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Evaluation of transmission potential and pathobiological characteristics of Mallard originated *Avian orthoavulavirus* 1 (sub-genotype VII.2) in commercial broilers

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Abstract

Newcastle disease (ND), caused by *Avian orthoavulavirus* 1 (AOAV-1), affects multiple avian species around the globe. Frequent disease outbreaks are not uncommon even in vaccinates despite routine vaccination and, in this regards, viruses of diverse genotypes originating from natural reservoirs (migratory waterfowls) play an important role in a disease endemic setting. Though genomic characterization of waterfowl originated viruses has been will-elucidated previously, there is a paucity of data on clinico-pathological assessment of mallard-originated sub-genotype VII.2 in commercial chickens. Hence, the current study was designed to evaluate its transmission potential, tissue tropism and micro- and macroscopic lesions in commercial broilers. Based on complete genome and complete *F* gene, phylogenetic analysis clustered the study isolate within genotype VII and sub-genotype VII.2 in close association with those reported previously from multiple avian species worldwide. The study strain was found to be velogenic on the basis for typical residue pattern in the F-protein cleavage site (^{112}R-RQ-K-R↓F^{117}), sever disease induction in chicken, tissue tropism and subsequent clinico-pathological characteristics. Giving a clear evidence of horizontal transmission, a 100% mortality was observed by 4th and 6th day post infection (dpi) in chickens challenged with the virus and those kept with the challenged birds (contact birds), respectively. The observed clinical signs, particularly the greenish diarrhea, and macroscopic lesions such as pinpoint hemorrhages in proventriculus and cecal tonsils were typical of the infection caused by an AOAV-1 in chickens. The virus exhibited a broad tissue tropism where genomic RNA corresponding to study virus was detected in all of the tissues collected from recently mortile and necropsied birds. The study concludes that mallard-originated *Avian orthoavulavirus* 1 is highly velogenic to commercial chicken and, therefore, ascertain continuous vaccine monitoring in vaccinates and routine

surveillance of migratory/aquatic fowls to better elucidate infection epidemiology and subsequent potential impacts on commercial poultry.

Key words: *Avian orthoavulavirus* 1; water fowls; greenish diarrhoea; phylogenetic analysis; macroscopic changes; sub-genotype VII.2

1. Introduction

Newcastle disease (ND), caused by *Avian orthoavulavirus 1 (*AOAV-1), is a contagious viral disease of a wide range of avian species worldwide [1]. First reported in Java (Indonesia,1926) [2] and England at Newcastle-upon-tyne [3, 4]. The disease is now endemic in six of the seven continents [5]. The virus is classified as a member of genus *Orthoavulavirus* within the family *Paramyxoviridae* [6]. It has a single-stranded, non-segmented pleomorphic RNA genome of 15186, 15192 or 15198 nucleotides in length that follow a rule of six in an order of 3′-*NP-P-M-F-HN-L*-5′. Depending upon pathogenicity in chickens, the virus exist as four pathotypes named velogenic, mesogenic, lentogenic and avirulent [7]. Two major classes, class I and II, of AOAV-1 are known. Class I viruses are isolated from waterfowls, comprised of a single genotype, carries a large genome (15198 bp) and are considered non-virulent for chickens. On the other hand, class II viruses are isolated from a wide range of avian species comprised of XXI genotypes [6], have relatively a short genome size (15186 bp and/or 15192 bp) and includes both virulent and non-virulent strains [8, 9].

*Avian orthoavulavirus*1 has a potential to evolve with the passage of time and this has fairly been evidenced with the emergence of novel genotypes and sub-genotypes followed by outbreaks in multiple avian hosts across many parts of the world [10-12]. In this regards, migratory birds play a vital role that themselves remain asymptomatic (reservoir); however, while their movement from one part of the world to another [13], shed viruses of different genotypes and varying

