agarose gel with the use of ethidium bromide staining in Tris-borate buffer (SPC "DNA Technology", Russia) at a voltage gradient of 10 V/cm. The results have been evaluated by means of visual observation of the gel after the finish of the electrophoresis with the use of trans-illuminator device. The gel have been illuminated with UV-spectrum light and either presence or absence of orange to reddish DNA fragments have been observed. The specificity of the amplicons have been identified by their size in the comparison to the fragments of a standard ladder marker.

3. Virus isolation in cell cultures and type cytopathic effect

1607 samples from clinically healthy pigs; sick pigs with symptoms of encephalomyelitis, gastroenteritis, pneumonia and pneumoenterytis; pigs that had recovered from mentioned sicknesses; tools that had been used in the treatment of animals; as well as synanthropic animals and birds, have been obtained on the territory of Ukraine in the period from 1996 to 2018. 410 *Teschovirus A* isolates (25.5% from the all samples that had been taken) have been obtained. Their position in the modern taxonomic system have been determined by means of studying physicochemical, antigenic and biological properties of virions as well as the organization of the genome. 3 isolates of 410 (T 3, Ch 863, Ch 878) have been found to be antigenically different from the reference strains of TV-A, SV-A and EV-G. Other isolates have belonged to the known serotype TV-A.

Viral strains T 3, Ch 863 and Ch 878 had been isolated from rectal and nasal washout samples, which have been taken from 3–4 months old pigs 2–4 passages (Table 3).

Table 3

Characterization of selected strains of viruses and their biological activity

Virus strains	Biological material from which the virus was isolated	Condition of animals from which materials have been taken	Age of pigs from which samples were selected, months	Passage selection	The titer of virus lg TCD_{50}/cm^3
T 3	rectal washings	Clinically healthy	4	3	7,0
Ch 863,	rectal washings	Gastroenteritis, pneumonia	4	4	5,5
Ch 878	nasal washings	Gastroenteritis, pneumonia	3	2	7,5

CPE characterized by degenerative changed PEKV culture cell, appearance of individuals rounded cell, followed by increasing their quantity of the complete destruction of a monolayer, which is typical for *Teschovirus A* (Fig. 1). A distinctive type of cytopathic effect of the reference strains *Teschovirus A* were found. After adaptation to cell cultures PEKV, RK-15, PTP and BHK-21 their titers were in the range of 5,5 to 7,5 lg TCD_{50}/cm^3. In cell cultures the virus is through CPE 24-72 hours depending on the dose of virus. Type cytopathic effect was typical for TV-A.

4. Pathogenic properties of viral strains

The study of pathogenic properties of viral strains T 3, Ch 863, Ch 878 showed that suspension of cultures of the viruses are not pathogenic for 2-month-old piglets. All pigs that were infected with these strains were clinically healthy during the observation period. Intracerebral injection of viral strains to the body of piglets stimulated leucopoesis and erythropoiesis. The total number of leukocytes increased by 1,3–3,7 times 15 days, and the number of red blood cells during the study period in 1,1–1,7 times in comparison with these indicators of animals in the control

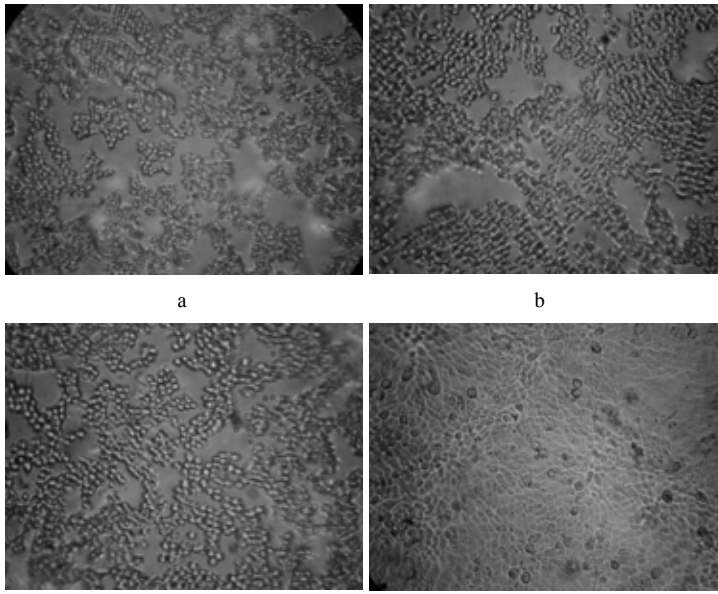


Figure 1. CPE in cell culture PEKV strains of viruses TV-A (a) T 3, (b) Ch 863, (c) Ch 878, (d) intact cell culture

group. The level of virus neutralizing antibodies in the blood serum of pigs was in the range of 1:16–1:512. When intracerebral infection viruses accumulate in the central nervous system in titer 3,5–4,5 lg TCD_{50}/cm^3. Reproduction of virus in the body of pigs does not cause these clinical and pathological complex.

