ISSN 0233-7657
Biopolymers and Cell. 2019. Vol. 35. N 6. P 486–494
doi: http://dx.doi.org/10.7124/bc.000A1B

UDC 578

Molecular characteristics of the *Porcine Epidemic Diarrhea Virus* strains isolated in different regions of Ukraine

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Aim. Research on the molecular characteristics of the PEDV strains isolated in different regions of Ukraine. **Methods.** PEDV was detected and differentiated in biological material by PCR using the EZ-RED / TGE / PDCoV MPX 1.0 Realtime RT-PCR test system. The complete S gene was sequenced for comparative genotyping of the PEDV strains using PCR. **Results.** The diagnosis of PED was confirmed in the pig farms under investigation. The PED strains isolated in different regions of Ukraine have a high similarity (99 %) to the strains from North America in 2013-2014 and China in 2011-2012 and a lower similarity (90 %) with the strains circulating in Europe before 1995. The PEDV_Cherkasy_UA_17 strain was clustered into group 2 (cognate to the Chinese strain BJ-2011-1) in the North American clade II of the PEDV. The Ukrainian PEDV strain was phylogenetically clustered together with the highly virulent North American PEDV strains, but differed from the strains circulating in European countries. **Conclusions.** The PEDV strains isolated in various regions of Ukraine have a high (>99.5 %) degree of homology and are phylogenetically clustered with the highly virulent North American PEDV strains.

Keywords: diagnostics, PCR, S gene, phylogenetic analysis, virulence.

Introduction

Porcine epidemic diarrhea (PED) is a highly contagious viral disease of pigs, characterized by vomiting, watery diarrhea and a high level of morbidity and mortality, especially among the suckling piglets [3, 7, 15].

The emergence of epizootic outbreaks of PED has caused catastrophic economic consequences for pork producers in the USA, China, South Korea, Thailand, Vietnam and other countries. Thus, the annual economic losses from the PED in the USA amounted from $ 0.9 to $ 1.8 billion and are associated with high mortality of piglets and a decrease in the productivity among breeding animals [10].

The causative agent of PED is an RNA virus that belongs to the order *Nidovirales* in

the subfamily *Coronavirinae*, the family *Coronaviridae* and the genus *Alphacoronavirus*. The Spike (S) gene in the *PEDV* genome, like in other coronaviruses, plays a key role in the molecular epidemiology and genetic variation of strains. This gene is the most suitable for studying genetic diversity of various isolates of the *PEDV* [6, 11, 12, 15].

In China during 2010-2011 years there was a sharp increase in the number of PED outbreaks, characterized by 80-100 % morbidity and 90 % animal mortality. The change in the intensity of the manifestation of the epizootic process was caused by a new highly virulent strain of the virus. It was found that the pathogen with altered biological properties appeared due to the mutations in the *PEDV* genome [7].

Molecular studies have shown that the occurrence and spread of epizootic outbreaks of PED since 2013 in the United States were caused by genetically related strains of the virus of 99.6-100 % identity of the strains). These data suggest that there is one source of the pathogen. At the same time, the Spike (S) gene sequencing showed the highest degree of homology (> 99.0 %) with some Chinese strains isolated in 2011–2012 [15]. Since 2014 the PED outbreaks have been reported in Italy, Austria, Portugal, Belgium, Serbia, France, Germany, Hungary, Ukraine [8]. The results of phylogenetic analysis of the *PEDV* strains isolated in the countries neighboring Ukraine (Slovenia, Romania, Hungary) showed their location in the cluster of European PEDV S-INDEL strains, circulating since 2014 [2, 16].

A comparative analysis of the pathogen genome showed that the isolates circulating in Germany are highly identical with the OH851 strain, discovered in the United States in 2014 [4].

In Ukraine the *PEDV* was first isolated in 2014. Genetic analysis of the Ukrainian virus strain revealed the highest homology (99.8 %) with the strains registered in 2013 in the United States (KJ645637.1, KJ645638.1) and a lower (98.5 %) — with the strains isolated in 2014 in Germany [3].