pathogenicity resulting in clinical disease in their susceptible host, the poultry [14-16]. Isolation, identification and genomic characterization of sub-genotype VII.2 has previously been evidenced from migratory birds in Pakistan. Indeed, during the recent years, viruses of genotype VII have been reported from many poultry rearing settings worldwide and are known to have a panzootic potential [11, 17, 18]. Virulent strains of genotype VII causes the epidemics not only in Pakistan but also in China, Japan, and Korea [19-21] are co circulating into ducks and chickens [22]. Vaccines routinely used in the country are based on genotype I and II strains such as LaSota and Mukteswer. Both the vaccine provide protective immunity but unable to block the virus shedding that lead to continuous presence of virulent strains in the environment. This is particularly more dangerous with genotype VII isolates, whose shedding after LaSota vaccinated birds is significantly higher than those of other genotypes [23]. Taken together, in a disease endemic setting worldwide such as Pakistan, it become utmost important not only to characterize biologic and genomic characteristics of waterfowl's-originated viruses in routine but also to evaluate their clinico-pathological assessment, transmission and tissue distribution pattern in susceptible hosts such as the commercial broiler. Genomic and biologic characteristic of study isolate has previously been documented [24] Here we report infection transmission potential, tissue tropism, macroscopic and microscopic lesions of duck-originated AOAV-1 in commercial broilers.

2. Materials and Methods

2.1. Ethical approval

All animal handling and sample processing procedures were carried out in strict accordance to institutional guidelines and regulations related to animal welfare and health. The used procedures were approved by the Ethical Review Committee for use of Laboratory Animals (ERCULA) of

92 the University of Veterinary and Animal Sciences, Lahore, Pakistan (Permit Number: ORIC/DR-
93 70).

94 **2.2. Virus strain**

95 The study used virus strain was isolated from clinically healthy mallard (*Anas platyrhynchos*) in
96 an influenza surveillance program at Chashma barrage during the period from June 2015 to
97 September 2016. The barrage is built in the river Indus and is considered one of the major
98 wildlife sanctuaries that accommodates a large variety of migratory and indigenous water birds
99 each year [25]. Typical biologic (mean death time = 49.5-50hr and EID_{50} = $10^{8.5}$/mL) and
100 genomic characteristics (F protein cleavage site, ^{112}R-RQ-K-R↓F^{117}) of the study virus
101 (KY967612; Mallard-II/UVAS/Pak/2016) revealed the virulent nature of the isolate [24].

102 **2.3. Phylogenetic analysis**

103 Complete genome and complete *F* gene of study isolate and other representative strains of
104 different genotypes of AOAVs reported from Pakistan and other parts of the world were
105 retrieved from GenBank database and were aligned in BioEdit® version 5.0.6 [26]. A
106 phylogenetic tree of both complete genome and *F* gene was constructed using neighbor-joining
107 method with 1000 bootstrap replicates through MEGA® version 6.0 software [27]. Labeling and
108 necessary interpretation of prevalent genotype was made as per newly proposed classification of
109 AOAV-1 [6].

110 **2.4. Experimental design**

111 One-day-old chicks (n=25) were procured from a commercial hatchery and raised at the animal
112 experiment unit in the Quality Operations Laboratory (QOL), University of veterinary and
113 animal sciences, Lahore, Pakistan. Feed and water were provided *ad libitum*. All chickens
114 included in the study were first screened for AOAV-1 and avian influenza virus specific

