Title Page:

Co-infection of emerging fifth panzootic genotype XIII of Newcastle disease virus with low pathogenicity avian influenza virus-H9N2 exacerbates the clinical disease in Newcastle disease vaccinated layer flocks

Vasudevan Gowthaman[1#]**, Shambhu Dayal Singh[1], Kuldeep Dhama[1], and Palani Srinivasan[2], and Muhammad Munir[3]

[1]Avian Diseases Section, Indian Veterinary Research Institute, Izatnagar -243 122, India

[2#]Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Namakkal -637 002, India

[3]The Pirbright Institute, Woking, Surrey, GU24 0NF, United Kingdom

**Corresponding Author:

Dr. Vasudevan Gowthaman

Avian Diseases Section, Division of Pathology, Indian Veterinary Research Institute, Izatnagar - 243 122, India; e-mail: vetgowth@yahoo.co.uk. Telephone-+91-9488764261

Current Address

Abstract:

Background:

Newcastle disease (ND) and avian influenza (AI) are infectious and economically important diseases of poultry, and multiple factors including secondary viral and bacterial infections contribute significantly in the ND/AI pathobiology. The disease pattern during co-infections of genotype XIII NDV and AI is not well understood. So far, there has been no strong explanation for such difference in mortality and severity of clinical disease. In our present case study, we describe clinico-pathological disease condition of genotype XIII NDV and its co-infection with LPAI-H9N2 subtype, which could further helpful to understand the interactions of these two economically important poultry viruses. To address interaction of two respiratory infections, ND virus (NDV) and avian influenza virus (AIV), a total of 37 commercial poultry flocks were investigated in the southern peninsular India.

Results:

Clinicopathological as well as molecular biological investigations identified simultaneous circulation of NDV and AIV in same flock/bird. Sequence analysis of hemagglutinin and neuraminidase genes revealed that all identified AIVs were belonging to low pathogenicity H9N2 subtype whereas the analysis of the fusion genes revealed that detected NDVs belong to virulent type, and revealed that NDV strains currently circulating in India are belong to NDV class II, genotype XIII. The NDV alone as well H9N2 and NDV co-infected flocks exhibited clinical signs and lesions similar to that of virulent NDV except the degree of severity, which was higher in H9N2-NDV co-infected flocks. Furthermore, E. coli and mycoplasma were

detected in all the ailing/dead birds from the co-infected flocks during progression of the clinical disease.

Conclusion:

The results demonstrate the importance of emerging fifth panzootic genotype XIII NDV in exacerbating clinical disease in vaccinated birds. The results not only present understanding on the virus-virus interaction and consequences on the bird's health but also highlight the multi-factorial disease complexity in commercial poultry. Further studies are needed to investigate the molecular mechanisms of these interactions and their cumulative impact on the poultry health.

Key words: Clinico-pathological patterns, Newcastle Disease, Low Pathogenic Avian Influenza; E. coli, mycoplasma, poultry.

Background

Newcastle disease (ND) and avian influenza (AI) are the most devastating diseases of poultry and remained the foremost constrain to the growth of poultry industry around the globe. The ND is caused by the avian paramyxovirus type-1 (APMV-1), which is a member of the genus Avulavirus in the Paramyxoviridae family under the order Mononegavirales [1,2]. The virulent and avirulent ND viruses (NDVs) have the sequence of ^{112}R/K-R-Q/K/R-K/R-R-F^{117} and ^{112}G/E-K/R-Q-G/E-R-L^{117} in fusion protein (F) cleavage site, respectively [3]. All APMVs can be divided into 13 serotypes (APMV 1-13) out of which APMV-1 is found to be associated with naturally occurring NDV infections with major economic consequences [4]. The APMV-1 can be divided into two distinct clades: class I and II, and class II is subdivided into 18 recognized genotypes [5]. The most recent fifth panzootic velogenic NDV (vNDV) belongs to a new sub-genotype VIIi, VIIh, XIIIa and XIIIb [1].

