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Infectious bursal disease virus: identification of the novel genetic group and reassortant viruses

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SUMMARY

The results of the phylogenic analysis of the nucleotide sequence of the IBDV A and B genome segments have been presented. Traditionally the IBDV isolates are classified based on the phylogenic analysis of the hypervariable region of the VP2 gene. The analysis of the VP2 gene segments of the isolates detected in the Russian Federation demonstrated that most of them belong to the genetic group comprising very virulent IBDV isolates. However, not all isolates belonging to one genetic group have the same phenotypic characteristics. This is related to the fact that the virulence is determined not only based on the characteristics of the VP2 gene (A segment) but on the characteristics of the VP1 gene (B segment) as well. The IBDV genome segmentation allows formation of reassortant viruses which can be identified as a result of the genome segment analysis. The phylogenic analysis of the nucleotide sequences of VP2 and VP1 genes of 28 IBDV isolates detected at RF, Ukrainian and Kazakh poultry establishments in 2007 and 2019 showed that 15 of them are reassortant viruses. Different combinations of the genome segments have been identified among these reassortant viruses. Detection of different combinations of IBDV genome segments is indicative of the fact that the heterogeneous virus population circulates on the poultry farms. Pathogenicity studies of the three IBDV isolates showed that the most virulent was an isolate having two genome segments characteristic of the very virulent virus. Two reassortant viruses having only one genome segment A or B, characteristic of the infectious bursal disease, demonstrated less pronounced virulent properties.

Keywords: infectious bursal disease virus, phylogenic analysis

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Вирус инфекционной бурсальной болезни: выявление новой генетической группы и реассортантов

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РЕЗЮМЕ

Представлены результаты филогенетического анализа изолятов вируса инфекционной бурсальной болезни по нуклеотидным последовательностям фрагментов геномных сегментов A и B. Традиционно изоляты вируса инфекционной бурсальной болезни классифицируют на основе филогенетического анализа гипервариабельной области гена VP2. Анализ фрагмента гена VP2 изолятов, выявленных на территории Российской Федерации, показал, что большинство из них относятся к генетической группе, объединяющей высоковирулентные изоляты вируса инфекционной бурсальной болезни. Но не все изоляты, относящиеся к одной генетической группе, обладают одинаковыми фенотипическими свойствами. Это связано, в частности, и с тем, что вирулентность определяется генетическими особенностями не только гена VP2 (сегмент A), но и гена VP1 (сегмент B). Сегментированная природа генома вируса инфекционной бурсальной болезни делает возможным образование реассортантов, которые можно выявить в результате анализа обоих геномных сегментов. Филогенетический анализ нуклеотидных последовательностей фрагментов генов VP2 и VP1 28 изолятов вируса инфекционной бурсальной болезни, выявленных в птицеводческих хозяйствах РФ, Украины и Казахстана в 2007–2019 гг., показал, что 15 из них являются реассортантами. Среди реассортантов выявлены различные комбинации геномных сегментов. Выявление разнообразия комбинаций геномных сегментов вируса инфекционной бурсальной болезни свидетельствует о том, что в птицеводческих хозяйствах циркулирует гетерогенная вирусная популяция. Изучение степени патогенности трех изолятов вируса инфекционной бурсальной болезни показало, что наиболее вирулентным был изолят, имеющий оба геномных сегмента, характерных для высоковирулентного вируса. Два реассортанта, имеющих только по одному геномному сегменту A или B, характерному для высоковирулентного вируса инфекционной бурсальной болезни, обладали менее выраженными вирулентными свойствами.

Ключевые слова: вирус инфекционной бурсальной болезни, филогенетический анализ

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INTRODUCTION

The infectious bursal disease virus (IBDV) is the causing agent of the acute, highly contagious and widely spread disease in the countries with commercial poultry farming. IBDV infects lymphocytes of the bursa of Fabricius, thymus, spleen, Peyer's patches of the intestine. The consequences of IBDV-associated immunosuppression is that vaccination against other diseases is less effective and the chicks become more susceptible to opportunistic pathogens. Very virulent IBDV (VV IBDV) can cause high death rate in poultry. High contagiousness and sustainability of the virus to the environmental factors and disinfectants as well as a considerable economic losses make IBD a serous problem for poultry farming in many countries.

