Kafkas Univ Vet Fak Derg
23 (3): 385-389, 2017
DOI: 10.9775/kvfd.2016.16753

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

Genetic Characterization and Evolutionary Analysis of Emerging Newcastle Disease Virus Isolated from Tibetan Chickens

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Article Code: KVFD-2016-16753 Received: 19.09.2016 Accepted: 11.12.2016 Published Online: 12.12.2016

Citation of This Article

Zhu H, Zhang H,Wang Y, Ciren D, Dong H, Wu Q, Rehman MU, Nabi F, Mehmood K, Xu J, Li J: Genetic characterization and evolutionary analysis of emerging Newcastle Disease virus isolated from Tibetan chickens. *Kafkas Univ Vet Fak Derg,* 23 (3): 385-389, 2017. DOI: 10.9775/kvfd.2016.16753

Abstract

Newcastle disease (ND) is one of the highly contagious disease responsible for devastating outbreaks and considerable economic losses to poultry industry in China. However, no information is available about NDV in Tibet region; the aim of this study was to determine the genetic characterization and evolutionary analysis of NDV in Tibetan chickens. Four NDVs were isolated from an outbreak in Tibetan chickens. The pathogenicity of the isolates was determined by mean death time (MDT), intracerebral pathogenicity index (ICPI) and amino acid sequencing (112 to 117) of F protein. While the genetic characterization of F gene was determined by sequencing the isolated NDVs and phylogenetic relationship was established with the help of reference strains. Pathogenicity experiments revealed that XZ-F10 and XZ-F20 strains were lentogenic pathotype, while the remaining XZ-F2-1 and XZ-F4-6 strains were velogenic pathotype. The deduced amino acid sequences of the cleavage site of the F protein confirmed our results. Phylogenic analysis of these strains indicated that both XZ-F2-1 and XZ-F4-6 belong to genotype VII. However, XZ-F10 and XZ-F20 strains were assigned to genotypes II. The present study highlights the need for continuous surveillance of NDV in Tibetan chickens; moreover, this study will provide a reference for the local government to make the strategies and control emerging NDV in Tibet.

Keywords: *Newcastle disease viruses (NDVs), Fusion protein (F) gene, Molecular characterization, Pathogenicity, Tibetan chickens*

Tibet Tavuklarından İzole Edilen Newcastle Hastalığı Virüsünün Genetik Karakterizasyonu ve Kalıtsal Analizi

Özet

Newcatle hastalığı (ND) Çin'de önemli salgınlara ve ekonomik kayıplara neden olan oldukça bulaşıcı bir hastalıktır. Ancak Tibet bölgesinde NDV'ye ait bir bulgu bulunmamaktadır. Bu çalışmanın amacı; Tibet tavuklarında NDV'nin genetik karakterizasyonunu ve kalıtımsal analizi yapmaktır. Meydana gelen bir salgında Tibet tavuklarından dört NDV izole edildi. İzolatların patojenitesi ortalama ölüm zamanı (MDT), intraserebral patojenite endeksi (ICPI) ve F proteininin amino asit sekansı (112'den 117'e kadar) yöntemleriyle belirlendi. F geninin genetik karakterizasyonu izole edilen NDV'lerin sekansı ile belirlendi ve filogenetik akrabalık referans suşun yardımıyla ortaya konuldu. Patojenite deneyleri ile XZ-F10 ve XZ-F20 suşlarının lentojenik patotip, geri kalan XZ-F2-1 ve XZ-F4-6 suşlarının ise velojenik patotip oldukları belirlendi. F proteininin ayrılma bölgesinin amino asit sekansı sonuçlarımızı doğruladı. Bu suşların filogenetik analizi hem XZ-F2-1 hem de XZ-F4-6 suşlarının genotip VII'ye ait olduğunu gösterdi. XZ-F10 ve XZ-F20 suşları genotip II'ye aitti. Bu çalışma; Tibet tavuklarında NDV için sürekli takibin gerekli olduğunu ve lokal sorumlular için kontrol stratejileri geliştirmeleri gerekliliğini göstermiştir.