Avian influenza is caused by various subtypes of influenza viruses of the family Orthomyxoviridae [6]. Two types of AI viruses (AIV) have been described based on their pathogenicity *viz.* high pathogenicity avian influenza viruses (HPAIV) that cause severe disease with high mortality, and low pathogenicity avian influenza virus (LPAIV) that generally causes asymptomatic infection or a mild disease [7]. ND and AI are the primary viral diseases of poultry worldwide and co-infection of NDV and AIV has been reported frequently [8–11]. However, reports are emerging on the detection of genotype XIII of NDV in vaccinated poultry flocks in several Asian countries [1,12–14]. Additionally, NDV co-associate with LPAIV-H9N2 leading to severe disease and the co-infection of genotype XIII NDV with LPAI results in high mortality compared to pure form of NDV [15]. The disease patterns during co-infections of genotype XIII

NDV and LPAI is not well understood. Currently, our understanding is limited on the impact of NDV-AIV confection on the mortality and severity of clinical disease in field conditions.

In this case study, we describe clinicopathological disease caused by the genotype XIII of NDV and its exacerbation with the co-infecting LPAI-H9N2 subtype in the field conditions. The information presented will not only help to understand the virus-virus interactions and consequences on the bird's health but would also highlight the multi-factorial disease complexity in the natural field conditions.

Methods

Case history, pathological studies and sample collection

The study was carried out in 37 commercial poultry flocks from southern India with a history of respiratory/neurological signs and production drops during the period between January 2010 - 2012. The age of the flocks affected ranged from four to 76 weeks and the size of the flocks ranged from 3000-50000. All the birds were reared under standard managemental conditions recommended by the national breeding companies. All poultry flocks were vaccinated against following respiratory pathogens *viz.,* Newcastle disease (F strain, LaSota, R2B, and Kumarov), infectious bronchitis, fowl pox and infectious coryza. Necropsy examination was carried out on dead and ailing birds. Tissue samples such as brain, trachea, lungs, kidneys, spleen and intestines were collected for virus isolation and histopathological studies. Heart blood, tracheal and airsac swabs were collected for bacterial and mycoplasmal isolation. The clinical disease pattern was monitored until the mortality decreased to average rates.

Virus Isolation

The tissue samples were homogenized in phosphate buffered saline (PBS, pH 7.4) to obtain a 10% suspension, and clarified at 12,000 ×g for 10 min. After 1 h incubation with antibiotics, 100 µL of suspension was inoculated into three 11 day-old specific pathogen free (SPF) embryonated chicken eggs via allantoic route. The eggs were incubated at 37 °C till embryo death or up to 5 days, and haemagglutination (HA) test was carried out on amnioallantoic fluid (AAF) [3]. Three blind passages were carried out before deciding the negativity of the samples.

Histopathology

The collected tissues were processed and embedded in paraffin. Sections of 4 µm thickness were made and stained with Haematoxylin & Eosin [16].

Screening of NDV and AIV

Viral RNA from HA positive AAF was extracted using QIAamp Viral RNA Mini Kit (Qiagen, Germany). cDNA was synthesized by random primer using SuperScript® First–Strand Synthesis System for RT-PCR (Invitrogen, Germany). The presence of AIV and NDV was confirmed by PCR targeting of M gene [17], and F gene [18]. Typing of influenza A viruses and NDV was done by further PCR amplification, sequencing of the HA, NA [19], F genes of AIV and NDV, respectively.

Phylogenetic Analysis

The consensus from each of the NDV positive samples were aligned with representative strain of known genotype for epidemiological investigations in the BioEdit. The alignment was

edited to equal length and was used to construct neighbor-joining tree in MEGA6 with 1000 bootstrap values. Simultaneously, the sequences under-study were also analyzed with the available sequence in the public domain to determine the global clustering pattern of these isolates

Partial nucleotide sequences for the HA (616 bp) and NA (570) were aligned with CLC 6.6.2 (CLC Bio, Aarhus, Denmark). Maximum-likelihood analyses were performed using the software PhyML 3.0 [20] with the general time reversible (GTR) evolutionary model, an estimation of the proportion of invariable sites (I) and of the nucleotide heterogeneity of substitution rates (α), as selected by ModelGenerator 0.85 [21]. Nodal supports were assessed with 1000 bootstrap replicates.