The infectious bursal disease virus (IBDV) is a member of the genus *Avibirnavirus*, family *Birnaviridae* [1]. These viruses have a genome that consists of two segments of double-stranded RNA designated A and B. Segment A contains partially overlapping open reading frames (ORFs). The small ORF1 encodes the non-structural VP5 virus protein, which contributes to the release of mature virions. The larger ORF2 encodes polyprotein from which VP2 (pVP2), VP4, and VP3 precursors are formed as a result of autoproteolysis [2]. VP2 protein takes part in formation of the external surface of the virion. This is the main immunogen carrying antigenic sites responsible for neutralizing the virus with antibodies. The VP2 protein is responsible for the antigenic variability and virulence of the virus. The VP3 protein interacts with all other components of the virion, so it plays a critical role in virion morphogenesis, encapsidation, and virus replication. The VP4 protein is a serine protease that plays an important role in VP2 maturation and is also involved in polyprotein proteolysis. Genomic segment B contains one ORF encoding RNA-dependent VP1 RNA polymerase responsible for viral genome replication and mRNA synthesis [3, 4].

Currently, two IBDV serotypes have been identified. IBDV strains, serotype 1, are classified into 3 types depending on the severity of the disease caused: subclinical, classical virulent and very virulent [5]. The serotype 2 virus has been isolated from turkeys and is non-virulent in chickens.

Traditionally, IBDV isolates are classified based on phylogenetic analysis of the VP2 gene segment, which includes the hypervariable region. The classification proposed by T. P. Van den Berg et al. [6], subdivides strains of serotype 1 into the following genotypes: attenuated, classical virulent, very virulent, antigenic variants, and Australian ones.

Very virulent IBDV was first detected in broilers in Europe in the late 1980s [7] and quickly spread to Africa, Asia and Latin America, causing high morbidity and mortality over 30% [8]. Antigenic variants have become predominant in the Americas. They usually cause a subclinical infection characterized by rapid atrophy of the bursa of Fabricius without significant inflammation, which can lead to immunosuppression. In 2019, a report appeared on the detection of a new IBDV antigenic variant in China

that differed from American variants and caused severe immunosuppression in chickens [9].

IBDV antigenic phenotype is determined by the VP2 hypervariable region, in particular by amino-acids located at the top of the loop and indicated as PBC, PDE, PFG, and PHI [10]. It was determined that even point mutations in these regions can cause IBDV antigenic drift [10, 11] and make vaccination against IBD ineffective.

Michel L. O. and Jackwood D. J. [12] suggested to use new IBDV classification by dividing the virus strains into 7 gene groups. Most IBDVs form three gene groups: 1 – classical, 2 – variant и 3 – VV IBDV or reassortant IBDV. The rest four genogroups include IBDVs detected in different regions of the world.

IBDV isolates belonging to one of the genogroups have several common genotypic and phenotypic properties. But not always the isolates, belonging to one and the same genogroup have the same phenotypic properties. It is related, in particular, to the fact that virulence is determined by genetic differences not only in the VP2 gene, but also in VP1 gene [13–15].

Phylogenetic analysis of the VP1 gene nucleotide sequences shows that all IBDV strains are divided into two large genetic groups: one of them contains the VV IBDV strain and the other one – all the rest IBDV strains [16].

The IBDV genome segmentation allows formation of reassortant viruses in case of host cell co-infection with different virus strains. There have been some publications on detection of reassortment between serotype 1 strains [17–22]. The pathogenicity of reassortant viruses whose A segment belongs to the VV IBDV and B segment belongs to the other non-very virulent group is lower than that of the very virulent parent strains [23]. On the other hand the reassortant virus Bpop/03 was detected in Poland which had segment A originating from VV IBDV and segment B originating from the classical attenuated strain D78 [24]. Despite the mosaicism the virus caused high mortality (80%) in experimentally infected SPF-chicks and IBD-associated lesions. These data contradict to the previous experimental studies where the natural reassortant viruses demonstrated the intermediate pathotype [23].