Anahtar sözcükler: *Newcastle hastalığı virüsü (NDV), Füzyon protein (F) geni, Moleküler karakterizasyon, Patojenite, Tibet tavuğu*

INTRODUCTION

Newcastle disease (ND) is one of the most contagious and devastating disease of poultry throughout the world [1]. NDV is the member of genus Avulavirus, family Paramyxoviridae, with six transcriptional proteins including fusion protein, phosphor protein, nucleocapsid protein, haemagglutinin-neuraminidase protein, matrix protein

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and polymerase protein [2,3]. It can cause severe economic losses to poultry industry, particularly chickens, and affects a range of other domestic species, including duck, wild bird, waterfowl and pigeon [4,5]. The main clinical signs are diarrhea, expiratory dyspnea, neurological symptoms, cloaca hemorrhage, focal glandular gastric bleeding or ulcers, intestinal mucosal bleeding and necrosis of the pancreas or spleen. The pathotype of NDV isolates are divided into three groups (lentogenic, mesogenic and velogenic) based on the intracerebral pathogenicity index (ICPI) and mean death time (MDT) [4]. Currently, basic amino acid sequence (from 112 to 117) of the fusion protein is used for classifying the virulence of NDV strains.

The Tibetan chickens have a very wide distribution at an altitude of 2.200 to 4.100 m on the Qinghai-Tibet Plateau, with a history of domestication of more than 1.000 year at high altitude [6,7]. Under special breeding conditions, this specie has been famous for disease resistance, easy breeding and increasingly vigorous market demand with high-quality meat production. This breed is of great economical importance to local herdsman, as the meat of Tibetan chickens is rich in protein, with a tender texture, and high content of amino acids [8]. In recent years, NDV has been reported with large-scale outbreaks and considerable economical losses to poultry industry in many provinces of China [9-11]. Until now, no information is available about the genetic characterization of NDV in Tibetan chickens. The present study reports the characterization of NDV isolates from the Tibetan chickens in Tibet.

MATERIAL and METHODS

Ethical Approval

All the experiments were approved by Animal Welfare and Research, Ethics Committee of Huazhong agricultural university and performed in accordance with the international guidelines for animal welfare.

The Study Site

The present study was carried out in Nyingchi Prefecture on Qinghai-Tibetan Plateau, China. This area is geographically isolated from Tibet and Sichuan Provinces by Himalayas, and shares border with Nepal in the south-west, and India and Myanmar in the south. The average elevation of the surveyed area is more than 3100 m above sea level, the largest continuous high elevation ecosystem *(Fig. 1)*.

Virus Isolation and Pathogenicity Test

Four NDVs were isolated from a flock of Tibetan chickens during an outbreak in Tibet, China and virus separation was carried out according to the guidelines provided by the World Organization for Animal Health [4]. Briefly, homogenized and clarified organ suspensions were made with streptomycin/penicillin in viral transportation media (VTM) overnight at 4°C and 0.2 mL suspensions were inoculated into the allantoic cavity of 9 to 11 day old specific pathogen free (SPF) chicken embryos, and incubated at 37°C for 72 h [12]. Allantoic fluid was harvested and the NDV was tested using haemagglutination inhibition (HI) and haemagglutination (HA) tests. The strains were designated names as XZ-F10, XZ-F20, XZ-F2-1 and XZ-F4-6. LaSota strain was used as a positive control in this study. The positive allantoic fluids were stored at -80°C until subsequent use and further analysis. The pathogenic potential for the isolates were evaluated by mean death time (MDT) using 10-days-old SPF embryonated chicken eggs and intracerebral pathogenicity index (ICPI) test using 1-day-old SPF chicks using standard procedures [13].

Viral RNA Purification, RT-PCR, and Sequencing of the F Gene

Viral RNA of the four isolated strains was extracted using TIANamp Virus DNA/RNA Kit (TianGen, China). Reverse transcription PCR amplification approach was performed using Quant One Step RT-PCR Kit (Tian Gen, China) with primers for the F gene (~1662bp). Based on the published F gene sequences in the GenBank database, we designed a pair of primers with the help of Primer Premier Software (version 5.0) and synthesized by Wuhan Qingke biotechnology CO., LTD (Wuhan, China). The primer forward: ATGGGCTCCAGAC CTTCTACCA and reverse CATTTTTGT AGT GGCTCTCATCTGAT was used and commercial RT-PCR Kit (TianGen, China) was used for reverse transcription. The RT-PCR mixture contained 14.2 µL RNase-