5. Properties of virions

Inhibitors of DNA have almost no effect on the reproduction viruses in cell culture, their infectious titers were 6,0–7,5 lg TCD_{50}/cm^3, indicating that they belong to the RNA-containing viruses (Table 4).

Established that lipid solvents and protein lytic enzymes did not significantly affect the infectious activity of viruses. This property is characteristic of viruses, which is absent lipids containing external shell and indicates their affiliation with intestinal viruses (Table 5).

Table 4

Activity strains of infectious viruses after contact with inhibitors of DNA (lg TCD_{50}/cm^3)

Virus strains	Actinomycin-D	5-bromo-2-deoxyuridine	Zero cycle	Control
T 3	6,5	6,0	3,5	6,5
Ch 863	6,0	6,0	3,5	6,0
Ch 878	7,5	7,5	3,5	7,5

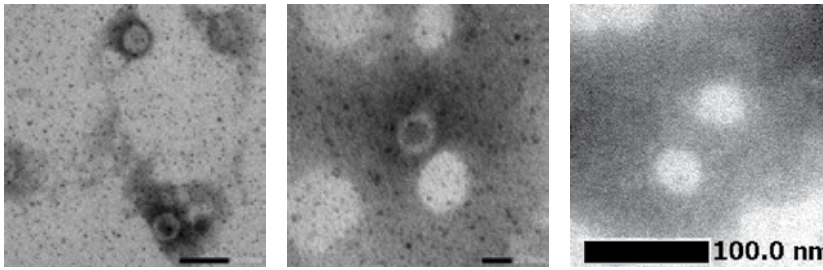
The studied strains of viruses partially inactivated by heating at 50°C for 1 hour, their infectious activity decreased to 1–2 lg TCD_{50}/cm^3. In the presence of 1 M $MgCl_2$ solution infectious virus titers almost unchanged compared with controls, indicating a stabilization of virions divalent cations of magnesium (Table 5). Different pH values in the interval 2,2–11,0 infectious titer viruses almost did not change (Table 5).

Table 5

Infectious activity of strains of viruses under the action of chloroform, diethyl ether, trypsin, magnesium ions and pH warming environment (lg TCD_{50}/cm^3)

Virus strains	Chloro-form	Diethyl ether	Trypsin	Warmed		Control	Values pH		
				without $MgCl_2$	with $MgCl_2$		2,2	7,2	11,0
T 3	6,5	6,5	6,5	5,5	6,0	6,5	6,0	6,5	6,5
Ch 863	5,5	5,0	6,0	4,5	5,5	5,5	4,5	5,5	5,0
Ch 878	7,5	7,5	8,0	5,5	7,5	7,5	7,5	7,5	7,0

As a result of electron microscopy of viruses which were prepared by purification in sucrose density gradient, it was found that the virions of strains virus T 3, Ch 863, Ch 878 have spherical shape with a diameter of 28–30 nm (Fig. 2). The morphological features of studied virus strains did not differ from virions reference strains of TV-A, SV-A and EV-G.



a b c

Figure 2. Electronic microscopy photography (a) TV-A strain T 3, (b) TV-A strain Ch 863, (c) TV-A Ch 878

6. Antigenic properties of viruses

By reference strains of TV-A, SV-A and EV-G strains studied and T 3, Ch 863, Ch 878 received specific hyper-immune serum. Their titles were 1:128–1:1024.

In the reaction of neutralization of viruses found that strains of virus T 3, Ch 863, Ch 878 is antigenically distinct from the reference strains of TV-A, SV-A and EV-G. Neutralized only homologous serum antigenically differ, therefore, create new serotypes (Table 6). We consider these strains to belong to new serotypes that were previously unknown.

Table 6

Results typing strains of viruses in cross-neutralization reaction

Antisera / Virus	T 3	Ch 863	Ch 878
T 3	+	–	–
Ch 863	–	+	–
Ch 878	–	–	+

7. Results of PCR study

PCR revealed that RNA of virus strains T 3, Ch 863, Ch 878 reacted only with primers to *Teschovirus A* and form amplification product length of 650 nucleotides and their genetic properties belonging to the genus *Teschovirus*

species *Teschovirus A*. With primers to *Enterovirus G* and *Sapelovirus A* pigs RNA virus strains T 3, Ch 863, Ch 878 amplification products do not form (Figure 3, Table 7).