115 antibodies in blood through hemagglutination inhibition (HI) assay [28]. and antigen in oral and
116 cloacal swabs through previously published RT-PCR assays targeting *F* gene for AOAV-1 [29]
117 and *M* gene for AIV [30] and egg inoculation [31]. Briefly, for egg inoculation, the swab
118 samples containing buffered saline were mixed with antimicrobials (Penicillin 2000 IU/mL,
119 Gentamicin 200 µg/mL and Fungizone 1.5 µg/mL) and centrifuged at 3000g for 5min. About 1.0
120 mL of each sample was filtered through 0.22µm syringe filter (EMD Millipore Millex^{TM},
121 Millipore Billerica MA, USA). A 0.2mL of filtrate was inoculated in 9 day-old chicken
122 embryonated egg as described previously.
123 At the age of day 30, chickens were randomly divided into three groups. Group A (n =10) served
124 as challenged birds, group B served as negative control (n=10), while birds in group C (n=5)
125 served as contact birds. The group A was inoculated with 10^8 EID_{50}/mL^{-1} of Mallard-
126 II/UVAS/Pak/2016 *via* intra-ocular and intranasal routes. The negative control group was
127 inoculated with 1mL phosphate buffered saline. After 24hr of challenge, the contact birds (group
128 C) were introduced with group A birds for an assessment of horizontal transmission of virus
129 from infected (group A) to healthy birds (group C). Chickens of groups A, B and C were housed
130 separately and monitored daily for clinical presentation. With the onset of generic and typical
131 clinical signs suggestive of ND infection and/or sudden death, diseased birds were euthanized
132 and tissue samples n=13 (brain, breast muscles, heart, tongue, trachea, hair follicles, caecal
133 tonsils, gizzard, proventriculus, small intestine, liver and lungs) were collected for tissue tropism
134 and histopathology.
135 **2.4.1. Genomic identification of AOAV-1**
136 Tissue samples were ground completely in a pestle-mortar, centrifuged at 2000g for 15min and
137 supernatant was collected and archived at -20 until used. Genomic RNA was extracted from the

cloacal and oral swabs, as well as tissue-extracted supernatant using a commercially available kit as per manufacturer's protocol (QIamp Viral RNA mini kit, Qiagen®, USA). Tissue distribution was determined using previously reported RT-PCR targeting *F* gene [29].

2.4.2. Histopathology

Tissue samples (liver, lung, spleen, brain, cecal tonsils and intestine) from recently mortile birds were collected, stored in 10% buffered formalin and processed for hematoxylin and eosin staining [32]. Tissue section (5µm) were prepared and observed for microscopic changes under light microscope (10X and 40X).

3. Results

3.1. Phylogenetic analysis

Phylogenetic analysis of complete genome clustered study isolate within genotype VII together with previously isolated strain of Pakistan-originated chicken (KX791187-88), duck (KU845252) and wild pigeon (KX496962) and vaccinated broilers (HQ697254) from Indonesia (Figure 1A). Based on complete *F* gene, phylogenetic analysis clustered study isolate within VII.2 together with AOAV-1 strains previously isolated from pheasant (JX854452) and backyard poultry (KX791187-88) from Pakistan (Figure 1B).

3.2. Infection potential, transmission and tissue tropism of mallard originated AOAV-1 in commercial chickens

All the birds in the challenged group died by 4^{th} dpi while the contact birds died by 6^{th} dpi clearly indicating velogenic nature of mallard-originated isolate. We observed generic as well as typical clinical signs that were suggestive of ND infection in both of the challenged and contact birds. Generic clinical signs included off-feed, general sickness (depression, isolation and lethargic), oculo-nasal discharge and coughing. On the other hand, typical signs included sudden death,

161 greenish diarrhea. Though macroscopic lesions such as pin point hemorrhages in proventriculus
162 and cecal tonsil were evident in each of the recently mortile bird, all the birds were devoid of any
163 nervous symptoms until their death. In challenged birds, the signs were appeared on 2nd dpi
164 along with sudden death in three of the birds, become aggravated further by 3rd dpi and all of
165 them get mortile by 4th dpi. Signs appeared in contact birds (group C) by 3rd dpi with no sudden
166 death throughout the experiment period; however, severity of infection aggravated by 5th dpi and
167 all the birds died by 6th dpi. Birds in group B (negative control) remained alive throughout with
168 no evident clinical signs. A brief description of mortalities observed for each of the group is
169 presented in Kaplan-Meier curve (Figure 2).
170 As for tissue tropism of AOAV-1 is concerned in diseased chicken originating from challenged
171 and contact bird's group, viral RNA corresponding to *F* gene was detected in all of the select
172 tissue samples (n =13) including brain, breast muscles, heart, tongue, trachea, hair follicles, cecal
173 tonsils, gizzard, proventriculus, small intestine, liver and lungs.
174 **3.2.1. Macro- and microscopic changes**
175 Necropsy of recently mortile birds in each of the challenged and contact birds showed
176 comparable macroscopic changes. These included swelling of inner lining of eyelid, enlargement
177 of lymphoid organs (liver, spleen) hemorrhages in trachea, liver, lungs, proventriculus, and small
178 intestine particularly cecal tonsils. Microscopic changes in liver included degeneration and
179 necrosis of hepatic cords, severe congestion along with emphysema and degeneration (Figure
180 3A). Congestion and hemorrhages with mononuclear inflammatory cells infiltration in sub-
181 mucosa of lungs were observed (Figure 3 B). Similarly, congestion, degeneration and necrosis of
182 lymphoid follicles were evident in cecal tonsils (Figure 3C). Microscopic lesion in small
183 intestine included mononuclear cells infiltration; necrotic intestinal villi and sloughing of luminal