Analysis of the VP2 gene segment of the IBDV isolates detected in the Russian Federation in 1993–2020 demonstrated that most of them don't belong to VV IBDV group. Analysis of two genome segments are more informative and allows detection of reassortant viruses. This research describes molecular characteristics of the IBDV isolates, detected in 2007–2019 based on the alignment of neucleotide sequences of the VP2 gene hypervariable region (segment A) and VP1 gene segment containing a phylogenetic marker (segment B).

MATERIALS AND METHODS

26 IBDV isolates detected in the samples of biological material from the RF poultry farms and 2 IBDV isolates from Ukraine and Kazakhstan were used in the research (Table 1).

RNA extraction. Total RNA was extracted from the clarified suspension prepared from the bursa of Fabricius using the SV 96 Total RNA Isolation System (Promega, USA) kit.

One step *RT-PCR* was performed. To perform the test Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT) and Taq-ДНК-polymerase (Promega, USA) were used according to the manufacturer's instruction. Conservative regions VP1 and VP2 were used for primer calculation. The 25 µL final dilution of the reaction mixture: 10 µL of the deionized water; 5 µL 5× buffer for RT-PCR; 2 µL of the 25 mM $MgCl_2$ solution; 1 µL of the 10 mM dNTP solution; 1 µL of the direct and reversed primer solution,10 pmol/µL concentration; 0.13 µL of the reversed transcriptase and 0.25 µL of polymerase; 5 µL of the total RNA solution. The reversed transcription was performed for 30 minutes at 50 °C. The following time-temperature parameters were

Table 1
IBDV isolates

No.	Sample receipt date	Subject where samples were collected	Isolate
1	25.06.2007	Krasnoyarsky Krai	IBDVRF02-2007
2	11.10.2007	The Republic of Dagestan	IBDVRF03-2007
3	29.10.2007	Ukraine	IBDVUkr04-2007
4	12.11.2007	Primorsky Krai	IBDVRF05-2007
5	04.06.2014	Novgorod Oblast	IBDVRF02-2014
6	28.07.2014	The Republic of Kazakhstan	IBDVKaz03-2014
7	11.08.2014	The Republic of Tatarstan	IBDVRF04-2014
8	16.10.2014	Orenburg Oblast	IBDVRF05-2014
9	08.04.2015	Vladimir Oblast	IBDVRF02-2015
10	11.10.2015	Yaroslavl Oblast	IBDVRF06-2015
11	27.10.2016	Novgorod Oblast	IBDVRF02-2016
12	09.11.2016	Chelyabinsk Oblast	IBDVRF03-2016
13	04.04.2017	Novgorod Oblast	IBDVRF01-2017
14	02.08.2017	The Chuvash Republic	IBDVRF02-2017
15	05.10.2017	Kursk Oblast	IBDVRF03-2017
16	24.11.2017	Kirov Oblast	IBDVRF06-2017
17	28.11.2017	The Mari El Republic	IBDVRF08-2017
18	21.08.2018	Leningrad Oblast	IBDVRF01-2018
19	03.08.2018	Kursk Oblast	IBDVRF02-2018
20	16.01.2019	Novgorod Oblast	IBDVRF01-2019
21	16.01.2019	Novgorod Oblast	IBDVRF02-2019
22	27.02.2019	Sverdlovsk Oblast	IBDVRF03-2019
23	07.03.2019	Leningrad Oblast	IBDVRF04-2019
24	06.05.2019	Ivanovo Oblast	IBDVRF05-2019
25	06.06.2019	Novgorod Oblast	IBDVRF06-2019
26	24.06.2019	Vladimir Oblast	IBDVRF07-2019
27	02.07.2019	Vladimir Oblast	IBDVRF09-2019
28	18.10.2019	Yaroslavl Oblast	IBDVRF10-2019

used for amplification: 95 °C – 10 min (polymerase activation), then – 40 cycles, each of them consisted of three steps: 95 °C – 50 sec., 55 °C – 50 sec., 72 °C – 60 sec. In order to increase the sensitivity the nested PCR using inner pair of primers was applied. To visualize the results of the reaction electrophoresis in 2% agarose gel with ethidium bromide was used.

Alignment of nucleotide sequences and phylogenetic analysis. Purified PCR products were used for identification of the nucleotide sequences of the VP1 and VP2 gene segments using automatic sequencer ABI Prism® 3100 (USA) and BigDye Terminator Cycle Sequencing kit (Applied Biosystems, USA) according to the manufacturer's instruction.

