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# INFLUENZA D VIRUSES - PATHOGENS FORMING A NEW GENUS IN THE ORTHOMYXOVIRIDAE FAMILY

**Abstract.** Influenza pathogens belong to the Orthomyxoviridae family and are divided into genera: Influenzavirus A, B, C, D, as well as Quaranjavirus, Thogotovirus, and Isavirus. For the first time, the influenza D virus was isolated from swine nasal swabs in 2011 in the United States, and its widespread distribution among cattle in France, China, Italy, Ireland, Japan, and several African countries, as well as its ability to infect ferrets, guinea pigs, is further shown. Antibodies to influenza D virus are found in the blood serum of horses, sheep, goats, and in people who have been in contact with cattle. The RNA genome of the influenza D virus is represented by seven fragments responsible for the synthesis of nine proteins. The longest three segments encode for polymerases PB2, PB1, and P3; the fourth and fifth segments encode for hemagglutinin-esterase fusion protein – HEF and nucleoprotein – NP, respectively. The sixth fragment is involved in the synthesis of membrane polypeptides DM1 and DM2, which, in accordance, lines the viral membrane from the inside and performs the function of proton channels. The seventh segment encodes the non-structural protein NS1 and the nuclear export protein NEP; NS1 helps to neutralize cellular interferon and NEP mediates the nuclear export of ribonucleoprotein. Three phylogenetic lines of the influenza virus D are described – D/OK, D/660, and D/Japan, which must be taken into account when preparing vaccines. It is concluded that from its epidemiological, pathological and biological characteristics, the potential ability to cause disease in humans and be transmitted from person to person, new, more in-depth studies are required using ecological-virological and molecular genetic methods.

**Key words:** virus, influenza D, genome, variability, cattle, phylogenesis, clade, HEF fusion protein, serology.

**Characteristics of the Orthomyxoviridae family.** Influenza occupies one of the first places among infectious diseases in terms of the number of species involved in the infectious process and is characterized by global distribution and high economic and social significance [1].

All currently known influenza pathogens belong to the family *Orthomyxoviridae* and are divided into genera: *Influenzavirus A, B, C, D*, as well as *Quaranjavirus, Thogotovirus* and *Isavirus*; the last two infect rabbit-like mammals and salmon fish [2,3,4]. Representatives of *Quaranjavirus* found among both invertebrates (ticks) and vertebrates (water birds) hosts [5].

Influenza A viruses are widespread in the environment and infect humans, mammals and birds. The global spread of influenza A pathogens is due to unique variability, which is based on point mutations and recombinations of eight segments of the genome. The classification of influenza A viruses is determined by a combination of the known subtypes of surface antigens of hemagglutinin (HA) and neuraminidase

(NA) – H1N1, H3N2, H5N1, H7N7, etc. [6]. In Kazakhstan, as a result of long-term studies of influenza A virus ecology, eight different subtypes of this pathogen were identified [7,8,9,10,11,12,13].

The causative agents of influenza A along with influenza B viruses, cause annual epidemics, accompanied by 3-5 million cases of severe morbidity and about 300,000 deaths, with a mortality rate reaching 16 % and 10 %, respectively [14,15].

Modern epidemic influenza B viruses are phylogenetically and antigenically divided into two lines - Yamagata-like and Victoria-like, which are easily differentiable in the hemagglutination inhibition assay (HI) [16]. The natural host of influenza B virus is human but sporadic infections of pheasants, horses, and dogs have been reported in 1960-1980 [17,18,19]. Further, this virus was isolated from a seal *(Phoca vitulina)* in the Netherlands in 1999, the B/Seal/Netherlands/1/99 [20]. Antibodies to influenza B virus were detected in pig sera in China in 2015, their sensitivity to experimental infection was also shown [21].

Representatives of the genus C predominantly infect the children's contingent and differ significantly in structural characteristics from A and B viruses. They possess an RNA genome consisting of seven fragments, one of which encodes hemagglutinin-esterase (HEF) synthesis, which combines the functions of HA and NA in the virion. Another feature of the influenza C virus (ICV) is the ability to infect pigs, which was first demonstrated in China in 1982 [22], in addition to this, antibodies to it were detected in 2015 in the horse sera of in the USA [23].

The Orthomyxoviridae family is represented by the so-called newly emerging infectious diseases, the number of which is growing in the world, and many of them pose a serious threat to wildlife, domestic animals, and public health [24,25].

