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MOLECULAR AND BIOLOGICAL PROPERTIES OF PATHOGENIC NEWCASTLE DISEASE VIRUSES ISOLATED IN KAZAKHSTAN

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Abstract

Currently, Newcastle disease (ND) is highly contagious viral infection of birds, characterized by pneumonia, encephalitis, multiple pointed hemorrhages and defeat of internals is spread in various regions of the world. To the present, all isolated ND viruses (*Paramyxoviridae*, *Paramyxovirus*) are divided into two classes, representing the diverse and constantly developing group of viruses. Despite universal vaccination, the disease is difficult to control, and in connection with this the ND causal agent is listed among the most important pathogens. In recent years, studies on the genetic variability of the ND strains in Kazakhstan were not conducted, although many transcontinental migratory routes of wild birds, the main carriers of the pathogens, are crossed exactly here. The present study was conducted to examine the characteristics of the circulation, as well as the isolation and characterization of isolates of Newcastle disease virus that caused the disease of poultry in different regions of Kazakhstan in 2010, 2012 and 2013. Using 10-day-old developing chicken embryos (DCE), we studied virus isolates from dead hens at ND outbreaks in poultry farms and private yards in Almatinskaya, North Kazakhstan and Zhambylskaya provinces of Kazakhstan. The mean death time of embryos (MDT) and intracerebral pathogenicity index (ICPI) were estimated. Viral RNA was isolated and used for PCR. Amplified products were further detected, purified and sequenced. The obtained nucleotide sequences were analyzed using Sequencher v. 4.5 (Gene Codes Corporation, USA). A set of nucleotide sequences from an international database GenBank was used to construct the dendrogram and determine the genotype. Phylogenetic analysis of the sequences was performed using Mega 6.06 and the following parameters: Statistical Method — Neighbor-joining; Test of Phylogeny — Bootstrap method; No. of Bootstrap Replications — 500; Model/Method — Kimura 2-parameter model. Studies have shown that the ND virus causes outbreaks both among vaccinated and non-vaccinated poultry. The ND isolates belong to velogenic strains. All of them had a proteolytic cleavage site ^{110}GGRRQKRF^{117} in the fusion protein, which is characteristic of the V-pathotype. The sequencing and phylogenetic analysis of the *F*-gene showed that the virus from dead birds of those vaccinated at the poultry farm in Almatinskaya Province belongs to VIId genotype, while the isolates from non-vaccinated birds of the private farms in Almatinskaya, Zhambylskaya and North Kazakhstan provinces belong to VIIb genotype. According to the obtained information, despite the geographical distance of outbreaks, the same ND virus genotypes are circulating in the territory of Northern Kazakhstan and in the southern regions of the country. Wide spread of the virus in Kazakhstan requires from veterinary services to develop effective control measures with regard to ND molecular epidemiology.

Keywords: Newcastle disease, strain, pathogenicity index, PCR, sequencing, phylogenetic analysis

Newcastle disease (ND) is a highly contagious poultry viral infection characterized by pneumonia, encephalitis, multiple pointed hemorrhages and failure of internal organs [1]. It was first registered in the island of Java in 1926 [2]. The causative agent (RNA virus belonging to family *Paramyxoviridae*, genus *Paramyxovirus*) was isolated and described as a filterable virus during an outbreak in the city of Newcastle (UK, 1926) [3], and the disease was called Newcastle disease. ND virus was found in 241 species from 27 orders of the *Aves* [4].

All ND viruses isolated to date are divided into two classes representing the diverse and constantly developing groups. Class I viruses are distributed worldwide among wild birds, are low virulent, and are currently divided into nine genotypes [5]. Class II viruses have been divided into 10 genotypes until recently. However, in 2012, D.G. Diel et al. [6] proposed a new genetic classification of ND viruses. The authors divided the class II viruses into 15 genotypes which included 10 previously known and 5 new ones. Later, having examined the viruses isolated in the Dominican Republic and Mexico, S.C. Courtney et al. [7] demonstrated the existence of genotype XVI, and C.J. Snoeck et al. [8] classified the viruses isolated in the West and Central Africa as genotypes XVII and XVIII.

