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Heterogeneity of avian infectious bronchitis virus population

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SUMMARY
Avian infectious bronchitis is one of the most common viral infections causing enormous economic losses in the global poultry industry. Due to the lack of mechanisms to correct errors during genome replication, the virus can quickly mutate and generate new strains. This is facilitated by widespread use of live vaccines, simultaneous circulation of field viruses belonging to different serotypes in one flock and rapid spread of the virus. Previous studies of avian infectious bronchitis virus strains and isolates identified in the Russian Federation poultry farms showed that 50% of samples tested positive for the 4-91, D274, H-120, Ma5 vaccine strains, and the other half of samples tested positive for the field viruses belonging to eight GI genetic lineages, while the G1-19 (QX) lineage was dominant. The paper presents identification and genotyping results of the avian infectious bronchitis virus in one of the poultry farms in the Saratov Oblast (the Russian Federation) in 2018–2019. The samples of internal organs and blood, as well as oropharyngeal and cloacal swabs were taken from chicks and layers of different ages in the parent and replacement flocks. The vaccine strain, GI-19 field isolates and variant isolates that do not belong to any of the known genetic lineages were detected. Analysis of test results within a two-year period showed that it is important to study samples taken from birds of different ages. The virus undergoes modification and adaptation inducing new genetic forms by infecting several poultry generations, due to which the heterogeneity of the virus population is observed not only in the poultry farm as a whole or in a separate department, but also within one organism. The identified isolates showed tropism for the tissues of intestine, reproductive organs, and, in rare cases, trachea and lungs. The data obtained indicate that, despite the vaccination used, a genetically diverse population of the infectious bronchitis virus circulates in the poultry farm, while the infection may not manifest itself at an early age, but may affect the flock productivity in the future due to pathological changes in the reproductive organs of laying chickens.

Key words: avian infectious bronchitis virus, genetic analysis, genetic lineage, virus population heterogeneity.

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Гетерогенность вирусной популяции при инфекционном бронхите кур

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РЕЗЮМЕ

Инфекционный бронхит кур является одной из наиболее распространенных вирусных инфекций, наносящих огромный экономический ущерб птицеводству во всем мире. По причине отсутствия механизмов коррекции во время репликации генома вирус может быстро мутировать и генерировать новые штаммы. Этому способствует широкое использование живых вакцин, одновременная циркуляция полевых вирусов, относящихся к разным серотипам в одном стаде, и быстрое распространение вируса. Проведенные ранее исследования выявленных на птицефабриках Российской Федерации штаммов и изолятов вируса инфекционного бронхита кур показали, что 50% положительных проб относятся к вакцинным штаммам 4-91, D274, H-120, Ma5, вторая половина положительных проб представлена полевыми вирусами, которые относятся к 8 генетическим линиям генотипа GI, при этом доминирующей является линия G1-19 (QX). В данной работе представлены результаты по выявлению и генотипированию вируса инфекционного бронхита кур на одной из птицефабрик Саратовской области Российской Федерации в 2018–2019 гг. Внутренние органы, ротоглоточные и клоакальные смывы, кровь отбирали от цыплят и кур-несушек разных возрастов из родительского стада и стада ремонтного молодняка. Выявлены: вакцинный штамм, полевые изоляты генетической линии GI-19 и вариантные изоляты, не относящиеся ни к одной из известных генетических линий. Анализ результатов исследований за двухлетний период показал, что важно исследовать пробы, взятые от птиц разного возраста. Инфицируя несколько поколений птиц, вирус изменяется и приспосабливается, порождая новые генетические формы, благодаря чему наблюдается гетерогенность вирусной популяции не только на птицефабрике в целом или в отдельном цехе, но и в одном организме. Выявленные изоляты обладали тропизмом к тканям кишечника, репродуктивных органов и, в единичных случаях, трахеи и легких. Полученные данные свидетельствуют о том, что на птицефабрике, несмотря на применяемую вакцинацию, циркулирует генетически разнородная популяция вируса инфекционного бронхита, при этом инфекция может не проявляться в раннем возрасте, но может повлиять на продуктивность стада в дальнейшем за счет патологических изменений органов репродукции кур-несушек.