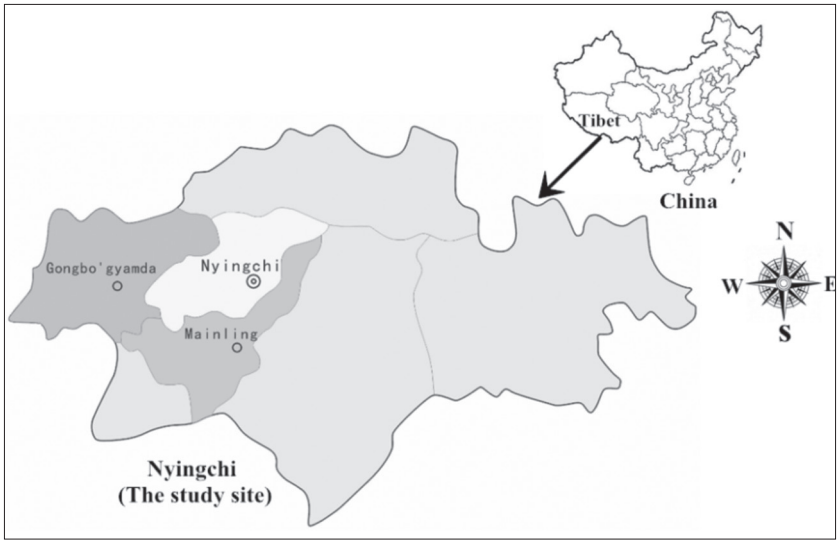

Fig 1. Collection sites of samples in Nyingchi, Tibet China

free water, 5 µL AMV Buffer, 0.5 µL dNTP, 0.8 µL $MgSO_4$, 0.5 µL Tfl DNA Polymerase, 2 µL RNA, 1 µL of each forward and reverse primer (working concentration: 10 µL mol/L) in a 25 µL reaction. For F gene: denaturation at 95°C for 40 s, and 72°C for 1.5 min of 35 cycles and annealing at 55°C for 50 s; and final extension at 72°C for 10 min. PCR products were separated on agarose gel (1%). The products were purified using a TaKaRa DNA Extraction Kit Ver.4.0 (Takara Biotechnology CO., LTD, Dalian, China) according to manufacturer's instructions, and then sequenced by a commercial company (Qingke Biosciences, Wuhan, China).

Sequence Analysis of F Protein Cleavage Site

Phylogenetic analysis, amino acid sequence prediction and nucleotide sequence similarity was performed by the Clustal W multiple alignment method (MegAlign program of the DNASTAR software, version 3.3.8) for the F gene (~1662bp). In addition to the 4 strains collected in this study, 15 previously reported F gene sequences representing different genotypes were obtained from GenBank database including vaccine strains, typically existing in China. The accession numbers of each of these NDVs are shown in the phylogenetic tree.

RESULTS

In present study, four NDVs were isolated from Tibetan chickens on SPF embryonated chicken eggs during different outbreaks at chicken's farm from 2012 to 2016. The details of the NDV isolates are shown in *Table 1*. As per classification, the virus of XZ-F10 and XZ-F20 strains were assigned to lentogenic pathotype, while XZ-F2-1 and XZ-F4-6 strains were classified as velogenic pathotype. As it is shown in *Table 2*, the F protein cleavage site motif sequence was 112G-R-Q-G-R-L117 in strain XZ-F10 and XZ-F20, which is the major determinant of lentogenic for NDV strains and 112R-R-Q-K-R-F117 in strain XZ-F2-1 and XZ-F4-6, that is considered as the velogenic.

Based on the complete F gene sequences derived through different years in Tibet, the XZ-F2-1 and XZ-F4-6 strains isolated in 2012, and XZ-F10 and XZ-F20 strains isolated in 2015 and 2016 were assigned to genotypes VII and II, respectively *(Fig. 2)*.