Currently, phylogenetic analysis has been performed for a large number of ND virus strains, and the main genotypes circulating in different parts of the world have been identified, a total of 18 [6, 7]. Group of scientists from the CIS countries have found that all ND virus isolates from poultry and commensal birds in the territory of Russia, Kazakhstan, Kyrgyzstan, and Ukraine from 1993 to 2007, belonged to genotypes VIIa, VIIb, and VIId [9-11]. It has also been shown that in the territory of Russia, class II genotype VI viruses circulate in pigeon populations [12, 13], genotype I viruses circulate in waterfowl populations [13, 14], and genotype VII viruses circulate in poultry [15, 16]. However, despite the studies of the virus in different countries, including the Republic of Kazakhstan, there are still mass poultry diseases followed by high mortality.

In recent years, genetic variability studies in ND strains have not been conducted in Kazakhstan. In this study, we studied the variations of ND virus circulating within the territory of the Republic of Kazakhstan that have been changed in the process of evolution, which was of particular interest as it is here many transcontinental migration ways of wild birds, the main carriers of the infection, intersect.

This work was aimed to study the specificity of circulation, isolation and characterization of Newcastle disease virus isolate that cause poultry disease in different regions of the Republic of Kazakhstan.

Technique. Virus isolates from dead poultry were studied at ND in farms and private yards in Kazakhstan in 2010, 2012, and 2013

10-Day-old developing chicken embryos (DCE) were used as a culture system. Mean death time (MDT) was estimated by dividing the sum of all embryos' death hours caused by the minimal lethal dose, by the number of embryos [17]. Intracerebral pathogenicity index [ICPI] was estimated using the standard method (18).

RNA was isolated using QIAAmp Viral RNA mini kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. For PCR products, a primer pair [19] (Fwd-upper-f1 — TTGCTTATAGTTAGTTCGCCTGTC, Rev-down-f2 — ACCCGTGTATTGCTCTTTGG) and One-step RT-PCR Kit (Qiagen GmbH, Germany) were used.

PCR products were detected in 1 % Tris-acetate buffer supplemented with ethidium bromide in Bio-Rad gel documentation system (Bio-Rad Laboratories Inc., USA). PCR products were purified using QIAquick PCR purification kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. Sequencing of PCR products was performed using BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems Inc., USA) and an automatic 3130xl Genetic Analyzer (Applied Biosystems Inc., USA; Hitachi, Japan)

The obtained nucleotide sequences were analyzed using Sequencher v. 4.5 (Gene Codes Corporation, USA). Mega 6.0 software [20] was used for nucleotide sequence alignment. A set of nucleotide sequences from GenBank was used to construct the dendrogram and determine the genotype. Phyloge-

netic analysis of sequences was performed using Mega 6.06 and the following parameters: Statistical Method — Neighbor-joining; Test of Phylogeny — Bootstrap method; No. of Bootstrap Replications — 500; Model/Method — Kimura 2-parameter model.

Results. Epizootic welfare in poultry farms in the Republic of Kazakhstan is maintained due to intensive poultry vaccination from the first days of life. Vaccination regimes are practiced in many farms to maintain a high titer of post-vaccination antibodies required for poultry immunity to Newcastle disease. But despite all the efforts, the Newcastle disease epizootic outbreaks damage the poultry industry in Kazakhstan

In November 2010, there was a mass death of 30-40-day-old broiler chickens at the Allele Agro poultry farm (Ili district, Almaty region). The farm had experienced a preventive vaccination program, poultry were vaccinated with a live vaccine (Nobilis ND Clone 30, Intervet international B.V., Netherlands). Despite vaccination, over 2,000 birds died within one week. In October 2012, mass poultry death was registered at the private yards of Aksuat settlement (Timiryazevskiy district, North-Kazakhstan region) where more than 900 individuals died. In June 2013, mass poultry death was observed at the private yards of Otar (Korday district, Zhambyl region) and Matybulak (Zhambyl district, Almaty region) settlements as well. Poultry of the private yards have not been vaccinated against ND.

We found that the disease and the death of poultry were caused by ND virus in all cases (Table).