Bacterial isolation and molecular detection

All the swabs were processed as per standard methods for bacterial isolation described by AAAP [22]. Bacterial DNA from the culture was extracted using Wizard® Genomic DNA Purification Kit (Promega, USA). The presence of *E. coli*, *Avibacterium paragallinarum* and *Ornithobacterium rhinotracheale* was screened by PCR targeting of 16S gene [23], followed by restriction fragment length polymorphism (RFLP) by using restriction enzymes such as *DdeI, RsaI*, and *EcoRI* (M/s Fermentas, USA).

Screening for Mycoplasma

Isolation of mycoplasmas form the tracheal/airsac swabs was carried out in Frey's modified mycoplasma broth and agar medium [24]. The mycoplasmal DNA from all the cultures was isolated by using Wizard® Genomic DNA Purification Kit (Promega, USA). The presence of mycoplasma was confirmed by PCR targeting of 16S gene of Mycoplasma[24].

Results

Virological and epidemiological findings

Out of 37 poultry flocks screened, NDV of virulent pathotype was detected in 22 farms (59.5%) and LPAI virus (H9N2) in 11 farms (29.7%) by virus isolation, RT-PCR and sequencing. Interestingly, all AIV positive flocks were simultaneously co-infected with NDV. Samples from all 22 flocks were further processed for genetic and phylogenetic analysis. Sequence analysis of HA and NA genes of AIV revealed that all were belonging to low pathogenic H9N2 subtype (RSSR*G in HA gene). Putative protein sequence analysis of F protein of the isolate revealed that presence of a motif, RRRKR*F, typical for the velogenic strains of NDV which complement the clinical picture of the disease.

To understand the epidemiological clustering and distribution patterns of the NDV co-infecting the commercial poultry along with influenza, F gene based phylogenetic analysis was performed. The clustering pattern indicates the grouping of the isolate into XIII genotype within Class II (Fig. 1A). Next we performed a higher resolution analysis of the under-study isolate with representative strains of the all known and characterized genotypes. The analysis of the 3' hypervariable region of the F gene suggests NDV strains currently circulating in India are belong to NDV class II, genotype XIII, and forming potentially a separate sub-clade which differs from previously known genotype XIII ancestral, XIIIa and XIIIb viruses (Fig. 1B).

Phylogenetic analyses of the partial nucleotides of the HA and NA genes revealed that multiple H9N2 genetic lineages are circulating in the poultry population of South Asia and in the Mideast during the last decade (Fig. 2 and 3). Although the H9N2 subtype have been isolated from chicken and ducks in Eastern Asia, analysis of the HA gene suggests that it is different

from the one documented in South Asia (India, Pakistan, Bangladesh). Nucleotide sequences obtained from the viruses isolated in this study were genetically related and formed independent genetic lineages. Based on the HA gene, these viruses may be closely related to AIVs that were isolated in India between 2003 and 2006, and also in Bangladesh, although low statistical support for internal nodes in the phylogenetic tree preclude definitive conclusion on their origin. The analysis performed on the NA gene did not provided more precise information on the geographic origin of the viruses isolated in the present study.

The mortality of the disease was comparatively high in the flocks co-infected with NDV and AIV (2.6 - 44.40%) than NDV alone infected flocks (0.8 -12 %). Flocks infected with only the NDV were found to recover as early as 10-15 days post infection, whereas the recovery period was prolonged to one month in NDV and AIV co-infected flocks. Further, E. coli and mycoplasma were detected in all the ailing/dead birds from the above co-infected flocks during progression of the clinical disease, (after 7 days of initial appearance of clinical disease); whereas detection rate of E. coli and mycoplasma was only in seven NDV alone infected flocks. The mortality attained peak after 2-3 days of initial detection of mycoplasma and E. coli in both co-infection and NDV alone infected flocks, thereafter it declined rapidly in NDV alone infected flocks, whereas it extended up to one month in LPAI co-infected flocks.

Clinical and pathological findings in young birds (growers) in LPAI co-infected flocks:

During initial stages of the outbreaks, sudden death without any premonitory signs of the disease was observed (1-4 days). Later, the signs progressed as dullness, depression, somnolascence (Fig. 4A), ruffled feathers, reduced feed intake, water consumption, stunted growth (Fig. 4B), watery/greenish/white diarrhoea and prostration. Gasping (Fig. 4C), coryza

and rales were suggestive of severe respiratory distress. Progressive neurological signs including of torticollis (Fig. 4D), head twitching, leg weakness, leg paralysis and loss of clutch reflex were consistently observed. The entire clinical course of the disease persisted for 3-4 weeks in all the complicated cases and the growers succumbed more rapidly than layers. Mortality rate was recorded between 15.4 and 44.4 %.