Ключевые слова: вирус инфекционного бронхита кур, генетический анализ, генетическая линия, гетерогенность вирусной популяции.

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INTRODUCTION

Avian infectious bronchitis (IB) is one of the most important viral infections causing significant economic losses in the global poultry industry. The causative agent of the disease is an RNA-containing virus belonging to order *Nidovirales*, family *Coronaviridae*, genus *Coronavirus*, which can quickly mutate due to the lack of correction mechanisms during genome replication, i.e. it is able to generate new viral strains.

IB prevention, along with appropriate biosafety measures, is based on routine vaccination. However, this approach is hindered by the high genetic diversity of the virus, leading to the constant emergence of new variants against which cross-protection may be absent. The protection level against a particular variant can be achieved by using either one vaccine based on a strain of the same virus genotype (homologous vaccination), or several vaccines based on different lineages to expand the scope of protection (heterologous vaccination) [1, 2]. For this purpose, various vaccines and immunization schedules are implemented. However, there are still difficulties in selecting attenuated heterologous vaccine virus strains that would provide effective ways to protect poultry from the disease. Despite its importance for disease control, the widespread use of IB vaccines has some significant drawbacks. Live attenuated vaccine strains can enter non-vaccinated herds, restore virulence and also participate in natural recombination. In addition, their application complicates the IB diagnosis, since many detectable field viruses are closely related to vaccine strains.

The analysis of IB virus strains and isolates identified in poultry farms of the Russian Federation showed that approximately 50% of positive samples belong to vaccine strains 4-91, D274, H-120, and Ma5. The second half of the positive samples is represented by the IB field viruses, which belong to 8 genetic lineages of the GI genotype: GI-1 (Mass), GI-12 (D274), GI-13 (793B), GI-14 (B1648), GI-16 (Q1), GI-19 (QX), GI-22, GI-23 (Variant-2). In addition, isolates that are natural recombinants and variant virus isolates that do not belong to any of the known genotypes were detected in poultry farms of the Russian Federation. The dominant group is the genetic lineage GI-19 (QX) [3].

The IB clinical manifestations depend on a number of factors, including virulence and tropism of the virus. The port of the IB virus entry is the respiratory tract, then it spreads systemically, affecting epithelial cells in many tissues. The severity of clinical signs depends on the virus strain and the poultry keeping conditions, such as the microclimate in the poultry house, dust, stocking density, age and type of birds, its immune status (vaccination, immune suppression, presence of maternal antibodies), presence of concomitant infections that are also important factors.

The IB mortality is usually very low but it may increase after secondary bacterial infections [4].

Field isolates of the GI-19 (QX) genetic lineage have tropism for epithelial cells of almost all organ systems, thereby inducing all possible syndromes of avian infectious bronchitis. Researchers describe viruses of this genetic lineage as respiratory, nephropathogenic, affecting reproductive organs, and there are also data indicating that some field strains damage the intestinal tract [5, 6]. Most of the IB virus field isolates were isolated and described during outbreaks with the acute course of the disease. However, there are known cases of the detection of the IB GI-19 genetic lineage virus with asymptomatic infection or with mild respiratory disorders [7].

The aim of this research was to study biological material from poultry of various ages kept in different units of one individual poultry farm for the presence of the IB virus genome, followed by phylogenetic analysis of the obtained nucleotide sequences of the S1 gene fragment.

MATERIALS AND METHODS

Samples of poultry biological material were tested using reverse transcription polymerase chain reaction (RT-PCR) and real-time RT-PCR according to methodical instructions [8, 9].

Comparative analysis of the S1 gene fragment of approximately 500 nucleotide bases (position 112–653 n.b. of S gene of H120 strain) was performed for IB virus genotyping. Nucleotide sequences of prototype strains proposed by V. Valastro et al. were used for the analysis [10].

The nucleotide sequences were determined according to Sanger method using fluorescence labeled chain-terminating nucleotides involving ABI Prism 3130 automated sequencer (Applied Biosystems, USA) according to the manufacturer's instructions.

The obtained nucleotide sequences were compared with IB virus sequences deposited in the international database NCBI (http://www.ncbi.nlm.nih.gov) using BioEdit software, version 7.0.5.3.