One of the aims of epidemiology is to find out the spread of *PEDV* strain on the basis of their genetic characteristics. Therefore, the purpose of our study was to find out the molecular characteristics of the *PEDV* strains isolated in various regions of Ukraine.

Materials and Methods

Samples collection. To confirm the diagnosis of PED the laboratory tests were carried out using the biological material from animals:

1. Pig-breeding No 1 in Zaporizhzhya region. Date of selection — June 2017. Biological material — feces of newborn piglets.

2. Pig-breeding No 2 in Dnipropetrovsk region. Date of selection — July 2016. Biological material – fragments of intestines of dead piglets 5–7days of age.

PEDV detection assays. The *PEDV* indication in biological materials was performed in the laboratory of immunochemical and molecular genetic analysis of the Scientific Research Centre of Biosafety and Environmental Control of the Agro-industrial Complex of the Dnipro State Agrarian and Economic University. Detection and differentiation of the *PEDV* in biological material was carried out by PCR using a test system EZ-RED/TGE/PDCoV MPX 1.0 Realtime RT-PCR of the companyTetracore (USA).

Amplification and detection were carried out using CFX 96 Real-Time System of the company BioRad (USA) with BioRad CFX Manager software.

Sequence analysis. For comparative genotyping of the *PEDV* strains by PCR, the complete S gene was sequenced. The reverse transcription reaction was carried out using random hexamers as primers.

The complete coding sequence of the gene was amplified by the long-range PCR method using a chain-displacing SD Polymerase and primers flanking the coding sequence of the gene that were used in the study of Huang YW *et al.*, 2013 [5]. For genotyping, the amplicons were taken from the samples 1 (*PEDV* S-F1, S-R1) and 2 (*PEDV* S-F2, S-R2), amplified with the primers:

*PEDV*S-F1: TGCTAGTGCGTAATAATGAC,

*PEDV*S-R1: CATCTTTGACAACTGTGT,

*PEDV*S-F2: GCCATTTGTGGTTTTTCTA ATCATT,

*PEDV*S-R2: CAATCGTGTATTGAAAAAG TCCAAG.

The amplified fragments were planted by the method of soft precipitation in ethanol, after which the fragments were cloned into the pGEM plasmid for subsequent transformation of *Escherichia coli* and growth of the obtained clones in LB media with ampicillin. Further, 4-5 clones were sequenced from each sample. The obtained plasmids were sequenced by the Sanger method using a set of ten primers given in the study of Steinriglet *et al.* [14]. In particular, 2 flanking/external primers:

*PEDV*S-F2: GCCATTTGTGGTTTTTCTA ATCATT,

*PEDV*S-F1: TGCTAGTGCGTAATAATGAC,

*PEDV*S-R2: CAATCGTGTATTGAAAAA GTCCAAG,

*PEDV*S-R1: CATCTTTGACAACTGTGT) and 8 internal primers:

*PEDV*_267_F: TCTGGTCAGGGCTTTG AGAT,

*PEDV*_796_F: TTTGTCCAATGATTCCAC TTTG,

*PEDV*_1296_F: CATGGCACTGACGATG ATGT,

*PEDV*_1777_F: GTGTTTCCACCAGCC TTTTG,

*PEDV*_2295_F: ATTGGCTACGTCCCAT CTCA,

*PEDV*_2768_F: GCTGTTCTAATGGTCG CTCTG,

*PEDV*_3274_F: CCGGCAGATTATCAG CACTT,

*PEDV*_3788_F: GCCCAATAGAACTGGT CCAA.

DNA sequencing was performed using the ABIPRISM® BigDye™ Terminatorv. 3.1 reagent kit followed by analysis of the reaction products on the Applied Biosystems 3730 DNA Analyzer automated sequencer.

The obtained chromatograms were analyzed with the Chromas 2.6.4 software (Technelysium, Australia). Primary sequences were fitting using the BLAST algorithm [1] in order to restore the complete primary structure of the S gene.