On postmortem examination, per-acutely died birds showed mild to marked congestion in the visceral organs. Birds died after 4-5 days of initial disease outbreak showed emaciated carcass, congestion of brain, haemorrhagic/catarrhal tracheitis, airsacculitis, diffuse pulmonary congestion and oedema (Fig. 4E), haemorrhagic proventriculitis, button ulcers in the small intestine, necrotic pancreatitis, spleenic atrophy and or mottling, nephritis-nephrosis complex and cloacal haemorrhages. The birds died one week after onset of clinical disease exhibited, thoracic airsacculitis, fibrinous adhesive pericarditis and fibrinous perihepatitis.

In histopathology, significant alterations were observed in brain, trachea, lung, proventriculus, intestine, pancreas, spleen, and kidney. Glial cell proliferation, neuronal shrinkage and neuronophagia were observed in the brain. Trachea showed extensive erosion of the epithelial layer which exposed the stromal vessels leading to the initiation and progression of haemorrhages. The lesions in the lungs consisted of peribronchiolar oedema, and infiltration of mononuclear inflammatory cells. Marked haemorrhages and degeneration were observed in the mucosal folds of proventriculus. The predominant lesions in the intestines included degeneration and sloughing off of the epithelial cells covering tips of villi and mononuclear infiltrations. The lesions in the pancreas consisted of multifocal acute cellular swelling resulting in necrosis and shrunken acinar cells devoid of zymogen granules. Focal vacuolation and depletion of the

follicular lymphocytes was observed in the spleen. In kidney, the lesions consisted of tubular degeneration and necrosis with mild interstitial haemorrhages.

Clinical disease and pathological findings in adult birds (Layers) in LPAI co-infected flocks:

Spread of the disease was comparatively slower in adult layers than young birds. The mortality rate ranged between 2.6-4.9%. Clinical signs consisted of depression, prostration, somnolascence, decreased feed intake and water consumption, facial swelling, cyanotic combs and wattles (Fig. 4F), drop/complete cessation of egg production, purulent conjunctivitis, watery diarrhea, gasping, loss of egg quality; including uneven sized and leathery eggs, small eggs etc. However, the respiratory signs were less severe in layers when compared to growers. Interestingly, the layers failed to exhibit any neurological signs, which were prominent in growers.

In necropsy examination, no lesions were detected form the birds that died during initial stage of the clinical disease (1-4 days), but the NDV and AIV could be detected from AAF. After 4 days the lesions were largely confined to proventriculus, visceral and parietal peritoneum and abdominal cavity. Cachexia, caseous plugs in the larynx, haemorrhagic/catarrhal tracheitis/clear trachea, pulmonary congestion and edema, airsacculitis, petechiae in serosal layer of heart and abdominal fat (Fig. 4G), haemorrhagic proventriculitis (Fig. 4H), catarrhal enteritis, necrotic pancreatitis, atrophy of spleen, egg peritonitis, oophoritis, flaccid, misshapen/ruptured/haemorrhagic follicles (Fig. 4I), salpingitis nephritis-nephrosis complex and cloacal haemorrhages were observed in entire course of the outbreaks. Fibrinous perihepatitis,

pericarditis and abdominal airsacculitis (Fig. 4J) were observed after one week of the clinical onset. The clinical course of the outbreak was continued for 2-3 weeks.

The prevailing histopathological alterations were restricted to the trachea, pancreas, spleen, peritoneal cavity, ovary and uterus. The lesions observed in respiratory, digestive tract, pancreas, spleen and kidneys were similar to that of HP lesions exhibited by growers. In addition to that diffuse serositis (peritonitis) including severe engorgement of vasculature, fibrous tissue proliferation and mononuclear cell infiltrations were observed in peritoneal cavity. Atretic follicles were characterized by the shrunken oocyte, separation of granulosa layer from thecal layer, and shrinkage of yolk spherules. Degeneration of granulosa and marked to severe haemorrhges in the follicular cavity of non-bursting type of follicles were also noticed. Oviduct showed atrophy and degeneration of tubular glands along with infiltrations of mononuclear inflammatory cells.