In April 2011, B.M. Hause et al. [26] in Oklahoma (USA), during the virological study of nasal swabs from pigs, isolated an influenza virus with an RNA genome consisting of 7 fragments that are 50% structurally similar in amino acid sequence to ICV. Originally designated as C/Oklahoma/1334/2011, it diverged from ICV in phylogenetic analysis to the same extent as influenza A viruses differ from representatives of genus B. No cross-reactivity was observed between the new isolate and ICV in HI. Antibodies to this strain were found in 9.5 % and 1.3 % of pig sera and staff, respectively; in addition, it was transmitted to healthy animals through direct contact and was able to infect pigs, ferrets, and guinea pigs in the experiment. Cell tropism of the new virus was superior to that of ICV, while conservative enzymatic and divergent receptor-binding sites were detected in the HEF fusion protein. In experiments with two human ICVs and two newly isolated swine and cattle viruses, no reassortment and production of viable generation were found. In experiments with specific polyclonal antibodies, cross-recognition was not observed in agar gel immunodiffusion. Based on the data obtained, the isolated virus was assigned to the new genus D [27], which was adopted by the decision of the International Committee on Taxonomy of Viruses (ICTV) in 2018 [28]. Subsequent molecular genetic studies have identified several characteristics of the causative agent of influenza D.

**Structural features of the influenza D virus.** The RNA genome of the influenza D virus (IDV) is represented by seven fragments responsible for the synthesis of 9 proteins. The longest three segments encode polymerases PB2, PB1, and P3, the fourth – protein HEF, the fifth – NP. The sixth fragment is involved in the synthesis of membrane polypeptides DM1 and DM2, one of which underlines the viral membrane from the inside, the other performs the function of proton channels [29]. The seventh segment encodes the non-structural protein NS1, and the nuclear export protein NEP; NS1 helps neutralize cellular interferon, NEP mediates the nuclear export of ribonucleoprotein [30]. IDV, like ICV, uses cellular 9-*O*-acetylated sialic acid as its receptor but exhibits a broader cellular and host tropism [31]. The reason for this lies in the structural features of the molecule of the HEF monomer, consisting of two subunits HEF1 and HEF2, As revealed by comparative X-ray crystallographic study and following the domain nomenclature previously used for ICV, H. Song et al. [31] divided the structure of IDV HEF into three domains: a receptor-binding domain (R), an esterase domain (consisting of subdomains E1, E' and E2) and a fusion domain (consisting of subdomains F1, F2, and F3). The general structure and structural folds of the individual HEF subdomains of both viruses were similar. E domains are the most conserved; the F1 and F2 subdomains are less identical, while the F3 subdomain contains the fusion peptide, which is essential for the viral membrane fusion.

The E domain of IDV HEF, harboring the receptor-destroying enzyme (RDE) activity, has a hydrolase fold that is highly similar to that of ICV HEF. The active-site architecture of the HEF sialate-9-O-acetylesterase is fully conserved in ICV and IDV HEF and has oxyanion hole. The pocket is

extremely conserved not only in IDV HEF and ICV HEF but also in some nidovirus hemagglutinin-esterase (HE) proteins such as bovine coronavirus, porcine torovirus and bovine torovirus HE [32]. Phylogenetic analysis also shows the relationship between HEF and HE to be much closer than the relationship between HEF and HA or hemagglutinin-neuraminidase (HN) proteins, implying common ancestral origins.

**Epidemiology of influenza D.** Following the first report on the isolation of IDV in 2013, L. Ferguson et al. [33] in 2014 recovered 15 IDVs from surveillance of bovine herds in Mississippi belonging to two genetic lines. Based on the serological analysis of "archived" sera, it was shown that the pathogen has been circulating in cattle stock in this region since at least 2014. Active transmission of the virus has been detected in places where newborn, weaned, and comingled calves were maintained. The detection rate of IDV in RT-PCR was 2.4 % of 82 healthy calves, which was significantly higher than in Chinese Shandong – 0.7 % of 453 calves [34], which indicates its possible participation in the etiology of respiratory cattle disease, as an infection with many potential pathogens. Continuing his research, L. Ferguson et al. [29] conducted an experimental intranasal infection of three dairy calves with the influenza virus D/bovine/Mississippi/C00046N/2014 followed by replanting of a seronegative contact calf. Seroconversion and the presence of the virus in the respiratory tract were detected in all animals. The infection of ferrets with nasal discharge from calves has not yielded results. These data are consistent with Koch's postulates and confirm the hypothesis that cattle are a natural reservoir of the virus.