DISCUSSION

Newcastle disease can lead to high mortality and morbidity rates, and creates a big potential threat to the poultry industry in terms of serious economic losses in China [10]. In recent years, although many dynamic measures have been adopted to prevent and control this disease, however, it is still the major infectious disease, and as a big potential threat to the chicken industry in China. As a very wide distribution at altitudes of 2.200 to 4.100 m on the Qinghai-Tibet Plateau [6,7], no information is available on the pathogenicity and genetic characterization of NDV in Tibetan chickens. Fusion (F) protein is the key protein that measures the NDV pathogenicity and therefore, F gene was analyzed by different laboratories in recent decades [14,15]. The virulent strength of NDV can be classified through basic amino acid sequences from 112 to 117 of F protein [16]. As it is shown in table 2, the F protein cleavage site motif sequence was 112G-R-Q-G-R-L117 in strain XZ-F10 and XZ-F20, which is the major determinant of lentogenic for NDV strains [16], and 112R-R-Q-K-R-F117 in strains XZ-F2-1 and XZ-F4-6, that is considered as the velogenic [17,18]. This confirms that not only MDT and ICPI can be used to validate the pathogenicity, but as previous reports, the cleavage site motifs is also reliable way to predict the pathogenicity and virulence of NDV strains [16].

Table 1. *The characteristics description of four NDVs isolated from Tibetan Chickens in this study*

NDV Isolates	Year of Isolation	Location	MDT [b]	ICPI [c]	Pathotype
XZ-F10	2015	Tibet	>120	0.2	Lentogenic
XZ-F20	2016	Tibet	>120	0.2	Lentogenic
XZ-F2-1	2012	Tibet	53	1.62	Velogenic
XZ-F4-6	2012	Tibet	49	1.56	Velogenic

[a] Amino acid sequence (112 to 117) of F protein; [b] Mean death time in 9-day-old SPF embryonated chicken eggs (hours) (velogenic, <60; mesogenic, 60-90; lentogenic, >90); [c] Intracerebral pathogenicity index in 1-day-old chickens (lentogenic, <0.7; mesogenic, 0.7–1.4; velogenic, 1.4-2.0)

Table 2. *Fusion protein cleavage site description of NDVs isolated from Tibetan chickens*

Fusion Protein Cleavage Site From 112 to 117												NDV Isolates
109	110	111	112	113	114	115	116	117	118	119	120	
S	G	G	G	**R**	**Q**	G	**R**	L	I	G	A	XZ-F10
S	G	G	G	**R**	**Q**	G	**R**	L	I	G	A	XZ-F20
S	G	G	**R**	**R**	**Q**	**K**	**R**	**F**	I	G	A	XZ-F2-1
S	G	G	**R**	**R**	**Q**	**K**	**R**	**F**	I	G	A	XZ-F4-6