Phylogenetic analysis.For phylogenetic analysis, the multiple alignment was performed using the Muscle algorithm, after which the phylogenetic tree was constructed by the Bayes`s methods using MrBayes software integrated into the U gene software version 1.26.1 [9]. The individual sequences identified during the study were compared with the

strains of the *PEDV* available in the GenBank database and described in our previous work (strain PEDv_Cherkasy_UA_17) [8].

Results and Discussions

At two pig farms in Ukraine, due to insufficient biosafety, inadequate compliance of the veterinary and sanitary requirements to prevent the penetration of the infectious agent and technological violations, PED outbreaks occurred in the form of a mild course of watery diarrhea mainly in gilts.

Within 3-5 days, the disease spread to most of the sows and was manifested by the complex of symptoms of intestinal disorder: watery diarrhea without acute pathophysiological processes, the temperature reaction was absent, the feed eat ability decreased, behavioral reactions were characterized by insignificant inhibition. In 5–6 days after the first cases of PED with the mild clinical course of diarrhea, the condition of the animals returned to norm.

Subsequently, the clinical signs of PED appeared in neonatal piglets. Diarrhea in piglets began one day after birth, in most animals on the second or third day, less often diarrhea appeared on the fourth or sixth day. Diarrhea was combined with vomiting. The feces were in the form of watery bowel movements, colorless or greenish-yellow, without any admixture of blood. Diarrhea led to dehydration, animals lost weight. The skin became wrinkled, inelastic, dry, rough. The inhibition of the physiological functions of the body of piglets rapidly increased, the animals noticeably weakened against the background of toxic phenomena in combination with metabolic acidosis. After three to six days of diarrhea syndrome, the pigs died due to hypothermia and progressive exhaustion. Mortality among piglets was 98-100 %.

During the autopsy, the characteristic signs were represented by a complex of pathological changes consisting of dehydration, exhaustion, anemia, severe intoxication in combination with the phenomena of metabolic acidosis and alimentary asthenia. In the gastrointestinal tract, the signs of catarrhal-hemorrhagic enterocolitis and desquamation of the secretory epithelium of the small and large intestines were noted. The liver, kidneys and heart muscle were in a state of protein-lipid dystrophy. The regional lymph nodes were swollen and hyperemic. The spleen was not enlarged, bright red. The mucous membranes and the skin were anemic, the blood in the vessels did not clot, and the fat depots of the internal organs were exhausted. Thus, in two farms, a preliminary diagnosis of PED was established.

Based on the results of laboratory studies using PCR, the diagnosis of PED in the tested pigs was confirmed.

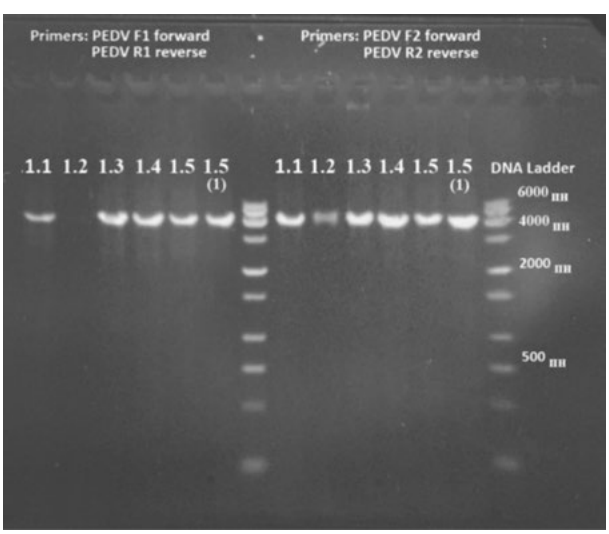

Fig. 1. Distribution of amplification products in agarose gel

For comparative genotyping of 2 *PEDV* strains (strain PEDv_Zaporizhzhya_UA_17 and strain PEDv_Dnipropetrovsk_UA_16), the S gene was sequenced. The theoretical size of the amplicon was 4243 bp. (in accordance with isolate sequence GER/L00721/2014, GenBank accession No LM645057). The observed size of amplicons obtained from the genome of the studied strains corresponded to the expected one (Fig. 1).