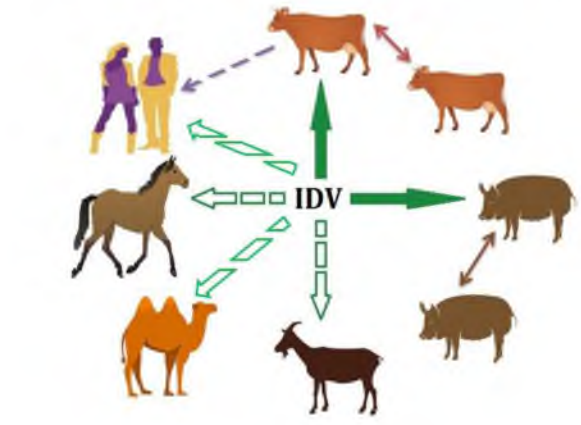
In subsequent years, IDVs were found in cattle in France, China, Italy, and Japan [35,34,36,37]. E. Collin et al. [38] studied 208 biological samples from cattle from 12 US states in real-time PCR and found 10 positives for IDV samples, using passages on cell cultures, they isolated six IDV strains.

O. Flynn et al. [38] examined 320 "archival" samples in the form of nasal swabs taken from cattle in Ireland during 2014–2016. Using RT-PCR on IDV and Sanger sequencing, five partial RNA sequences suitable for phylogenetic analysis were obtained from 18 positive samples.

In Japan, according to a national serological study, the average level of seropositivity in cattle for the influenza virus is 30%, and the values vary depending on the region [37,40]. At the same time, a molecular epidemiological analysis of cattle samples with symptoms of respiratory disease collected from 23 different farms in this country revealed 2.1 % (8/377) influenza D infection [41].

E. Salem et al. [42] conducted a serological search for antibodies to influenza C and D viruses among ruminants and camelids in Africa in 1991–2015, and for this purpose, they collected 2083 blood serum samples from a cattle, pigs, small ruminants, and dromedary camels in Morocco (n=200), Togo (n=540), Côte d'Ivoire (n=203), Benin (n=308) and Kenya (n=1231). The percentage of positive samples from cattle in Morocco was 35 %, Togo – 10.4 %, Côte d'Ivoire – 0 %, Benin – 1.9 %, Kenya – 0 %. Noteworthy is the high percentage (99 %) of positive blood serum from camels in Kenya (287 out of 293), but the serum was not pre-adsorbed by ICV in this test. The results indicated a wide circulation in Africa of IDV, and its significant tropism to all the hosts studied.

H. Nedland et al. [23] examined 364 serum samples from horses of 141 farms in the Midwestern United States and showed that animals are susceptible to two IDV lines represented by D/swine/Oklahoma/1334/2011 (D/OK line) and D/bovine/Oklahoma/660/2013 (line D/660), as 12 % and 11 % of sera, respectively, were seropositive in HI.




The hosts of the influenza D virus. IDV can be transmitted between cattle and also between pigs as indicated by the solid line. Antibody specific for IDV were also detected in sera from small ruminants (sheep and goat), horses, camels and humans, especially in people with cattle exposure, but no virus was isolated (indicated by the broken line), indicating that IDV may be transmitted from cattle to humans (broken line)

M. Quast et al. [43] in a serological analysis of 648 sheep and goat blood serum samples collected in 2014 in the USA and Canada, detected antibodies to IDV in 5.2 % (29/557) of the studied sheep from 13.5 % (17/126) of farms. In turn, 8.8 % (8/91) of goats from 13.3 % (2/15) of the tested farms also contained antibodies against IDV.

IDV was first isolated from pigs and then from cattle. Since cattle seropositivity is much higher than that of pigs, it is believed that it is the main natural reservoir. Currently known IDV hosts are shown in figure. In addition, the ability of D/OK to infect ferrets, guinea pigs, and transmit them to native animals by direct contact is shown [26,44], which allows them to be used as models in the study of IDV.