Biological characteristics of Newcastle disease virus isolates from different regions of the Republic of Kazakhstan ($X \pm m$, n = 3, culture on 10-day-old developing chicken embryos)

Isolate (ID GenBank)	Site (year of isolation), host	BA	HA	MDT	ICPI	CS
Chicken/KZ/Almaty/11/2010 (KT719396)	Almaty region (2010), domestic chicken	9.20±0.047	8.06±0.12	56	1.76	^{110}GGRRQKRF^{117}
Chicken/KZ/SKO/12/2012 (KT719397)	North-Kazakhstan region (2012), domestic chicken	8.80±0.080	8.53±0.12	52	1.82	^{110}GGRRQKRF^{117}
Chicken/KZ/Kordai/06/2013 (KT719398)	Zhambyl region (2013), domestic chicken	9.26±0.074	8.20±0.08	56	1.82	^{110}GGRRQKRF^{117}
Chicken/KZ/Almaty/07/2013 (KT719399)	Almaty region (2013), domestic chicken	9.58±0.069	8.73±0.05	56	1.80	^{110}GGRRQKRF^{117}

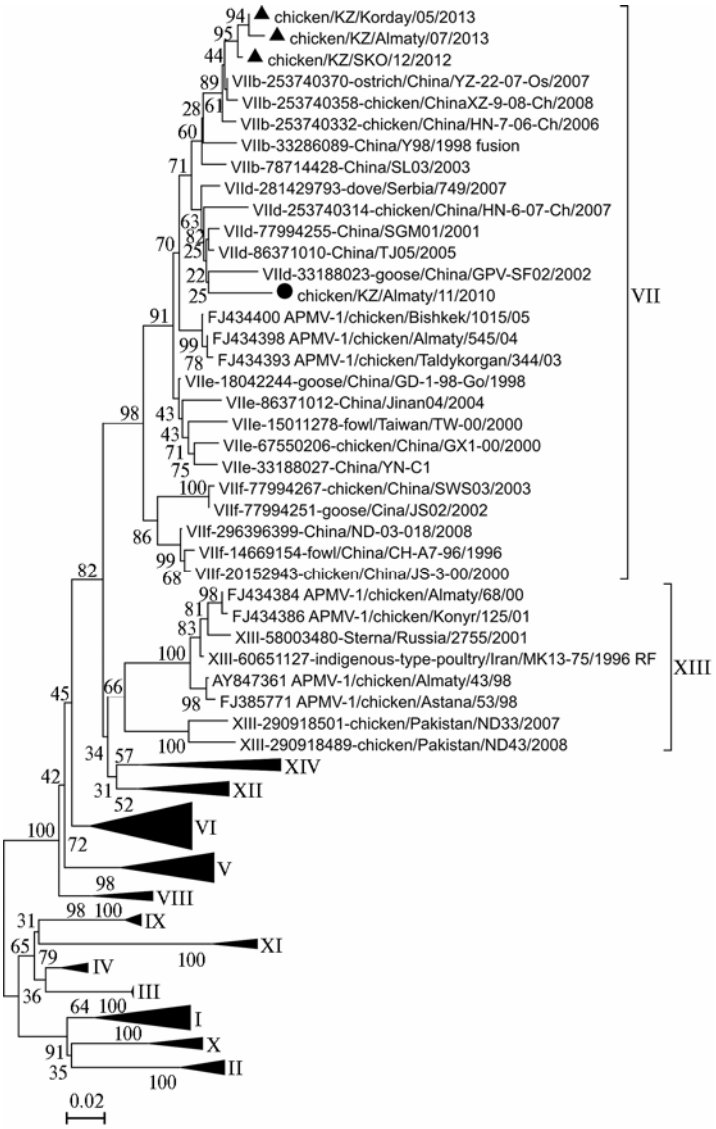
N o t e. BA — biological activity, lg $EID_{50}/см^3$ (embryo infectious dose); HA — hemagglutinating activity, log_2; MDT — mean chicken embryo death time, h; ICPI — intracerebral pathogenicity index; CS — gene *F* product proteolytic cleavage site.

Mean embryo death time for isolated ND was 52-56 hours which corresponds to the velogenic ND virus strains. ICPI was in the range of 1.76-1.82 and confirmed the virulence of ND viruses isolated in Kazakhstan.