We did not detect differences among the clones from one isolate. As a result of sequenc-

Table1. **The results of the multiple nucleotide alignment of Spike (S) gene of the strain PEDv_Cherkasy_UA_17 with the sequences of the virus strains presented in GenBank**

Strain/isolate of PEDV	Accession	Country, collection date	Total score	Identity, %
strain USA/Iowa96/2013	KJ645688.1	USA (Iowa),Dec-2013	1238	99
strain HEN/ZMD/2012/6	KJ503833.1	China, Jun-2012	1238	99
strain USA/Illinois333/2014	KR265804.1	USA (Illinois), May-2014	1238	99
isolate CH-SDDZ-2012	KU133240.1	China , Jan-2012	1232	99
isolate CH-SDLY-1-2012	KU133249.1	China, Feb-2012	1232	99
isolate BJ-2011-1	JN825712.1	China, Apr-2011	1232	99
isolate MN	KF468752.1	USA (Minnesota), Jun-2013	1227	99
strain MEX/124/2014	KJ645700.1	Mexico, Jan-2014	1227	99
strain CHGD-01	JX261936.1	China, Feb-2011	1199	99
strain CH/FJND-3/2011	JQ282909.1	China, May-2011	1160	98
isolate KNU-0902	GU180145.1	South Korea, Feb-2009	1149	97
isolate KNU-0802	GU180143.1	South Korea, Dec-2008	1140	97
isolate KNU-0903	GU180146.1	South Korea, Feb-2009	1038	95
strain NK	AB548623.1	Japan, 2010	1022	94
isolate KNU-0901	GU180144.1	South Korea, Feb-2009	1011	94
isolate KNU-0801	GU180142.1	South Korea, Dec-2008	1011	94
isolate KNU-0904	GU180147.1	South Korea, Jul-2009	994	93
strain Chinju99	AY167585.1	SouthKorea,Oct-2002	979	93
strain KH	AB548622.1	Japan,Mar-2010	977	93
strain DR13	JQ023161.1	South Korea, 2009	891	91
strain USA/Iowa106/2013	KJ645695.1	USA (Iowa), Dec-2013	885	90
strain USA/Iowa107/2013	KJ645696.1	USA (Iowa), Dec-2013	885	90
strain LJB/03	DQ985739.1	China, Sep-2006	885	90
strain DX	EU031893.1	China,Jul-2007	885	90
strain JS-2004-2	AY653204.1	China,Jun-2004	880	90
isolate Br1/87	Z25483.1	Europe,Aug-1993	869	90
strain CV777	AF353511.1	Europe,1993	863	90
isolate SM98	GU937797.1	SouthKorea,Feb-2010	848	90
strain: MK	AB548624.1	Japan,Mar-2010	846	89
strain: 83P-5	AB548618.1	Japan,Mar-2010	841	89
strain DR13, (attenuated)	JQ023162.1	SouthKorea,Nov-2011	833	89
strain LZC	EF185992.1	China,Dec-2006	824	89

ing of the S gene, it was found that the strain PEDv_Dnipropetrovsk_UA_16 has a high identity (99 %) with the strains from North America 2013-2014 (No. KJ645688.1, KR265804.1, KF468752.1, KJ645700.1) and China 2011-2012 (KJ503833.1, KU133240.1, KU133249.1, JN825712.1, JX261936.1), and a lower similarity (90 %) with the strains circulating in Europe until 1995 (Z25483.1, AF353511.1) (Tab.No. 1).

As can be seen in the cladogram (Fig. 2), the sequence of strain PEDv_Cherkasy_UA_17 belong to the group 2 (related to the Chinese strain BJ-2011-1) in the North American clade II of the *PEDV* together with the strains USA/Illinois/333/2014(No. KR265804.1), USA/Iowa/2013 (KJ645688.1), *etc.* The investigated strain differs from these sequences by one nucleotide substitution (for example, KJ645688.1:n.20965C>T).