**Phylogenesis of influenza D. viruses.** With the accumulation of IDV strains of various origins and places of isolation, the possibility of their comparative antigenic and phylogenetic studies appeared. E.A. Collin et al. [38] performed full genome sequencing and phylogenetic analysis of 7 viral RNA segments of six new strains and four previously registered IDVs and revealed two different circulating lines – D/OK and D/660, which often reassorted with each other. Antigenic analysis using representative viruses D/swine/Oklahoma/1334/2011 and D/bovine/Oklahoma/660/2013 and their antisera in the HI showed an approximately 10-fold loss of cross-reactivity. An important finding was that one of the isolates (D/bovine/Texas/3-13/2011) belonged to the D/bovine/Oklahoma/660/2013 cluster, but was characterized by high titers with a serum to the heterologous variant D/swine/Oklahoma/1334/2011. Molecular modeling of the HEF fusion protein of strain D/bovine/Texas/3-13/2011 made it possible to identify the mutation at position 212 responsible for unusual serological test titers. The obtained data indicate the widespread prevalence of at least two genetically different IDV lines in cattle, and also the vital role of lysine (K212) in antigenic recognition of D/swine/Oklahoma/1334/2011-like viruses. IDVs related to D/bovine/Oklahoma/660/2013 clade carried arginine in this position (R212).

Phylogenetic analysis of the HEF sequence of the USA IDV isolates performed by L. Ferguson et al. [26], aligned them into two genetic clusters: viruses D/bovine/Mississippi/C00046N/2014 and D/bovine/Mississippi/C00030P/2014 were genetically close toD/swine/Oklahoma/1334/2011 (D/OK), while D/bovine/Mississippi/C00013N/2014 and D/bovine/Mississippi/C00014N/2014 were genetically related to D/bovine/Oklahoma/660/2013 (D / 660).

O. Flynn et al. [39] found that five IDV strains isolated in Ireland in 2014–2016 were grouped in the same clade together with the virus from Europe D/swine/OK/1334/2011.

S. Murakami et al. [37] using reverse transcription-PCR successfully amplified the complete genome sequence of the first Japanese IDV strain (D/bovine/Ibaraki/7768/2016), sequenced its seven segments and built their phylogenetic trees. The results indicated that it occupies a separate position from strains from other countries, and only the M gene is included in one cluster with isolates from France (D/bovine/France/2986/2012) and China (D/bovine/Shandong/Y127/2014, D/bovine/Shandong/Y217/2014, D/bovine/Shandong/Y125/2014). Later on, in Japan, from a cow with signs of respiratory disease, the D/bovine/Yamagata/10710/2016 strain highly related D/bovine/Ibaraki/7768/2016 according to the HEF gene was isolated, the homology of the nucleotide and amino acid sequences was 99.8 % and 100 %, respectively [45]. H. Mekata et al. [41] performed analysis of 46 nasal swabs from cattle with signs of respiratory disease from 26 different farms in Japan, using the Next Generation Sequencing, the nucleotide sequence of the complete IDV genome was determined, and it was shown that it forms a separate cluster. According to the authors, the virus could develop uniquely over a long period, and its pathogenic properties differ from the strains found in other countries. In 2018 T. Odagiri et al. [46] studied IDV phylogenesis and established the presence of three genetically distinct lines: D/OK, D/660, and D/Japan. In addition, in the cross-linked HI, it was found that representatives of three lines in the composition of the surface protein HEF present both common for all and line-specific antigens.

The most conserved PB1 viral protein gene is often used to evaluate the evolutionary relationships of influenza viruses. S. Su et al. [2] based on the nucleotide sequences of the PB1 genes of influenza A, B, C, and D viruses, constructed a phylogenetic tree to determine the relationship between them. IDV clusters turned out to be the most closely related to ICV, which suggests their common ancestor. Sequence analysis of the PB2, P3, NP, M, and NS genes also confirmed the origin of IDV from human ICV [26]. To study the origin and evolutionary history of IDV, the authors conducted a Bayesian analysis of the HEF ICV gene sequences, indicating 1896 as the average time (t-MRCA) of the most recent common ancestor, which is consistent with previously obtained data [47]. For the HEF genes of ICV and

IDV, the t-MRCA value was 482 A.D. It is shown that two IDV lines – D/OK and D/660 had the last common ancestor (t-MRCA) about 44.6 years ago. The average frequency of substitution for the HEF IDV gene was calculated using Bayesian analysis and amounted to $1.54 \times 10^{-3}$, which exceeds the frequency of ICV [47]. From this point of view, the causative agent of influenza D has great epidemic potential, and in the future, it is necessary to monitor its development constantly.