IB specific antibodies were detected in blood sera using the FGBI "ARRIAH" ELISA test kit for determining antibodies to avian infectious bronchitis virus when testing sera in single dilution in accordance with the instructions. The test results were recorded using Tecan spectrophotometer plate reader (Austria) at a wavelength of 405 nm using the SINKO-IFA software. The test was considered positive if the antibody titer was 725 or higher.

RESULTS AND DISCUSSION

Live poultry (9 replacement chickens and 12 layers of the parent stock) from one of the poultry farms in the Saratov Oblast were received and tested for the presence of IB virus genetic material in the FGBI "ARRIAH" (Table 1).

All tests in animals were carried out in accordance with the requirements of Directive 2010/63/EU of the European Parliament and of the Council of September 22, 2010 on the protection of animals used for scientific purposes.

No abnormalities were detected in live chickens at 19–290 days of age, received in December 2018, which were visually examined and subjected to autopsy. Multiple cysts of reproductive organs with a watery liquid of more than 100 ml were observed during autopsy of chickens at 361 days of age, received in October 2019.

Oropharyngeal and cloacal swabs, as well as samples of tissues of internal organs (trachea, lungs, kidneys, intestines, reproductive organs) were collected from each chicken for PCR testing. At the first stage the tissue samples of the internal organs, pooled within each group (i.e. 8 samples) were tested (Table 1). Pooled sample No. 1 from 19-day-old chicks from Unit 4 was negative in RT-PCR and real-time RT-PCR, despite the fact that all chicks were vaccinated with a bivalent vaccine based on strains H120 and D274 at two days of age and with the vaccine based on strain 4–91 at 12–14-days of age according to the manufacturer's instructions. The IB virus genome was detected in other pooled samples (No. 2–8) (Table 2). Similar results were obtained inblood sera tests using enzyme-linked immunosorbent assay (ELISA), when IB virus-specific antibodies were detected in poultry of all groups except for Group 1 (Table 3).

The 4-91 IBV45-18 vaccine derivative was detected in sample No. 4 (the nucleotide sequence of the S1 gene fragment was 99% homologous with the vaccine strain and differed from it by 4 bp). The data in the scientific literature indicate that the passaging of vaccine virus strains in live poultry and chicken embryonated eggs sometimes leads to single nucleotide and amino acid substitutions. It is interesting to note that one and the same vaccine virus

Table 1
Characteristics of poultry received for testing

Таблица 1
Характеристика поступившей для исследования птицы

Date of Submission	Group No.	Poultry	Unit No.	Age, days
12.2018	1	Replacement chickens (3 birds from each unit)	4	19
	2		7	60
	3		1	124
	4	Laying chickens of the Ross-PM3-cross parental stock (2 birds from each unit)	21	192
	5		7	228
	6		15	290
10.2019	7	Laying chickens of the Ross-PM3-cross parental stock (3 birds from each unit)	1	361
	8		18	361

Table 2
Genotyping results of avian infectious bronchitis virus

Таблица 2
Результаты генотипирования вируса инфекционного бронхита кур

Date	Group No.	Poultry age, days	Genetic relation	Isolate name
12.2018	2	60	GI-19	IBV42-18
	3	124	GI-19	IBV42-18 IBV43-18
	4	192	GI-13, 4-91 vaccine strain derivative	IBV45-18
	5	228	Variant isolate	IBV44-18
	6	290	GI-19	IBV43-18
10.2019	7	361	Variant isolate	IBV43-19
	8	361	GI-19	IBV44-19

strain used for production of different vaccines batches by one or more manufacturers mutates differently after passaging. These differences may indicate different sources of the original virus strain obtained for the production of this vaccine, as well as the methods and number of the virus passages carried out for its attenuation [11].

The following field viruses were detected in the other remaining positive samples: three isolates of the S1 gene fragment of the GI-19 genetic lineage (IBV42-18, IBV43-18, IBV44-19) and two variant isolates with a unique primary structure (IBV44-18, IBV43-19), not related to any genetic lineage (Table 2, Fig. 1).