Phylogenetic analysis revealed that the studied strain PEDv_Zaporizhzhya_UA_17 and strain PEDv_Dnipropetrovsk_UA_16 are the closest to the group of American highly pathogenic strains of the *PEDV*. Two

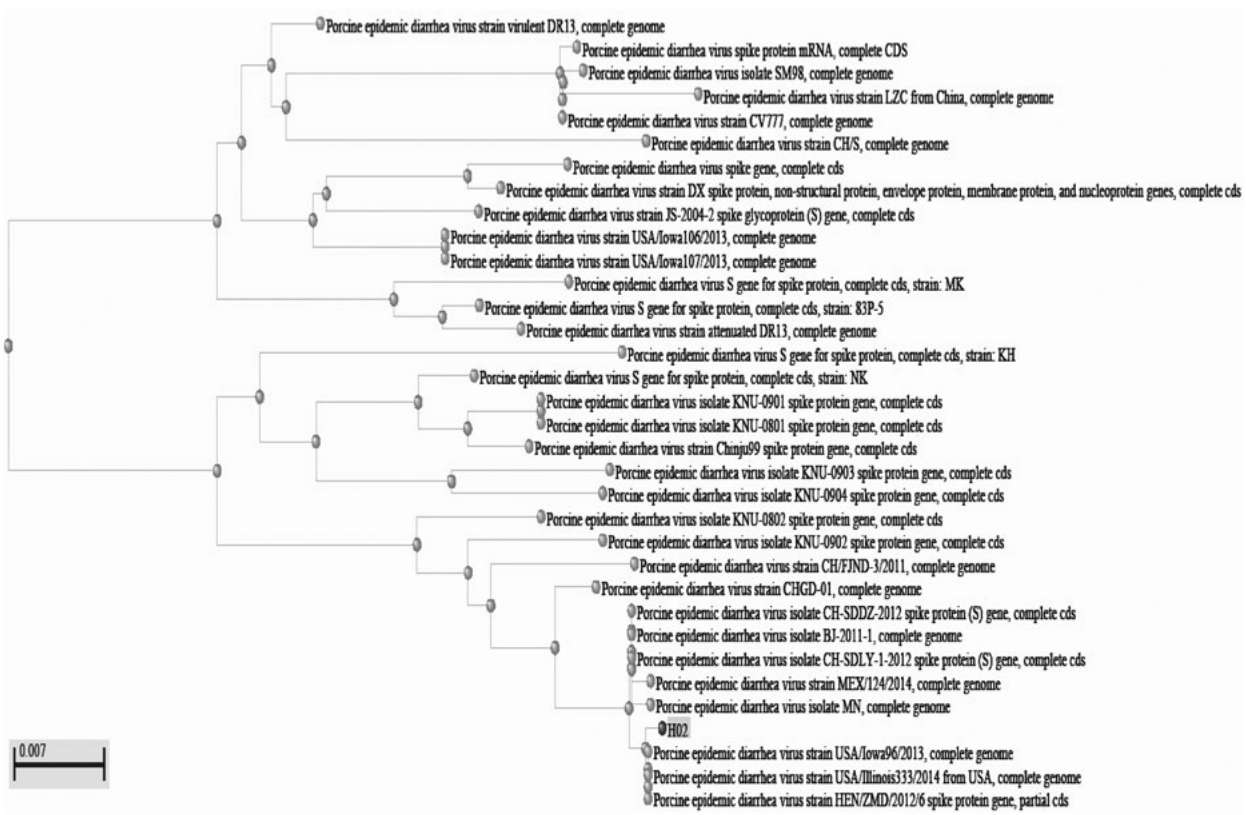

Fig. 2. Position of the genome fragment of the strain PEDv_Cherkasy_UA_17 on the phylogenetic tree. The sequence identified in this study is highlighted in yellow