epithelial cells (Figure 3D). Hemorrhages and degenerative changes were evident both in the spleen (Figure 3E) and brain tissues (Figure 3F). However, no microscopic changes were observed in control group.

4. Discussion

The study revealed disease causing potential of mallard-originated AOAV-1 in its susceptible host (chicken) and, therefore highlight potential role of such natural reservoirs in disease epizootology, particularly in a setting where ND is endemic and frequent occurrence of outbreaks is not unusual. Phylogenetic analysis of independent complete genome and *F* gene revealed clustering of study isolate with previously reported viruses from different host (duck, backyard poultry and pigeon) suggesting its interspecies potential. A close association with an Indonesian isolate is interesting that not only is a clear indicator of its potential ancestral link bit also it does provide an evidence on potential role of wild birds in dissemination of pathogens across the borders. This is not exclusively limited to transboundary transmission of AOAV-1 through their natural reservoirs but also has been evidenced previously for other respiratory pathogens of birds in Pakistan such as highly pathogenic avian influenza strain H5N1 [33, 34].

As per recently proposed classification, the study isolate classified as VII.2. Indeed, AOAV-1 within genotype VII are considered to be genetically diverse group of viruses and are found associated with recurrent outbreaks in their susceptible hosts, particularly the commercial poultry, in Middle East, Asia, Africa and South America [5, 35-38]. Among genotype VII viruses, sub-genotype VII.2 has demonstrated an intercontinental spread and, therefore has a global significance in the perspective of potential fifth panzootic [11]. Hence, an extensive surveillance of wild birds for AOAV-1 is very much necessary to monitor circulating genotypes in the country and/or region.

We used oculonasal route for virus exposure in birds simply because it is considered a natural route of infection and the same has previously been employed in various other experimental infection of AOAV-1 under captivity [39, 40]. Clinico-pathological findings of duck originated AOAV-1 revealed a highly pathogenic nature of virus to their susceptible host, the chicken. Given a broad range of host susceptibility to AOAV- 1 across many part of the globe [41-44], particularly sub-genotype VII.2, clinical implications such as observed in this study were not very much unexpected. However, it were disease progression, clinical signs and their severity that may be taken as unusual particularly in birds representing a disease-endemic region. An occurrence of disease in contact birds was found to be a clear indication of horizontal transmission of virus from birds in challenged group to contact birds. A lack of nervous sign in both group of challenged and contact birds indicated a viscerotropic nature of study pathotype [5, 37]. Sudden death observed exclusively in challenged birds is another typical feature of ND infection [45]. A high mortality rate (100%) observed in both challenged and contact birds was found to be in agreement to observation made previously where, post infection with velogenic virus, all the challenged birds died within a week [37]. The clinical infection was found to be more pronounced and severe between 2^{nd} to 4^{th} dpi with an exhibition of ocular and nasal discharge, conjunctivitis, ruffled feathers, greenish diarrhea and sudden death. Consistent to this observation, a similar pattern of clinical sign progression has previously been documented with an exposure of wild-bird originated velogenic strain in chicken [39, 46]. Similarly, macroscopic lesion upon necropsy as well as microscopic changes observed under microscope were found suggestive of virulent AOAV-1 in a pattern similar to those reported previously during experimentally infected commercial chickens with wild bird originated velogenic isolates [37, 39].