**Conclusion.** To date, an intensive study of IDV has shown that it infects pigs, cattle, and small ruminants, but the full spectrum of its susceptible hosts remains to be determined. To better understand the causative agent of influenza D from epidemiological, pathological and biological characteristics, in particular, the ability to cause disease in humans and be transmitted from person to person, new, more in-depth studies using molecular genetic tools are required. A significant stock of broad species range of susceptible farm animals needs a study of IDV prevalence in Kazakhstan.

Taken together, the epidemiological and metagenomic data, as well as experimental infection of animals, showed that the influenza virus D is the causative agent of respiratory disease of cattle, and therefore there is the task of appropriate vaccination. Moreover, the antigenic heterogeneity of HEF IDV explains the need to take into account which strains of which line circulate in a given region to prepare the most effective vaccine against this infection.

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## ТҰМАУ D ВИРУСЫ – ORTHOMYXOVIRIDAE ТҰҚЫМДАСЫНДАҒЫ ЖАҢА ТҮР ҚАЛЫПТАСТЫРАТЫН ПАТОГЕНДЕР

**Аңдатпа**. Тұмау қоздырғыштары Orthomyxoviridae тұқымдасына жатады және A, B, C, D тұмауы болып, сондай-ақ Quaranjavirus, Thogotovirus және Isavirus туыстықтарына бөлінеді. D тұмауы вирусы алгаш рет 2011 ж. АҚШ-та шошқаның кеңсірік шайындысынан бөлінін алынды, кейінірек Франция, Қытай, Италия, Ирландия, Жапония, бірқатар Африка елдерінде ірі қара малдыц арасында кең тараганы, сонымен қатар оның күзендер мен теңіз шошқаларына жұгатыны анықталган. D тұмауы вирусына қарсы антиденелер жылқылардың, қой мен ешкілердің және малмен байланыста болган адамдардың қан сарысуынан кездеседі. D тұмауы вирусының РНҚ геномы 9 ақуыз синтезіне жауап беретін жеті фрагменттен тұрады. Ең ұзын үш сегмент PB2, PB1 және P3 полимеразаларын кодтайды, төртіншісі – HEF гемагглютинин-эстераза тұтасу ақуызы, бесінші – NP нуклеопротеині. Алтыншы фрагмент мембрана полипептидтерінің DM1 және DM2 синтезіне қатысады, олардың бірі вирустық мембрананы ішінен қаптап жатады, екіншісі протон арналарының қызметін орындайды. Жетінші сегмент NS1 құрылымдық емес ақуызды және NEP ядролық экспорт ақуызын кодтайды; NS1 жасушалық интерферонды бейтараптандыруга көмектеседі, NEP рибонуклеопротеиннің ядролық экспортын жүзеге асырады. Тұмау D вирусының үш – D/OK, D/660 және D/Japan филогенетикалық желісі сипатталган, олар вакцина дайындау кезінде ескерілуі керек. Оның эпидемиологиялық, патологиялық және биологиялық сипаттамалары тұргысынан, адамдарда ауру тудыруы және адамнан адамга берілуі мүмкін болатын қазіргі замангы экологиялық-вирусологиялық және молекулалық-генетикалық әдістерді қолдана отырып, жаңа, тереңірек зерттеулер қажет деген тұжырым жасалды. Аса консервативті вирустық PB1 ақуыз гені тұмау вирустарының эволюциялық қатынастарын багалау үшін жиі қолданылады. S. Su et al. [2] A, B, C және D вирустарының PB1 генінің нуклеотидтік тізбегі негізінде олардың арасындагы байланысты анықтау үшін филогенетикалық дарақ құрастырылды. IDV кластерлері ICV-мен ең жақын байланыста болды, бұл олардың ата-бабаларының ортақ екенін білдіреді. PB2, P3, NP, M, NS гендерінің тізбегін талдау нәтижесіде, олардың адам ICV-нен IDV шыққанын растады [26]. IDV-ның пайда болуы мен эволюциялық тарихын зерттеу үшін авторлар HEF ICV гендік тізбегіне байесовский талдауын жүргізді, бұл 1896 ж., ортақ туыстастықтың орташа уақыты (t-MRCA) деп көрсетеді, бұл алдыңгы деректермен сәйкес келеді [47]. HEF гендері, ICV және IDV үшін t-MRCA мәні 482 н.т., болды. Екі линия IDV-D/OK және D/660 шамамен 44.6 жыл бұрын соңгы жалпы ата-бабалары (t-MRCA) болганын көрсетеді. HEF IDV гені алмасуының орташа жиілігі Байес анализінің көмегімен есептелді және 1,54×10-3 құрады, бұл ICV жиілігінен асып түсті [47]. Осы тұргыдан алганда, D тұмауының қоздырғышы үлкен эпидемиялық потенциалга ие және заманауи экологиялық-вирусологиялық және молекулалық-генетикалық әдістерді қолдану арқылы болашақта оның дамуын үнемі бақылау қажет.