For analysis, we used the nucleotide sequences of IBDV isolates and strains published in the GenBank database of the NCBI electronic resource (https://www.ncbi.nlm.nih.gov/nucleotide) (Table 2). Nucleotide and corresponding amino acid sequences were analyzed using the BioEdit program, version 7.0.5.3. Sequence alignment was performed using the ClustalW multiple alignment program. The phylogenetic tree was constructed using the UPGMA algorithm in the implementation of the MEGA package, version 6.06.

BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for comparing nucleotide and amino-acid sequences with the database of sequences and calculation of statistical significance.

RESULTS AND DISCUSSION

The nucleotide sequences of the VP2 and VP1 gene segments were identified for 28 IBDV isolates detected on the poultry farms of the Russian Federation, Ukraine, and Kazakhstan in the period from 2007 to 2019. For comparative analysis, we used published nucleotide sequences of IBDV strains belonging to different genogroups according to the L. O. Michel and D. J. Jackwood classification [12] (Table 2).

Phylogenetic analysis of the VP2 gene segment. As a result of comparative analysis of the IBDV VP2 gene nucleotide sequences it was determined that 22 isolates belong to Genogroup 3 (VV IBDV), IBDVRF07-2019 isolate – to Genogroup 1, 2 isolates IBDVRF03-2017 and IBDVRF02-2018 are variant, 3 isolates IBDVRF05-2014, IBDVRF03-2016 and IBDVRF03-2019 differ from all isolates by 5% and form a new Genogroup 8 (Fig.).

Genogroup 3 includes VV IBDV and is the largest group. IBDV strains belonging to this group were detected during acute IBD outbreaks on poultry farms in the world. Recently the number of published nucleotide sequences of the VP2 gene of the Genogroup 3 strains has increased.

The sequence analysis shows that the hypervariable VP2 region accumulates mutations inside the group which makes it possible to identify subgroups 3-1, 3-2, 3-3 [12]. The amino-acid composition of the isolates belonging to these subgroups differ at positions 212, 222, 254, included in the loop structure and responsible for IBDV antigenic properties (Table 3).