5. Conclusion

We determined the infection potential of migratory duck originating AOAV-1 in commercial chickens. Clustered within the viruses of genotype VII.2, the study isolate was found to be highly pathogenic to chicken and had a broad tissue tropism. Hence, in a disease endemic setting worldwide including Pakistan, continuous surveillance of migratory birds coupled with genomic, biologic and clinico-pathologic assessment is ascertained for its effective control and management.

Competing interest statement

All authors declared no competing interest with data presented in this manuscript

Contributors

MH and MZS conceived and designed the work. MH, TY and AR performed laboratory procedures and relevant methods. MH, AR and MZS involved in data analysis. MZS, WS, MS and TY provided necessary laboratory resources and consumables. MH, AR, MM and MZS wrote the draft and edited.

Acknowledgement

None

Figure legends

Figure 1: A phylogenetic tree based on complete genome (1A) and complete F *gene* (1B) of study isolate, duck origin and other representatives of A*vian orthoavulavirus* 1. The tree was constructed in MEGA 6.0 software using neighbor joining method with 1000 bootstraps. The study isolate is marked with black circle.

Figure 2: Percent survival of infected chicken with duck isolate along with control (non-infected) and contact group. All birds in infected and contact group were died at 4 and 6 dpi respectively. No mortality was observed in control group**.**

Figure 3: Microscopic examination of histopathological changes at different resolutions (10 & 40X) in different tissues of experimentally infected broiler with Mallard I/UVAS/Pak/2016 isolate. Arrows indicate pathological lesions in liver (A), lung (B), caecal tonsil (C), villi (D), spleen (E) and brain (F).

References

[1] Kaleta EF, Baldauf C. Newcastle disease in free-living and pet birds. Newcastle disease: Springer; 1988. p. 197-246.

[2] Kraneveld F. A poultry disease in the Dutch East Indies. Ned Indisch Bl Diergeneeskd. 1926;38:448-50.

[3] Doyle T. A hitherto unrecorded disease of fowls due to a filter-passing virus. J Comp Pathol Ther. 1927;40:144-69.

[4] Alexander DJ. Newcastle disease. British poultry science. 2001;42:5-22.

[5] Diel DG, Susta L, Garcia SC, Killian ML, Brown CC, Miller PJ, et al. Complete genome and clinicopathological characterization of a virulent Newcastle disease virus isolate from South America. Journal of clinical microbiology. 2012;50:378-87.

[6] Dimitrov KM, Abolnik C, Afonso CL, Albina E, Bahl J, Berg M, et al. Updated unified phylogenetic classification system and revised nomenclature for Newcastle disease virus. Infection, genetics and evolution. 2019:103917.

[7] Khan MI. Newcastle disease. Handbook of Zoonoses. 1994:473-81.

[8] Czeglédi A, Ujvári D, Somogyi E, Wehmann E, Werner O, Lomniczi B. Third genome size category of avian paramyxovirus serotype 1 (Newcastle disease virus) and evolutionary implications. Virus research. 2006;120:36-48.

[9] Zhu J, Xu H, Liu J, Zhao Z, Hu S, Wang X, et al. Surveillance of avirulent Newcastle disease viruses at live bird markets in Eastern China during 2008–2012 reveals a new sub-genotype of class I virus. Virology journal. 2014;11:211.

[10] Ewies SS, Ali A, Tamam SM, Madbouly HM. Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. Beni-Suef University journal of basic and applied sciences. 2017;6:232-7.

[11] Miller PJ, Haddas R, Simanov L, Lublin A, Rehmani SF, Wajid A, et al. Identification of new sub-genotypes of virulent Newcastle disease virus with potential panzootic features. Infection, genetics and evolution. 2015;29:216-29.

[12] Wajid A, Dimitrov KM, Wasim M, Rehmani SF, Basharat A, Bibi T, et al. Repeated isolation of virulent Newcastle disease viruses in poultry and captive non-poultry avian species in Pakistan from 2011 to 2016. Preventive Veterinary Medicine. 2017;142:1-6.