Clinical disease and pathological findings in NDV alone flocks:

The young and adult birds exhibited similar clinical signs and lesions, but the severity of symptoms and lesions were less and recovery was faster when compared to LPAI co-infected flocks.

Discussion

Although, several reports are available on concurrent infections of poultry with various viral and bacterial agents, especially in respiratory disease complex (RDC) cases [8,25–28] , studies on co- occurrence of LPAI and NDV in chicken is very limited [11,29] and no description about the clinic-pathological patterns of these two agents during natural co-infection. This study demonstrated the natural co-infection of NDV, LPAI, mycoplasma and E. coli

resulted in severe clinical disease and high mortality when compared to pure form of NDV infection. These findings support the hypothesis that NDV infection makes the signs of LPAI-H9N2 infection more severe and predispose the animals for superinfection with E.coli and mycoplasma and vice versa. Newcastle disease and LPAI -H9N2 can produce severe disease depending on the type of secondary pathogen present [30,31]. The previous studies of [32] revealed that dual infection of APV with either NDV or E. coli resulted in increased morbidity rates. Mixed infections of H9N2 and other respiratory pathogens such as IBV and M. gallisepticum, can cause mortality between 20% and 60% in affected flocks [11,33]. Previous experimental studies of [34] revealed that co-infection of the broilers with ORT and H9N2 virus isolates induced higher mortality than infection with ORT or H9N2 virus alone. Co-infection of AIV and NDV demonstrated that co-infections can exacerbate clinical disease, affect virus replication, serological conversion and virus transmission [35,36] .

In the current study the course of infection in NDV-LPAI co-infections extended up to 3-4 weeks, while compared to pure form of NDV infections, which lost up to 2 weeks. This could be due to potentiation of LPAI by NDV and its complicating agents like mycoplasma and E. coli. The mortality attained peak after 2-3 days of initial detection of mycoplasma and E. coli, suggesting that bacteria may play some role in the exhibition of the clinical syndrome. Co-infection of infectious bronchitis live vaccine and H9N2 avian influenza virus led to an extension of the shedding period of H9N2 virus, increasing the severity of clinical signs and mortality rates, causing macroscopic lesions in the embryos [37]. Experimental co-infection of poults with APV/NDV had longer periods of virus recovery, and a higher percentage of positive birds at each sampling[32]. This finding supported the hypothesis that the APV disease pattern in the field may be linked to concurrent NDV infections.

The clinic-pathological alterations were in concurrence with the previous studies on LPAI and/or NDV [38,39]. Since H9N2 produces minimal or no lesions when inoculated in to SPF chickens[38], other viruses and bacteria might have exacerbated LPAI in field conditions. The pathological alterations were almost similar (except degree of severity) in NDV-LPAI co-infected and NDV alone-infected flocks. This suggests that the NDV might play a triggering role in induction of clinic-pathological alterations. Protein cleavage site (HA0) of LPAI is only cleaved by trypsin-like proteases present in respiratory, gastrointestinal and reproductive tracts. When birds are co-infected with H9N2 and bacteria, the latter may release bacterial proteases into the other tissues which will cleave the HA0 and enhance its replication in multiple sites [37,40,41]. For instance, it was demonstrated that the protease of S. aureus activated the HA of influenza virus, allowing multiple cycles of virus replication in the lungs of mice [42]. Alternatively, the stress of bacterial infection might have affected the immune system of chickens and exacerbated the pathogenicity of H9N2 influenza virus infection[41].

NDV cause severe lymphocytolysis leading to immuno-suppression, which paves way for replication of LPAI and both virus cause damage in respiratory epithelial cell lead to colonization of bacterial agent [43,44]. Therefore, our results suggest that the genotype XIII NDV triggered a severe clinical disease in co-infection with LPAI. It is also assumed that mycoplasma and E. coli might have played a key role in this exacerbating effect. However, these finding needs to be evaluated carefully since no experimental attempts/demonstration of organisms in tissues were made. In order to fully prove the hypothesis about the existence of synergism after simultaneous infection with genotype XIII NDV, LPAI and other pathogens, an experimental study is highly desirable.