Analysis demonstrated the presence of the similar sequence in the proteolytic cleavage site (^{110}GGRRQKRF^{117}) in the fusion protein F, which is characteristic of the velogenic ND V-pathotype [21]. Consequently, the results of genetic studies were consistent with those of biological tests (ICPI and MDT) and confirmed the virulence of ND isolates studied.

An in-depth study of genetic relationships between the viruses isolated in different various areas provided important information on ND molecular epidemiology. It was necessary to find out the genotype belonging of the strains that have caused epizootic Newcastle disease outbreaks in Almaty, Zhambyl, and North Kazakhstan regions. For this purpose, we performed alignment of nucleotide sequences of isolated viruses and the sequences of ND virus available in GenBank, and constructed a phylogenetic tree. All ND viruses isolated in Kazakhstan were close to the ones circulating in Asia and belonged to genotype VII. The VII genotype viruses were first isolated in Taiwan in 1984 [22]. Later, they were found in Europe [23], China [24], Africa [25, 26], and in the CIS

countries — in Russia, Ukraine, Kazakhstan, and Kyrgyzstan [9-11, 27].



Phylogenetic tree of gene *F* which encodes the fusion protein of Newcastle disease virus class II: I-XIV — genotypes; ▲ and ● — isolates from Kazakhstan (VIIb and VIId genotype viruses, respectively; dendrogram constructed using nucleotide sequences deposited in GenBank, Neighbor-joining method, bootstrap = 500).

Using the reference strains proposed by D.G. Diel et al. [6], ND virus Chicken/KZ/Almaty/11/2010 isolate obtained from vaccinated poultry that have died at the poultry farm was assigned to the genotype VIId, and Chicken/KZ/SKO/12/2012, Chicken/KZ/Kordai/06/2013, Chicken/KZ/Almaty/06/2013 isolates from non-vaccinated poultry from private yards in Almaty, Zhambyl, and North Kazakhstan regions were assigned to genotype VIIb.

So, despite the geographical distance of outbreaks, the same genotypes were circulating in the territory of Northern Kazakhstan and in the southern regions of the country. Our data confirm that geographically, any genetic group had its own area, and its distribution may have been due to the migration routes of wild birds. The same opinion is shared by I.S. Korotetskiy et al. [11] who found that the presence of a short migration route between the North Europe

countries and Ukraine resulted in the local spread of genotype VIIa ND viruses within the territory of Ukraine only, and massive migration routes from Southeast Asia through Russia, Kazakhstan, and Kyrgyzstan to the Caspian and beyond resulted in the spread of genotype VIIb and VIId viruses.

The research has demonstrated that ND caused outbreaks among both vaccinated and non-vaccinated poultry. As noted above, preventive vaccination with live vaccine was carried out at the Allele Agro poultry farm (Almaty region). It is noteworthy that ND virus strain used for vaccine preparation is assigned to genotype II, according to the available publications [28]. Perhaps, the outbreak was due to low immunity, as with well-established vaccination regimes the classical vaccines related to genotype II provide 100 % poultry protection irrespective of antigenic differences in the epizootic strain [24, 29, 30]. Vaccination against ND is assumed to provide immunity against infection and suppression of viral replication. But according to some authors, the existing vaccines prevent the disease, but can not stop the replication and spread of the virus [31-33]. Analysis of these studies demonstrates that virus production was significantly lower only when using a particular genotype based vaccine [34, 35].

Thus, the disease and death in poultry in Almaty, Zhambyl, and North Kazakhstan regions of the Republic of Kazakhstan in 2010, 2012, and 2013 were caused by the Newcastle disease (ND) virus. Our findings have shown that ND virus causes the outbreaks both in vaccinated and non-vaccinated poultry. An outbreak of the disease in vaccinated poultry may have occurred as a result of a decrease in immunity. Biological characteristics (mean death time, intracerebral pathogenicity index), and the sequence of proteolytic F protein activation site in the ND viruses studied correspond to those of velogenic strains. Phylogenetic analysis demonstrated that the virus isolated from poultry that died at the poultry farm among the vaccinated population belonged to genotype VIId, and the isolates obtained from non-vaccinated poultry of private yards in Almaty, Zhambyl, and North Kazakhstan regions belonged to genotype VIIb. Wide spread of Newcastle disease virus in the Republic of Kazakhstan requires to develop effective control measures with regard to ND molecular epidemiology.

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