[13] Kang Y, Li Y, Yuan R, Li X, Sun M, Wang Z, et al. Phylogenetic relationships and pathogenicity variation of two Newcastle disease viruses isolated from domestic ducks in Southern China. Virology journal. 2014;11:147.
[14] Miller PJ, Koch G. Newcastle disease. Diseases of poultry. 2013;13:89-138.
[15] Onapa MO, Christensen H, Mukiibi G, Bisgaard M. A preliminary study of the role of ducks in the transmission of Newcastle disease virus to in-contact rural free-range chickens. Tropical animal health and production. 2006;38:285-9.
[16] Brown VR, Bevins SN. A review of virulent Newcastle disease viruses in the United States and the role of wild birds in viral persistence and spread. Veterinary research. 2017;48:68.
[17] Dimitrov KM, Ferreira HL, Pantin-Jackwood MJ, Taylor TL, Goraichuk IV, Crossley BM, et al. Pathogenicity and transmission of virulent Newcastle disease virus from the 2018–2019 California outbreak and related viruses in young and adult chickens. Virology. 2019;531:203-18.
[18] Wajid A, Dundon WG, Hussain T, Babar ME. Pathotyping and genetic characterization of avian avulavirus-1 from domestic and wild waterfowl, geese and black swans in Pakistan, 2014 to 2017. Archives of virology. 2018;163:2513-8.
[19] Liu X, Wan H, Ni X, Wu Y, Liu W. Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985–2001. Archives of virology. 2003;148:1387-403.
[20] Kim LM, King DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, et al. Phylogenetic diversity among low-virulence Newcastle disease viruses from waterfowl and shorebirds and comparison of genotype distributions to those of poultry-origin isolates. Journal of virology. 2007;81:12641-53.
[21] Umali DV, Ito H, Shirota K, Katoh H, Ito T. Characterization of complete genome sequence of genotype VI and VII velogenic Newcastle disease virus from Japan. Virus genes. 2014;49:89-99.
[22] Meng C, Rehman ZU, Liu K, Qiu X, Tan L, Sun Y, et al. Potential of genotype VII Newcastle disease viruses to cause differential infections in chickens and ducks. Transboundary and emerging diseases. 2018;65:1851-62.
[23] Roohani K, Tan SW, Yeap SK, Ideris A, Bejo MH, Omar AR. Characterisation of genotype VII Newcastle disease virus (NDV) isolated from NDV vaccinated chickens, and the efficacy of LaSota and recombinant genotype VII vaccines against challenge with velogenic NDV. Journal of veterinary science. 2015;16:447-57.
[24] Habib M, Yaqub T, Nazir J, Shehzad W, Sohail T, Mukhtar N, et al. Genomic and biological characterization of Newcastle disease viruses isolated from migratory mallards (Anas platyrhynchos). Archives of virology. 2018:1-10.
[25] Akbar M, Mushtaq-Ul-Hassan M, Mahmood-Ul-Hassan M, Hassan M. Waterfowl diversity at Chashma barrage (Wildlife Sanctuary Mianwali) and Marala headworks (Game Reserve Sialkot), Pakistan during 1996-2005. International Journal of Agriculture and Biology. 2009;11:188-92.
[26] Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic acids symposium series: [London]: Information Retrieval Ltd., c1979-c2000.; 1999. p. 95-8.
[27] Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular biology and evolution. 2013;30:2725-9.
[28] Alexander D, Chettle N. Procedures for the haemagglutination and the haemagglutination inhibition tests for avian infectious bronchitis virus. Avian Pathology. 1977;6:9-17.