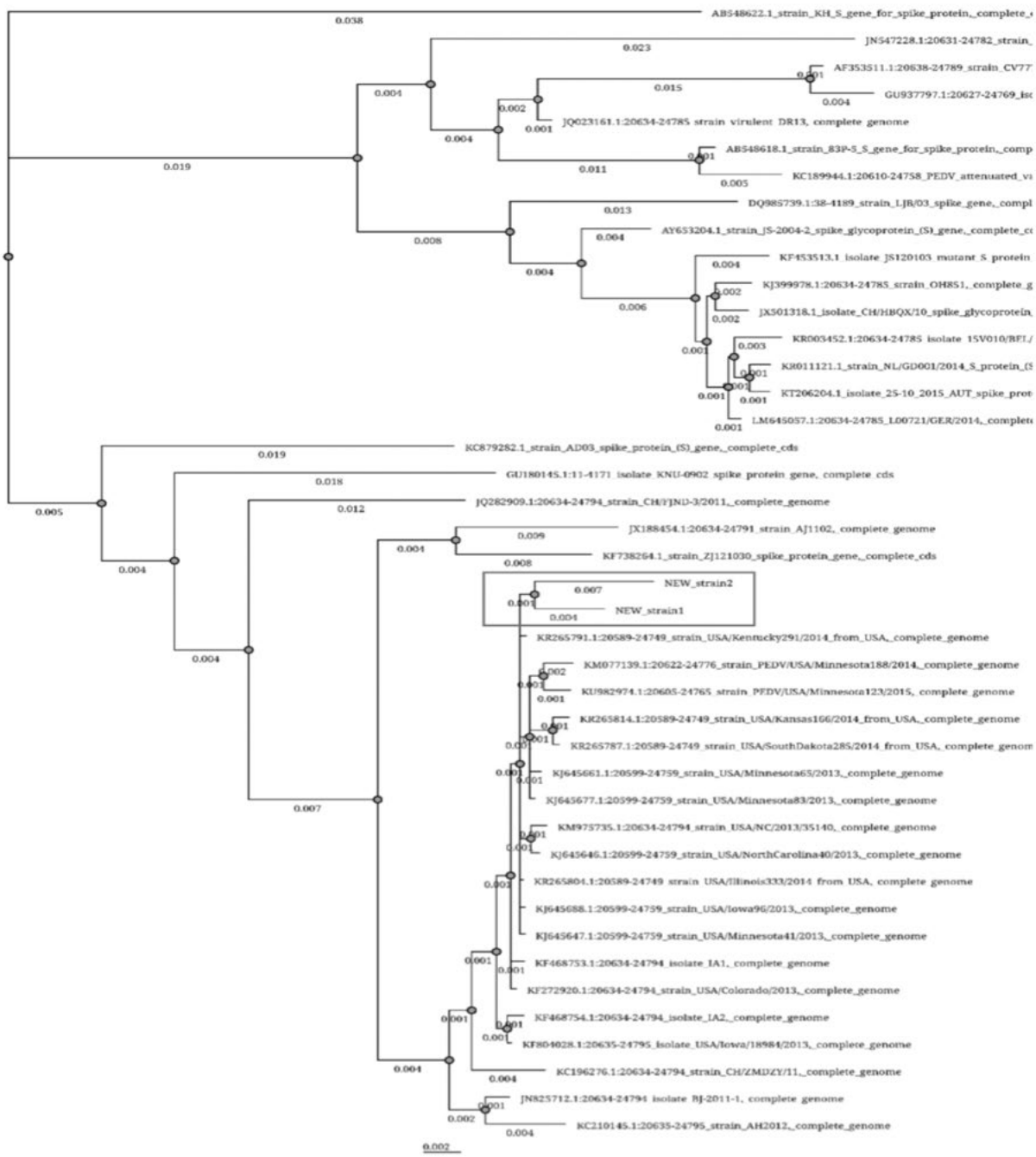

Fig. 3. Phylogenetic tree constructed using Bayes`s phylogeny methods with S gene sequences of 40 known isolates and strains of the *PEDV*, as well as the sequences of the studied isolates (indicated in the cladogram as NEW_strain 1 (strain PEDv_Zaporizhzhya_UA_17) and NEW_strain 2 (strain PEDv_Dnipropetrovsk_UA_16), circled in a red square). The numbers along the branches of the cladogram are posterior probabilities

Ukrainian isolates differ from each other, that is, they are two different strains of the *PEDV*. At the same time, a separate clade is formed on the cladogram, which probably originated from the American strains of 2013-2014 (Fig. 3).