The phylogenetic analysis showed that IBV44-19 isolate of GI-19 lineage, identified in 2019, formed a separate branch and differed in amino acid composition from isolates IBV42-18 and IBV43-18 identified in 2018, by 7.5 and 10.3% respectively. Whereas the isolates IBV42-18 and IBV43-18 differed only by 2.7% (Fig. 2) and were found in birds at 60, 124 and 290 days of age, and a mixture of these two isolates was detected in Group 3 (at 124 days of age).

The IBV variant isolates IBV44-18 and IBV43-19 having a unique structure and not related to any genetic lineage were identified in Samples No. 5 and 7. These isolates had 13.7% differences in amino acid composition. Earlier, in March 2018, the other two similar variant isolates (IBV08-18 and IBV09-18) were identified in the material from laying hens at the age of 359 and 285 days kept in different units on this poultry farm. These isolates differed from isolates IBV44-18 and IBV43-19 by 10.3% in amino acid composition, which indicates their different origin (Fig. 1, 3). Variant isolates result from the accumulation of mutations (point substitutions, deletions, insertions, and recombinations) that occur due to IB virus replication errors. A variant isolate may appear non-viable, or it may circulate for a long time on the farm and continue changing.

At the next stage of research process the virus tropism was determined. For this purpose, oropharyngeal, cloacal swabs and samples of tissues of internal organs were taken from each bird and then individually tested using real-time RT-PCR (Table 3). The IB virus isolate of the genetic lineage GI-19 (QX) IBV42-18 was recovered in the pooled sample from three 60-day-old chickens using RT-PCR and sequencing analysis. The samples of intestines and testes from two chicks were positive in real-time RT-PCR.

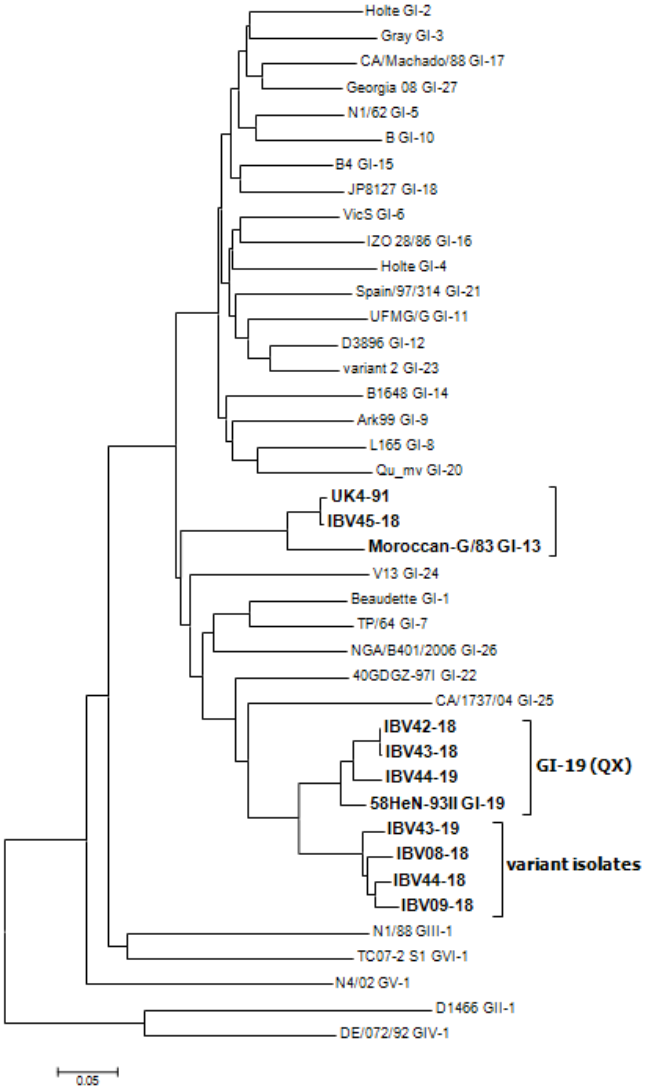

Fig. 1. Phylogenetic relationships based on analysis of the S1 gene fragment of IB virus isolates identified in a poultry farm in Saratov Oblast in 2018–2019

Рис. 1. Филогенетические связи на основе анализа фрагмента гена S1 изолятов вируса ИБК, выявленных на птицефабрике Саратовской области в 2018–2019 гг.