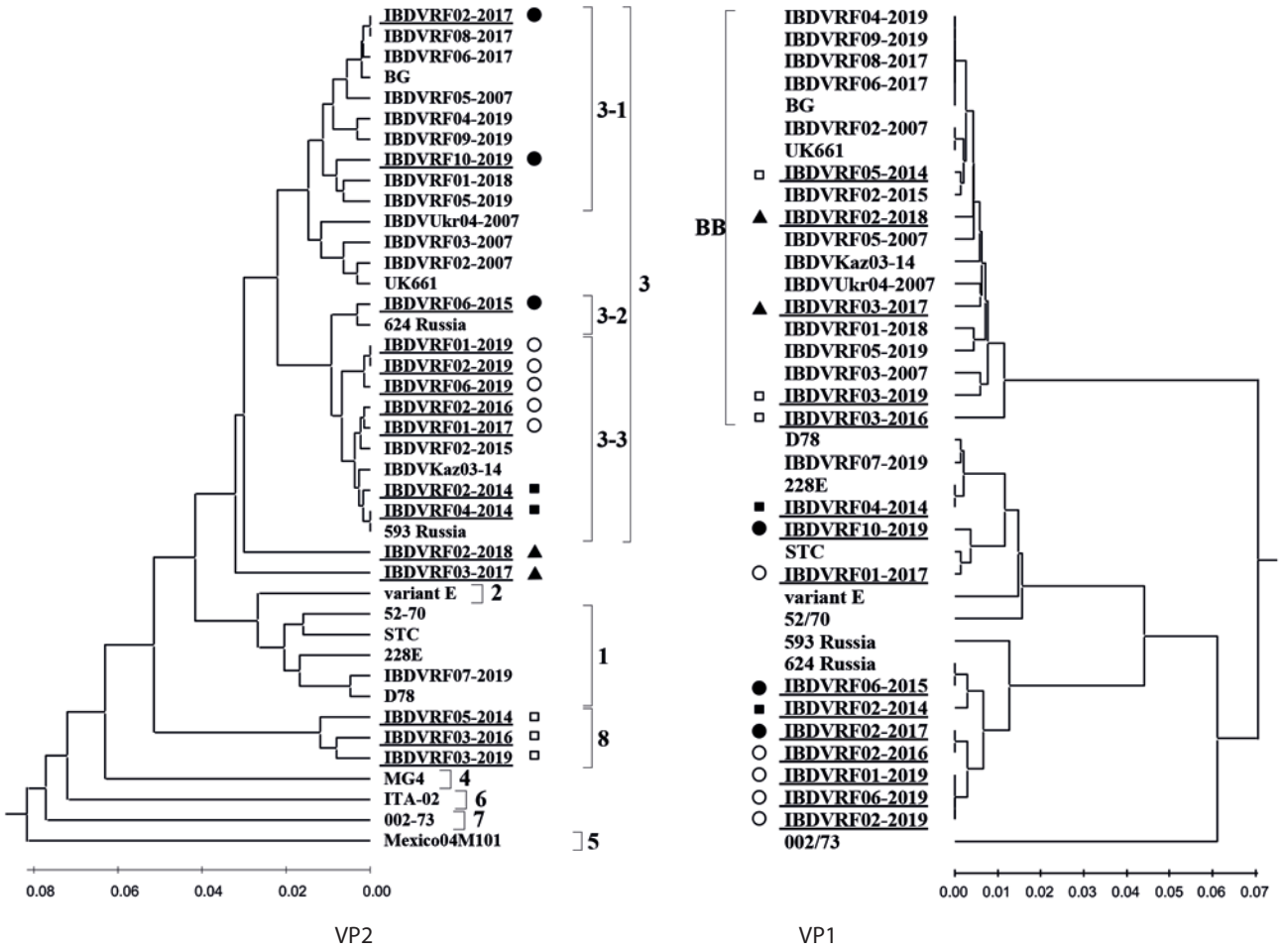
IBDVRF07-2019 isolate, belonging to Group 1, is a derivative of the D78 vaccine strains. Nucleotide sequences of the VP1 and VP2 gene segments are 99.36% homologous to D78 strain. The attenuated vaccine based on this strain has been widely used on the RF farms for 20 years.

Two variant isolates IBDVRF03-2017 and IBDVRF02-2018 were detected in samples from one of the poultry farms of the Kursk Oblast. The VP2 gene nucleotide sequences different from each other and from the isolates of other genogroups.

Three isolates: IBDVRF05-2014, IBDVRF03-2016, IBDVRF03-2019, detected in Orenburg, Chelyabinsk and Sverdlovsk Oblasts differ from all tested and published strains and are likely to form Genogroup 8. The nucleotide sequences of VP2 gene strains and IBDV isolates, contained in the GenBank differed from the consequences of this group of isolates by more than 5%. Genogroup 8

Table 2
Nucleotide sequences of the IBDV isolates used for analysis

No.	Strain	Number in the GenBank VP2	Number in the GenBank VP1	Country	Genogroup
1	D78	AF499929	EU162090	Luxembourg	1
2	228E	AF457104	AJ878657	The Netherlands	1
3	52/70	HG974565	HG974566	Great Britain	1
4	STC	D00499	JQ619639	The USA	1
5	Variant E	AF133904	AF133905	The USA	2
6	UK661	X92760	X92761	Great Britain	3
7	624Russia	MF142552	MF142481	Russia	3-2
8	593Russia	MF142550	MF142479	Russia	3-3
9	MG4	JN982252	–	Brazil	4
10	Mexico04M101	DQ916210	–	Mexico	5
11	ITA-02	JN852986	–	Italy	6
12	002-73	AJ878908	AJ878639	Australia	7



VP2 VP1

Fig. Phylogenic trees built on the basis of the alignment of the nucleotide sequences of the VP2 gene hypervariable region and VP1 gene segment.
Numerals represent genotypes, underlining and geometric figures represent reassortant isolates

isolates have VV IBDV associated amino-acid residues in the hypervariable region of the VP2 region (Table 3): 242I, 256I, 294I, 299S, as well as a number of amino acid residues peculiar only to them: 213E, 220F, 222V, 269S, 280T, 317R, 324P. It is worth noting that the amino acid change at position 222 is important because this residue is located in the PBC loop. It is believed that the transition from Pro to Thr at position 222 played a significant role in the significant change in the antigenic properties of variant IBDV strains in the 1980s [25], which led to the ineffective vaccination.