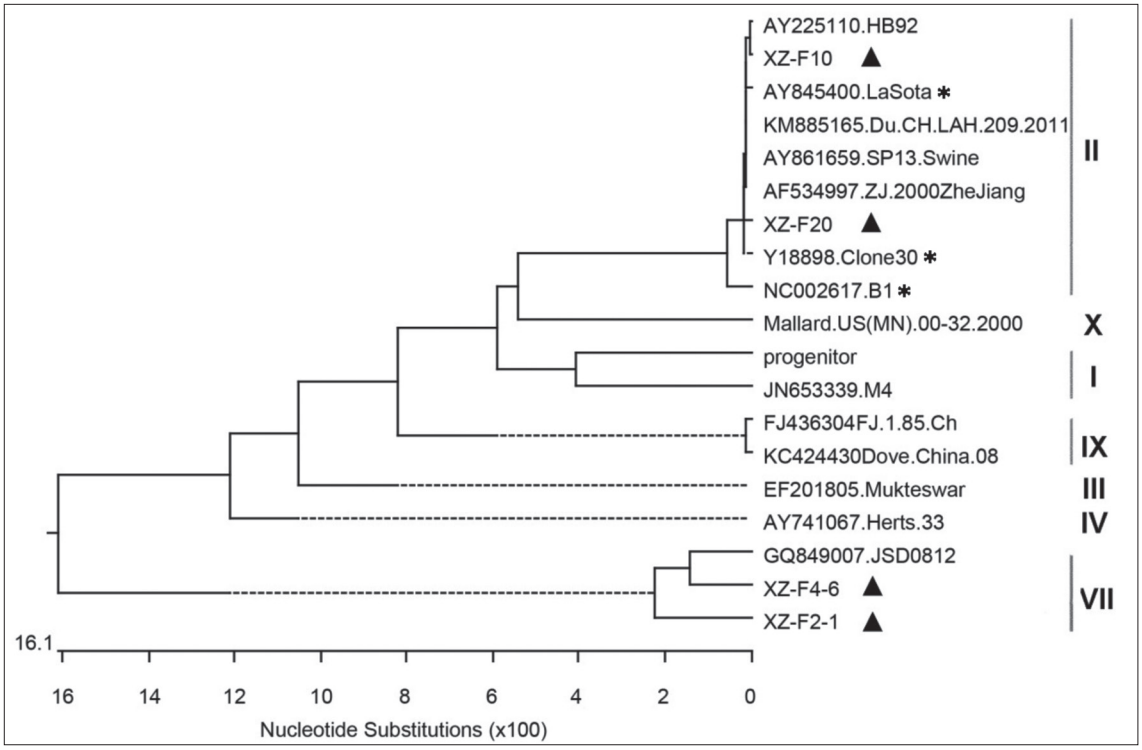

Fig 2. Phylogenetic tree constructed by the neighbor joining method using MegAlign software. * =Vaccine strains

The genotype VII NDVs have become the most prevalent strains since 1990s in most parts of China [19]. Because of the unique natural environment and high altitude in Tibet, NDV genotype and distribution is different from surrounding regions and countries. However, in recent years, with the continuous enhancement in tourism and transportation, more and more Tibetan chickens are exposing to the virus, this is an important reason that genotype VII was found in Tibetan chickens in 2012. In the past two years, genotypes II NDVs are reported from many countries including China, from numerous species [20]. We also found that XZ-F10 and XZ-F20 strains had a close genetic relationship with LaSota, HB92, SP13, CH.LAH209, ZJ.2000, Clone 30 and B1 (Accession number: AY845400, AY225110, AY861659, KM885165, AF534997, Y18898 and AF309418, respectively) strains that belongs to genotype II in terms of F gene analysis. The LaSota, Clone 30 and B1 were the attenuated strains, NDV attenuated strains (LaSota, Clone-30 and B1) are still used to make attenuated vaccine at a large scale in China [20-22]. Our results revealed that the NDVs isolated in Tibetan chickens showed a close phylogenetic relationship and evolutionary distance with strains of LaSota, Clone 30 and B1. There was significant similarity between the Tibetan chickens NDV strains and the current vaccine strains in their serology and genetics, which might be considered as the reasons for the ND outbreaks in Tibetan chickens.

In conclusion, we identified two genotypes (II and VII) in Tibetan chickens for the first time in our studied strains; moreover, results provided clear evidence that the non-standard use of vaccine may be the important reason that leads to epidemic outbreak of NDV in Tibetan chickens on Qinghai-Tibet Plateau, China. Furthermore, our study also highlights the need for continuous surveillance of NDV in Tibetan chickens, which will provide a reference for the local government to make the strategies for the control of emerging NDV in Tibet.

Acknowledgments

This study was supported by Key laboratory of clinical veterinary medicine in Tibet, The National Natural Science Foundation of China (No.31460682) and Provincial Natural Science Research Project of Anhui University (KJ2016A180).

Conflict of Interest

The authors declare that they have no competing interests.

REFERENCES

1. Aldous EW, Alexander DJ: Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathol*, 30, 117-28, 2001. DOI: 10.1080/03079450120044515

2. Mayo MA: A summary of taxonomic changes recently approved by ICTV. *Arch Virol,* 147, 1655-1663, 2002. DOI: 10.1007/s007050200039

3. Okwor E, Chukwudi E, Didacus C, Mary U: Newcastle disease virus shedding among healthy commercial chickens and its epidemiological importance. *Pak Vet J*, 8318, 2074-7764, 2011.

4. OIE: "Newcastle disease, chapter 2.3.14," in OIE Terrestrial Manual 2012:

Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Paris: World Organisation for Animal Health), 576-589. http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.14_NEWCASTLE_DIS.pdf; *Accessed:* 10.12.2016.

5. Sinkovics JG, Horvath JC: Newcastle disease virus (NDV): Brief history of its oncolytic strains. *J Clin Virol*, 16, 1-15, 2000. DOI: 10.1016/S1386-6532(99)00072-4

6. Zhang H, Wu C, Chamba Y, Ling Y, Luo Z: Adaptability to high altitude and NOS activity of lung in Tibetan chicken. *J China Agric Univ*, 11, 35-38, 2006.

7. Zhang H, Wu C, Chamba Y, Ma X, Tang X, Po B: Curve analysis of embryonic mortality in chickens incubated at high altitude. *J China Agric Univ*, 10, 109-114, 2005.