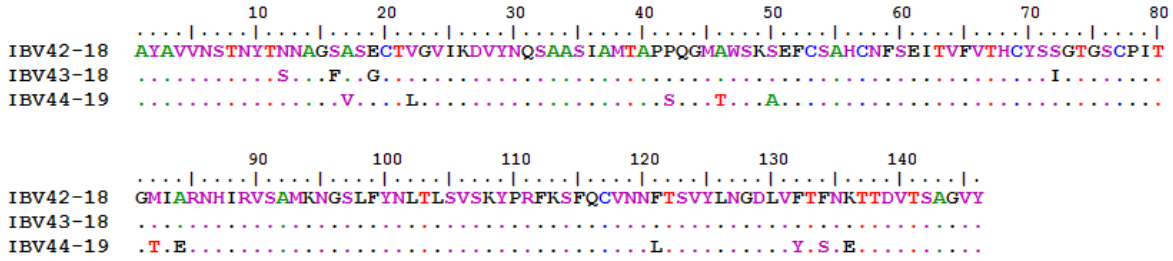

Fig. 2.Comparison of amino acid sequences of identified GI-19 isolates

Рис. 2. Сравнение аминокислотных последовательностей выявленных изолятов генетической линии GI-19

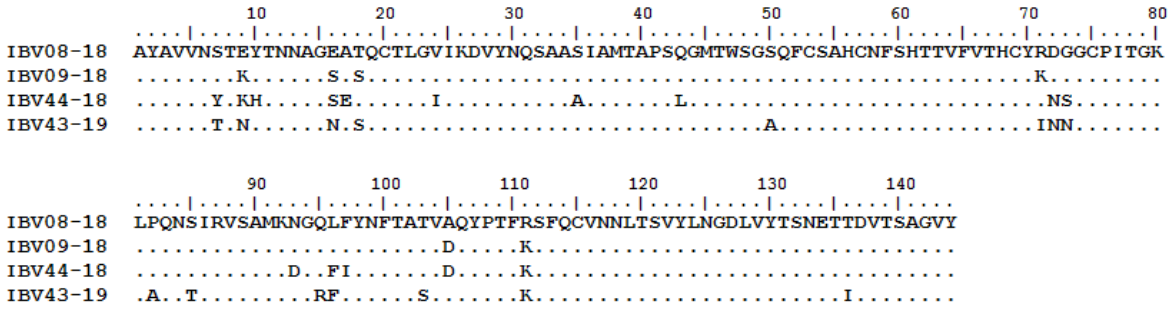

Fig. 3. Comparison of amino acid sequences of identified variant isolates

Рис. 3. Сравнение аминокислотных последовательностей выявленных вариантных изолятов

A combination of field viruses IBV42-18 and IBV43-18 of the IB virus genetic lineage GI-19 (QX) was recovered in Group 3 with replacement chicks (124 days of age) using RT-PCR and sequencing analysis. The samples taken from each organ and the pooled tissue sample from one of the three chicks tested negative in real-time RT-PCR. The IB virus genome was detected in the biological material of trachea and lungs in two other chicks of this group, as compared with other age groups, where only samples of intestinal tissues and reproductive organs were positive. Sequencing of the fragment of the S1 gene of the virus detected in samples obtained from the tissues of the trachea and intestines of chick No. 8 was carried out. It was found that the field virus IBV42-18 persisted in the intestine, and the vaccine strain 4-91 was detected in the trachea of the same chick.

Vaccine strain 4-91 (IBV45-18), two variant isolates (IBV44-18, IBV43-19) and two field virus (IBV43-18, IBV44-19) of the IB virus genetic lineage GI-19 (QX) were recovered in samples of tissues of internal organs from poultry of the parent flock of the ages of 192, 228, 290 and 361 days using RT-PCR and sequencing methods. The samples of intestinal tissues, reproductive organs, and one cloacal swab were positive for IB virus in real-time RT-PCR (Table 3).