**Түйін сөздер:** вирус, тұмау D, геном, өзгергіштік, мүйізді ірі қара, филогенез, клайд, тұтасу ақуызы HEF, серология.

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# ВИРУСЫ ГРИППА D – ПАТОГЕНЫ, ОБРАЗУЮЩИЕ НОВЫЙ РОД В СЕМЕЙСТВЕ ORTHOMYXOVIRIDAE

**Аннотация.** Возбудители гриппа относятся к семейству Orthomyxoviridae и разделяются на роды: Influenzavirus A, B, C, D, а также Quaranjavirus, Thogotovirus и Isavirus. Впервые вирус гриппа D выделили из назальных смывов свиней в 2011 г. в США, в дальнейшем показана его широкая распространенность среди крупного рогатого скота во Франции, Китае, Италии, Ирландии, Японии, ряде африканских стран, а также способность инфицировать хорьков, морских свинок. Антитела к вирусу гриппа D обнаружены в сыворотках крови лошадей, овец, коз и у людей, контактировавших с крупным рогатым скотом. РНК-геном вируса гриппа D представлен семью фрагментами, ответственными за синтез 9 белков. Самые длинные три сегмента кодируют полимеразы PB2, PB1 и P3, четвертый – белок слияния гемагглютинин-эстеразу HEF, пятый – нуклеопротеин NP. Шестой фрагмент участвует в синтезе мембранных полипептидов DM1 и DM2, один из которых выстилает вирусную мембрану изнутри, другой осуществляет функцию протонных каналов. Седьмой сегмент кодирует неструктурный белок NS1 и белок ядерного экспорта NEP; NS1 способствует нейтрализации клеточного интерферона, NEP опосредует ядерный экспорт рибонуклеопротеина. Описаны три филогенетические линии вируса гриппа D – D/OK, D/660 и D/Japan, что необходимо учитывать при приготовлении вакцин. Делается вывод о том, что с точки зрения его эпидемиологических, патологических и биологических характеристик, потенциальной способности вызывать заболевание у людей и передаваться от человека человеку требуются новые, более углубленные исследования. Наиболее консервативный ген вирусного белка PB1 часто используется для оценки эволюционных взаимоотношений вирусов гриппа. S.. Su et al. [2] на основе нуклеотидных последовательностей генов PB1 вирусов гриппа A, B, C и D построили филогенетическое древо для определения взаимосвязи между ними. Кластеры IDV оказались наиболее тесно связаны с ICV, что предполагает их общего предка. Анализ последовательности генов PB2, P3, NP, M и NS также подтвердил происхождение IDV из ICV человека [26]. Чтобы изучить происхождение и эволюционную историю IDV, авторы провели байесовский анализ последовательностей генов HEF ICV, указав 1896 год как среднее время (t-MRCA) самого последнего общего предка, что согласуется с ранее полученными данными [47]. Для генов HEF ICV и IDV значение t-MRCA было 482 н.э. Показано, что две линии IDV – D/OK и D/660 имели последнего общего предка (t-MRCA) около 44,6 лет назад. Средняя частота замещения гена HEF IDV была рассчитана с использованием байесовского анализа и составила 1,54 × 10-3, что превышает частоту ICV [47]. С этой точки зрения возбудитель гриппа D обладает огромным эпидемическим потенциалом, и в будущем необходимо постоянно следить за его развитием. использованием современных эколого-вирусологических и молекулярно-генетических методов.

**Ключевые слова:** вирус, грипп D, геном, изменчивость, крупный рогатый скот, филогенез, кластер, белок слияния HEF, серология.

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