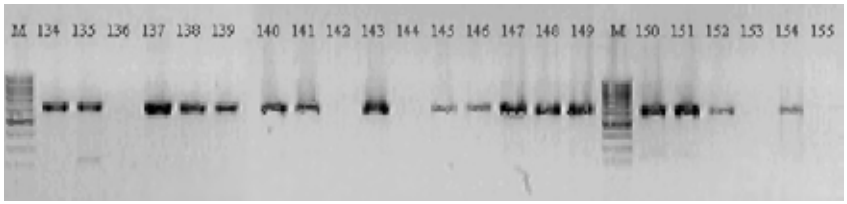


Figure 3. Elektroforehrama products of nucleic acids in PCR with primers to the TV-A

Table 7

The results of identification of viruses in PCR with primers to TV-A

№ tracks	Virus strains	Species belonging viruses	№ track	Virus strains	Species belonging viruses
134	Techen 199	TV-A	145	Ch 878	TV-A
135	Kontaric	TV-A	146	Ch 863	TV-A
136	V 13 – D	–	147	T 3	TV-A
137	V 13 – UK	TV-A	148	P 642	TV-A
138	P 501	TV-A	149	G 73	TV-A
139	I 59	TV-A	150	T 95	TV-A
140	K 422	TV-A	151	B 151	TV-A
141	Ch 2372	TV-A	152	P 142	TV-A
142	Ch 756	–	153	UKG 410/73	–
143	G 31	TV-A	154	brain suspension containing virus Ch 2372	TV-A
144	Ch 881	–	155	control cultures cells PEKV	–

Properties of strains of T 3, Ch 878 confirmed State Scientific Control Institute of Biotechnology and strains and deposited in the collection by this institution numbers 487 and 488 respectively. Strain Ch 863 – the community process to deposit. A patent of Ukraine is obtained on strain T 3 [9, p. 1]. Strains Ch 863, Ch 878 are in the process of patenting.

Despite the fact that nowadays Teschoviruses are studied extensively in different countries and o different continents, new serotypes are rare to be found. However, Chinese scientists report natural recombination between different serotypes, which is believed by them to be driving force of the evolution of the *Teschovirus* [39, p. 138; 28, p. 209].

Recently, new strains of *Teschovirus* have been also identified in Europe. Thus, a new strain WB2C-TV / 2011 / HUN (JQ429405) has been isolated from fecal samples of young wild boars in Hungary. The results of PCR revealed that this strain differs from the available referent strains in the region of the genome, which encodes VP1 protein (66–74% of similarity). Authors of the study have proposed the strain WB2C-TV / 2011 / HUN (JQ429405) to be used as a new referent serotype of *Teschovirus* [6, p. 1573; 34, p. 831].

Additionally, the identification of new serotypes have been reported in Shanghai, China by Sun H. and co-authors. They had genotyped fecal specimens from various pig herds and found three known serotypes (PTV 4, PTV 8 and PTV 10). They have also isolated some stains, which do not correspond to any of currently known serotypes. Phylogenetic analysis revealed that strain PTV SH8 is related to serotypes PTV 4 and PTV 6 but do not correspond to them. This strain do not also belong to new serotype WB2C / 2011 / HUN, which has been discovered by Boros A. et al. in Hungary [6, p. 1573].

8. Conclusions

1. As a result of a survey of farms in Ukraine in the period from 1996 to 2018 we have examined 1607 samples of materials, which have been taken from clinically healthy pigs and those that exhibited symptoms of encephalomyelitis, gastroenteritis, pneumonia and pneumoenterytis; pigs that have recovered from the mentioned sicknesses; tools that have been used in working with animals; as well as synanthropic animals and birds in Ukraine. 410 isolates of porcine teschovirus (25,5% of the total samples taken) were identified. 3 viral strains T 3, Ch 863 and Ch 878 new serotypes of *Teschovirus*.

2. Strains of viruses T 3, Ch 863, Ch 878 with morphological, physicochemical and biological properties inherent *Teschovirus A*. The titer of virus strain T 3 was 7,0 lg TCD_{50}/cm^3, Ch 863 – 5,0 lg TCD_{50}/cm^3, Ch 878 – 7,5 lg TCD_{50}/cm^3. Type cytopathic effect was typical for TV-A.

3. As a result of the study of pathogenic properties of viral strains T 3, Ch 863, Ch 878 found that of the viruses are not pathogenic for 2-month-old piglets. These strains are viruses not cause clinical and clinical symptoms inherent in Teschen disease.

4. As a result of electron microscopy of viruses, it was found that the virions of strains virus T 3, Ch 863, Ch 878 have spherical shape with a diameter of 28–30 nm.

5. In the reaction of neutralization of viruses found that strains of virus T 3, Ch 863, Ch 878 is antigenic ally distinct from the reference strains of TV-A, SV-A and EV-G. Neutralized only homologous serums. These strains to belong to new serotypes that were previously unknown.

6. In PCR amplification these strains products form only with primers derived to TV-A.

7. So, strains of T 3, Ch 863, Ch 878 belonging to the family *Picornaviridae*, genus to *Teschovirus*, species *Teschovirus A* and can be attributed to new serotypes.

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