An analysis of study results obtained at one poultry farm over a two-year period showed that the detection of IB virus using molecular methods depended on the type of sampling, the number of samples, the poultry age, tested organs etc. While infectious bronchitis causes reproductive and nephropathogenic syndromes, the clinical manifestations are often observed later, many months after infection, and the virus itself may no longer be found in the tissues of the bird, so it is important to examine samples from birds of different ages. On the other hand, the IB virus can persist for a long time in the environment. By infecting several poultry generations, the virus changes and adapts, causing new genetic forms, due to which the heterogeneity of the viral population is observed not only in the poultry farm as a whole or in a separate unit, but also in one organism. The virus can be represented via a set of virions containing both slightly modified but closely related genomes and genomes belonging to different genetic lineages even within one bird, contributing to the rapid adaptation and survival of the virus in the host organism. In addition, the diversity of the virus in one poultry farm, in particular, the appearance of new isolates with a high percentage of amino acid differences (8–10% and higher), indicates an external route of the virus introduction.

CONCLUSION

Phylogenetic analysis of the nucleotide sequences of the avian infectious bronchitis virus isolates recovered in one of the poultry farms in the Saratov Oblast of the Russian Federation in 2018–2019 was carried out. The heterogeneity of the viral population was shown in the layer parent flock and the replacement flock, where, besides the vaccine strain used, three field viruses of the GI-19 genetic lineage and two variant isolates not belonging to any of the known genetic lineages were identified. The data obtained indicate that, despite the vaccination implemented, a genetically heterogeneous IB virus population is circulating in the poultry farm, and it may not manifest itself at an early age, but may affect flock productivity due to pathological changes in layer reproductive organs.

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Table 3
Test results for tissue samples of internal organs from birds of different ages for presence of avian infectious bronchitis virus

Таблица 3
Результаты исследований проб тканей внутренних органов птиц разных возрастов на наличие вируса ИБК

Poultry number and age			Antibody titer	Real-time RT-PCR results, Ct							
				Organ pooled sample	Trachea	Lungs	Kidney	Intestine	Oviduct/ testes*	Oropharyngeal swabs	Cloacal swabs
1	Replacement stock	19	negative	negative	not tested	not tested	not tested	not tested	not tested	negative	negative
2			negative	negative	not tested	not tested	not tested	not tested	not tested	negative	negative
3			negative	negative	not tested	not tested	not tested	not tested	not tested	negative	negative
4		60	**3,220**	**33.9**	negative	negative	negative	**32.0**	negative	negative	negative
5			equivocal	negative	negative	negative	negative	negative	negative	negative	negative
6			**2,474**	**35.7**	negative	negative	negative	**36.6**	**35.0***	negative	negative
7		124	**3,600**	**33.7**	**35.6**	**39.8**	negative	**30.3**	negative	negative	negative
8			**5,012**	**34.7**	**38.5**	negative	negative	**34.7**	negative	negative	negative
9			**3,373**	negative	negative	negative	negative	negative	negative	negative	negative
10	Parent stock	192	not tested	**34.5**	negative	negative	negative	**29.9**	negative	negative	negative
11			**7,542**	**34.4**	negative	negative	negative	**31.8**	**38.5**	negative	negative
12		228	**4,925**	**34.4**	negative	negative	negative	**32.2**	negative	negative	negative
13			**5,857**	**35.2**	negative	negative	negative	**32.3**	**36.7**	negative	**38.4**
14		290	**3,341**	**33.8**	negative	negative	negative	**30.5**	**39.7**	negative	negative
15			**2,602**	negative	negative	negative	negative	negative	negative	negative	negative
16		361	**2,894**	not tested	negative	negative	negative	negative	negative	negative	negative
17			**5,454**	**37.9**	negative	negative	negative	**37.6**	negative	negative	negative
18			**3,828**	not tested	negative	negative	negative	negative	negative	negative	negative
19			**7,916**	**36.2**	negative	negative	negative	**34.1**	negative	negative	negative
20			**4,609**	not tested	negative	negative	negative	negative	negative	negative	negative
21			**2,931**	not tested	negative	negative	negative	negative	negative	negative	negative

negative – negative result;
equivocal – equivocal result;
not tested – not subjected to testing.

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