Phylogenetic analysis of the VP1 gene segment. The analysis of the published nucleotide sequences of the VP1 gene showed that IBDV strains are divided into 2 large groups. The first one includes VV IBDV, and the second includes all other genetic groups: attenuated, classical variant, antigenic and Australian variants, as well as serotype 2 [26].

It was determined that VP1 contains a TDN marker triplet characteristic of VV IBDV at positions 145/146/147 [27, 28]. Low variability of this triplet in VP1 was observed in VV IBDVs.

Phylogenetic analysis of the VP1 gene segment of the IBDV isolates under study showed that they are also divided into 2 groups: the group with the VV IBDV-like segment included 17 isolates, and the group with the non-VV IBDV-like B segment included 11 isolates. In the first group, with the exception of the isolate IBDVRF03-2017, the VP1 sequence carries the IBDV characteristic TDN marker sequence (Table 3).

Phylogenetic analysis of the VP1 gene segment shows that the group with a non-VV IBDV-like segment is divided into two subgroups. The first includes attenuated strains, classical virulent and antigenic variants, and 4 isolates under study: IBDVRF07-2019, IBDVRF04-2014, IBDVRF10-2019, IBDVRF01-2017. The second subgroup consists of 4 isolates: IBDVRF02-2014, IBDVRF02-2016, IBDVRF01-2019, IBDVRF06-2019, detected on one poultry farm in the Novgorod Oblast and three isolates: IBDVRF02-2017, IBDVRF06-2015, IBDVRF02-2019, detected in Chuvashia, Yaroslavl and Novgorod Oblasts. The same subgroup includes isolates 593 Russia and 624 Russia, detected in the RF. The origin of the IBDV isolates included in this endemic subgroup is interesting, since it includes isolates found exclusively in the territory of the Russian Federation. The comparison with the published VP1 sequences showed that the difference between IBDV nucleotide sequences of the VP1 gene and other isolates and strains was 6%. All isolates in this subgroup have NEG marker triplet.

Table 3
Difference between the amino acid sequences in the hypervariable VP2 region and VP1 marker sequence

Strain/isolate	Genogroup	VP2													VP1
		212	213	220	222	242	254	256	269	280	294	299	317	324	145-146-147
228E	1	D	D	Y	S	V	G	I	T	N	L	N	S	Q	NEG
D78	1	D	D	Y	P	V	G	V	T	N	L	N	S	Q	NEG
52-70	1	D	D	Y	P	I	G	V	T	N	L	N	S	Q	NEG
STC	1	D	D	Y	P	V	G	V	T	N	L	N	S	Q	NED
Variant E	2	D	N	Y	T	V	S	V	T	N	L	N	S	E	NEG
UK661	3	D	D	Y	A	I	G	I	T	N	I	S	S	Q	TDN
IBDVRF02-2017	3-1	D	D	Y	A	I	G	I	T	N	I	S	S	Q	NEG
IBDVRF06-2015	3-2	N	D	Y	T	I	D	I	T	N	I	S	S	Q	NEG
IBDVRF02-2016	3-3	N	D	Y	A	I	D	I	T	N	I	S	S	Q	NEG
IBDVRF04-2014	3-3	N	D	Y	A	I	D	I	T	N	I	S	S	Q	NEG
MG4	4	–	D	Y	S	V	S	V	T	T	L	N	S	Q	–
Mexico04M101	5	–	N	Y	T	V	N	V	T	N	L	N	K	Q	–
ITA-02	6	–	-	H	Q	V	S	K	S	N	L	S	S	Q	–
002-73	7	D	D	Y	P	V	G	V	T	N	L	S	S	Q	TES
IBDVRF05-2014	8	D	E	F	V	I	D	I	S	T	I	S	R	P	TDN
IBDVRF03-2017	Variant isolate	D	D	Y	T	I	D	I	T	N	I	S	R	Q	TDS
IBDVRF02-2018	Variant isolate	N	D	Y	A	I	D	I	T	N	I	S	S	Q	TDN

Identification of the reassortant viruses. Ten isolates in which the VP2 gene belongs to VV IBDV (Genogroup 3) are reassortants. In addition, Genogroup 8 isolates are also reassortants. The phylogenetic position determined by the analysis of the VP1 gene indicates that all three isolates are included in the VV IBDV group. Two variant isolates have VP1, also related to VV IBDV. Thus, 15 out of 28 tested VV IBDV isolates are reassortants.