Similar results were obtained by other authors. Accordingly, Dastjerdi A. *et al.* [3], have shown that the PEDV Ukraine/Poltava01/2014 is phylogenetically clustered with highly virulent North American *PEDV* strains in genetic clade II (together with No. KJ645708.1, KJ645637.1, KJ645701.1, KF272920.1, KJ645638.1), but differed from strains circulating in different European countries.

Conclusion

The *PEDV* strains isolated in different regions of Ukraine have a high (>99.5 %) degree of homology and are phylogenetically clustered with the highly virulent North American *PEDV* strains, which indicates the introduction of *PEDV* into the territory of Ukraine from the countries of Asian or American continents.

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Молекулярна характеристика штамів вірусу епідемічної діареї свиней, ізольованиху різних регіонах України

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Мета. Вивчення молекулярних характеристик штамів вірусу ЕДС, ізольованих у різних регіонах України. **Методи.** Виявлення та диференціацію вірусу ЕДС у біологічному матеріалі здійснювали методом ПЛР за допомогою тест-системи EZ-RED/TGE/PDCoV MPX 1.0 Realtime RT-PCR фірми Tetracore (США). Для порівняльного генотипування штамів вірусу ЕДС проведено повне секвенування гену S. **Результати.** Було підтверджено діагноз на ЕДС у досліджених свинарських господарствах. У результаті секвенування встановлено, що штами ЕДС, ізольовані в різних регіонах України мають високу(99 %) ідентичність зі штамами з Північної Америки 2013-2014 рр. та Китаю 2011-2012 рр. і низьку (90 %) – зі штамами, циркулюючими в Європі до 1995 г. Штам PEDv_Cherkasy_UA_17 кластеризовано до групи 2 (спорідненої китайському штаму BJ-2011-1) у північноамериканській кладі II вірусу ЕДС. Показано, що український штам вірусу ЕДС філогенетично кластеризовано разом з високо вірулентними північноамериканськими штамами ЕДС, однак відрізнявся від штамів, що циркулюють у різних країнах Європи. **Висновки.** Штами вірусу ЕДС, виділені у різних регіонах України мають високий (>99,5 %) ступінь гомологічності та філогенетично кластеризуються разом з високовірулентними північноамериканськими штамами вірусу ЕДС.

Ключові слова: діагностика, ПЛР, S ген, філогенетичний аналіз, вірулентність.

Молекулярная характеристика штаммов вируса эпидемической диареи свиней, изолированных в разных регионах Украины

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Цель. Изучение молекулярных характеристик штаммов вируса ЭДС, изолированных в разных регионах Украины. **Методы.** Выявление и дифференциацию вируса ЭДС в биологическом материале осуществляли методом ПЦР с помощью тест-системы EZ-RED/TGE/PDCoV MPX 1.0 Realtime RT-PCR фирмы Tetracore (США). Для сравнительного генотипирования штаммов вируса ЭДС проведено полное секвенирование гена S. **Результаты.** Был подтвержден диагноз на ЭДС в исследуемых свиноводческих хозяйствах. В результате секвенирования установлено, что штаммы ЭДС, изолированные в разных регионах Украины, имеют высокую (99 %) идентичность со штаммами из Северной Америки 2013-2014 гг и Китая 2011-2012 гг и низкую (90 %) – со штаммами, циркулирующими в Европе до 1995 г. Штамм PEDv_Cherkasy_UA_17 кластеризировано в группу 2 (родственной китайскому штамму BJ-2011-1) в североамериканской клади II вируса ЭДС. Показано, что украинский штамм вируса ЭДС филогенетически кластеризовано вместе с высоковирулентными североамериканскими штаммами ЭДС, но отличался от штаммов, циркулирующих в разных странах Европы. **Выводы.** Штаммы вируса ЭДС, выделенные в разных регионах Украины имеют высокую (>99,5 %) степень гомологичности и филогенетически кластеризуются вместе с высоко вирулентными североамериканскими штаммами вируса ЭДС.

Ключевые слова: диагностика, ПЦР, S ген, филогенетический анализ, вирулентность.

Received 19.09.2019