8. Zhang H, Wu CX, Yangzom C, Ling Y, Tang XH: Cardiac enzymes related to high-altitude hypoxic adaptation in tibetan chicken. *Chinese J Appl Physiol*, 24, 233-236, 2008.

9. Qin ZM, Tan LT, Xu HY, Ma BC, Wang YL: Pathotypical characterization and molecular epidemiology of Newcastle disease virus isolates from diffenrent hosts in China from 1996 to 2005. *J Clin Microbiol*, 46, 601-611, 2008. DOI: 10.1128/JCM.01356-07

10. Kang Y, Xiang B, Yuan R, Zhao X, Feng M: Phylogenetic and pathotypic characterization of Newcastle disease viruses circulating in South China and transmission in different birds. *Front Microbiol*, 7, 119, 2016. DOI: 10.3389/fmicb.2016.00119

11. Zhang R, Pu J, Su JL, Zhao JX, Wang XT, Zhang SP, Li XJ, Zhang GZ: Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001-2009. *Vet Microbiol*, 141, 246-257, 2010. DOI: 10.1016/j.vetmic.2009.09.020

12 Alexander DJ: Newcastle disease and other avian paramyxoviruses. *Rev Sci Tech*, 19, 443-462, 2010.

13. Kim SH, Xiao S, Shive H, Collins PL, Samal SK: Replication, neurotropism, and pathogenicity of avian paramyxovirus serotypes 1-9 in chickens and ducks. *PLoS One*, 7, e34927, 2012. DOI: 10.1371/journal.pone.0034927

14. Diel DG, Silva LHAD, Liu H, Wang Z, Miller PJ: Genetic diversity of avian paramyxovirus type 1: Proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. *Infect Genet Evol*, 12, 1770-1779, 2012. DOI: 10.1016/j.meegid.2012.07.012

15. Samal S, Khattar SK, Kumar S, Collins PL, Samal SK: Coordinate deletion of N-glycans from the heptad repeats of the fusion F protein of Newcastle disease virus yields a hyperfusogenic virus with increased replication, virulence, and immunogenicity. *J Virol*, 86, 2501-2511, 2012. DOI: 10.1128/JVI.06380-11

16. Kim LM, King DJ, Guzman H, Tesh RB, Travassos DA: Biological and phylogenetic characterization of pigeon Paramyxovirus serotype 1 circulating in wild North American pigeons and doves. *J Clin Microbiol*, 46, 3303-3310, 2008. DOI: 10.1128/JCM.00644-08

17. Mehrabanpour MJ, Khoobyar S, Rahimian A, Nazari MB, Keshtkar MR: Phylogenetic characterization of the fusion genes of the Newcastle disease viruses isolated in Fars province poultry farms during 2009-2011. *Vet Res Forum*, 5, 187-191, 2014.

18. Sun C, Wen H, Chen Y, Chu F, Lin B, Ren G, Song Y, Wang Z: Roles of the highly conserved amino acids in the globular head and stalk region of the Newcastle disease virus HN protein in the membrane fusion process. *Biosci Trends*, 9, 56-64, 2015.

19. Wu S, Wang W, Yao C, Wang X, Hu S, Cao J, Wu Y, Liu W, Liu X: Genetic diversity of Newcastle disease viruses isolated from domestic poultry species in Eastern China during 2005-2008. *Arch Virol*, 156, 253-61, 2010. DOI: 10.1007/s00705-010-0851-5

20. Gaikwad S, Kim JY, Lee HJ, Jung SC, Choi KS: Genetic characterization and evolutionary analysis of Newcastle disease virus isolated from domestic duck in South Korea. *Gene*, 579, 34-40, 2015. DOI: 10.1016/j.gene.2015.12.040

21. Hu S, Ma H, Wu Y, Liu W, Wang X: A vaccine candidate of attenuated genotype VII Newcastle disease virus generated by reverse genetics. *Vaccine*, 27, 904-910, 2015. DOI: 10.1016/j.vaccine.2008.11.091

22. Kim SH, Xiao S, Collins PL, Samal SK: LaSota fusion (F) cleavage motif-mediated fusion activity is affected by other regions of the F protein from different genotype Newcastle disease virus in a chimerical virus: implication for virulence attenuation. *J Gen Virol*, 97, 1-8, 2016. DOI: 10.1099/jgv.0.000439