[29] Munir M, Linde A-M, Zohari S, Ståhl K, Baule C, Holm K, et al. Complete genome analysis of an avian paramyxovirus type 1 strain isolated in 1994 from an asymptomatic black-headed gull (Larus ridibundus) in southern Sweden. Avian diseases. 2010;54:923-30.
[30] Ali M, Yaqub T, Mukhtar N, Imran M, Ghafoor A, Shahid MF, et al. Prevalence and phylogenetics of H9N2 in backyard and commercial poultry in Pakistan. Avian diseases. 2017;62:416-24.
[31] Stear M. OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (Mammals, Birds and Bees) 5th Edn. Volumes 1 & 2. World Organization for Animal Health 2004. ISBN 92 9044 622 6.€ 140. Parasitology. 2005;130:727-.
[32] Feldman AT, Wolfe D. Tissue processing and hematoxylin and eosin staining. Histopathology: Springer; 2014. p. 31-43.
[33] Fiebig L, Soyka J, Buda S, Buchholz U, Dehnert M, Haas W. Avian influenza A (H5N1) in humans: new insights from a line list of World Health Organization confirmed cases, September 2006 to August 2010. 2011.
[34] Uyeki TM. Human infection with highly pathogenic avian influenza A (H5N1) virus: review of clinical issues. Clinical infectious diseases. 2009;49:279-90.
[35] Perozo F, Marcano R, Afonso CL. Biological and phylogenetic characterization of a genotype VII Newcastle disease virus from Venezuela: efficacy of field vaccination. Journal of clinical microbiology. 2012:JCM. 06506-11.
[36] Miller PJ, Kim LM, Ip HS, Afonso CL. Evolutionary dynamics of Newcastle disease virus. Virology. 2009;391:64-72.
[37] Aziz-ul-Rahman, Yaqub T, Imran M, Habib M, Sohail T, Furqan Shahid M, et al. Phylogenomics and Infectious Potential of Avian Avulaviruses Species-Type 1 Isolated from Healthy Green-Winged Teal (Anas carolinensis) from a Wetland Sanctuary of Indus River. Avian diseases. 2018;62:404-15.
[38] Yang C-Y, Shieh HK, Lin Y-L, Chang P-C. Newcastle disease virus isolated from recent outbreaks in Taiwan phylogenetically related to viruses (genotype VII) from recent outbreaks in western Europe. Avian diseases. 1999:125-30.
[39] Shabbir MZ, Akhtar S, Tang Y, Yaqub T, Ahmad A, Mustafa G, et al. Infectivity of wild bird-origin avian paramyxovirus serotype 1 and vaccine effectiveness in chickens. Journal of General Virology. 2016;97:3161-73.
[40] Ferreira HL, Taylor TL, Dimitrov KM, Sabra M, Afonso CL, Suarez DL. Virulent Newcastle disease viruses from chicken origin are more pathogenic and transmissible to chickens than viruses normally maintained in wild birds. Veterinary microbiology. 2019.
[41] Dimitrov KM, Bolotin V, Muzyka D, Goraichuk IV, Solodiankin O, Gerilovych A, et al. Repeated isolation of virulent Newcastle disease viruses of sub-genotype VIId from backyard chickens in Bulgaria and Ukraine between 2002 and 2013. Archives of virology. 2016;161:3345-53.
[42] Perozo F, Marcano R, Afonso CL. Biological and phylogenetic characterization of a genotype VII Newcastle disease virus from Venezuela: efficacy of field vaccination. Journal of clinical microbiology. 2012;50:1204-8.
[43] Munir M, Chowdhury M, Ahmed Z. Emergence of new sub-genotypes of Newcastle disease virus in Pakistan. J Avian Res. 2016;2:1-7.
[44] Ghalyanchilangeroudi A, Hosseini H, Jabbarifakhr M, Fallah Mehrabadi MH, Najafi H, Ghafouri SA, et al. Emergence of a virulent genotype VIIi of Newcastle disease virus in Iran. Avian Pathology. 2018;47:509-19.

[45] Rehman H, Fawad N, Abbas G, Naheed G, Siddique B, Afzal F, et al. Surveillance of poultry diseases in Punjab province, Pakistan; special reference to Newcastle disease. Res j vet pract. 2013;1:1-4.
[46] Desingu P, Singh S, Dhama K, Kumar OV, Malik Y, Singh R. Clinicopathological characterization of experimental infection in chickens with sub-genotype VIIi Newcastle disease virus isolated from peafowl. Microbial pathogenesis. 2017;105:8-12.