Among isolates that do not belong to VV IBDV in terms of the structure of the VP1 gene, only the isolate IBDVRF07-2019 (Genogroup 1) is not a reassortant and has 99.36% homology with the vaccine strain D78 in both genes.

Isolate IBDVRF04-2014 (subgroup 3-3) has 100% homology with the vaccine strain 228E in the VP1 gene segment. The production of such a reassortant virus can be explained by the use of a vaccine based on the 228E strain.

An unusual group of reassortants was detected on the poultry farm of the Novgorod Oblast during 6 years. According to the structure of the VP2 gene segment, they are closely related (have 99% homology) and belong to subgroup 3-3. According to the structure of the VP1 gene, with the exception of IBDVRF01-2017, they are included in the endemic subgroup. The VP1 gene segment of the IBDVRF01-2017 isolate from the Novgorod Oblast has a high degree of homology with the STC strain. The origin of segment B of this isolate from a virus of a different genetic subgroup indicates that on this poultry farm there is a heterogeneous viral population, including IBDV from different genetic groups.

It has been shown that certain IBDV genetic forms can circulate on poultry farms for a long time. On the other hand, there is a change of virus isolates. So, on one of the poultry farms in the Kursk oblast, variant isolates IBDVRF03-2017, IBDVRF02-2018 were identified for 2 years, differing from each other in the nucleotide sequence of the hypervariable region of the VP2 gene by 4.7%. According to the structure of the VP1 gene segment, both isolates belong to VV IBDV and differ from each other by 1.8%.

Two different isolates IBDVRF06-2015 (Genogroup 3-2) and IBDVRF10-2019 (Genogroup 3-1) were detected on the poultry farm of the Yaroslavl Oblast. Both of them are reassortant viruses. The VP1 gene of these isolates belongs to different genetic subgroups: IBDVRF06-2015 belongs to the endemic subgroup, and IBDVRF10-2019 is closely related to the STC strain.

Previously, the degree of pathogenic action of three IBDV isolates was studied [29]. The study used the isolate IBDVRF08-2017, which has the VP1 and VP2 genes related to VV IBDV, and two reassortants: IBDVRF06-2019 characterized by VV VP2 and non-HV VP1; IBDVRF03-2017 with variant VP2 and VV VP1. It was found that in relation to the reference strain 52-70, the isolate IBDVRF08-17 (80.5%) had the highest virulence score. The least virulent isolates were IBDVRF06-19 (44.3%) and IBDVRF03-17 (43.9%).

Thus, the isolate, both genomic segments of which belonged to VV IBDV, showed the highest virulence. While reassortant viruses with only one segment related to VV IBDV showed a lower degree of pathogenicity.

CONCLUSION

Based on the analysis of the hypervariable region of the VP2 gene, the majority (22 out of 28) of the studied IBDV isolates belong to Genogroup 3 (VV IBDV). Three isolates detected in geographically close regions form a new Genogroup 8. Two isolates found on the poultry farm of the Kursk Oblast in 2017 and 2018 are variant. One isolate is a derivative of the D78 vaccine strain.

Only some of the isolates of Group 3 have VP1, which belongs to the VV IBDV. On the other hand, variant isolates and isolates of the new Genogroup 8 have VP1 characteristic of VV IBDV. Of the 28 isolates studied, 15 are reassortants. Among the reassortants, various combinations of genomic segments were identified (segment B – segment A): VV – endemic, VV – classical virulent, VV – attenuated strains, variant – VV, Genogroup 8 – VV. Identification of the diversity of combinations of genomic segments of IBDV indicates that a heterogeneous viral population circulates on poultry farms.

The study of the degree of pathogenicity of IBDV isolates showed that reassortant viruses are less pathogenic than VV IBDV.

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