Conclusions

In conclusion, emerging fifth panzootic Genotype XIII NDV causes disease in vaccinated birds. Clinical disease and mortality of genotype XIII NDV in vaccinated flocks were exacerbated by LPAI-H9N2 co-infection or vice versa. The co-infection leads to severe disease in young grower than adult layer birds. Moreover, secondary bacterial infection with mycoplasma and *E.coli* caused exacerbation of clinical disease leading to huge mortality. The co-infection with LPAIV and NDV present an overlapping clinical and pathological picture often misleading the identification and diagnosis of both of these viruses. Several AIV infected countries including India not practicing vaccination against AIV, and they only impose culling followed by enhanced biosecurity and surveillance for control of AIV. In this context, prevention of genotype XIII NDV is most needed. Additional studies are needed to identify the mechanism in genotype XIII NDV evading protective antibody and formulate proper vaccination strategy. Further research is warranted in order to ascertain the individual and cumulative possible contributions in the disease intensity and how a virus-virus interaction influences the clinical outcome in these economically important diseases of poultry.

Declaration:

Availability of Data and Materials: All reported data has been submitted to the public domain of NCBI and are accessible under accession numbers KF750607.1 , KF750608.1, KF750609.1, KF750610.1, KF750611.1, KF750612.1, KF750613.1, HG780871.1, HG780867.1, HG780868.,1 HG780869.1, HG780870.1, CY099336.1, CY099337.1, CY099338.1, CY099339.1, CY099340.1, CY099341.1, CY099350.1, CY099351.1, CY099352.1, CY099353.1, CY099354.1, CY099355.1, CY099356.1, CY099357.1,

Ethical Statement: All animal handling procedures were carried out in strict accordance with the guidance and regulations of Animal Welfare and Health as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), of Government of India. As part of this process the work has been approved by the ethics committee (F/No. 1209/IVRI/AEC/2010-2011) at the Indian Veterinary Research Institute, India.

Abbreviations: APMV-1: Avian Paramyxovirus serotype I, NDV: Newcastle disease virus, Newcastle disease: ND, avian influenza: AI, fusion protein: F, velogenic NDV: vNDV, high pathogenicity avian influenza viruses: HPAIV, low pathogenicity avian influenza virus: LPAIV, Haemagglutination: HA, specific pathogen free: SPF, amnioallantoic fluid: AAF, restriction fragment length polymorphism: RFLP, respiratory disease complex: RDC

Competing interests

The author(s) declare that they have no competing interests.

Authors' contribution

VG carried out the field and laboratory work, and drafted the manuscript. SDS and KD played a critical role in project development, manuscript preparation and obtained the funding to carry out this research. KS helped for pathological studies, MM did phylogenetic analysis. All the authors edited and approved the manuscript prior to submission.

Author Details

[1]Avian Diseases Section, Indian Veterinary Research Institute, Izatnagar -243 122, India

[2#]Poultry Disease Diagnosis and Surveillance Laboratory, Veterinary College and Research Institute Campus, Namakkal -637 002, India

[3]The Pirbright Institute, Woking, Surrey, GU24 0NF, United Kingdom

Acknowledgments

Authors are thankful to Indian Veterinary Research Institute and Indian Council of Agricultural Research for providing facilities and funds for carrying out the study

References

1. 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. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 2015;29:216–29.

2. Susta L, Jones MEB, Cattoli G, Cardenas-Garcia S, Miller PJ, Brown CC, et al. Pathologic characterization of genotypes XIV and XVII Newcastle disease viruses and efficacy of classical vaccination on specific pathogen-free birds. Vet. Pathol. 2015;52:120–31.

3. OIE. Newcastle Disease. Man Diagn Tests Vaccines Terr Anim Chapter 2314. 2012;

4. Terregino C, Aldous EW, Heidari A, Fuller CM, De Nardi R, Manvell RJ, et al. Antigenic and genetic analyses of isolate APMV/wigeon/Italy/3920-1/2005 indicate that it represents a new avian paramyxovirus (APMV-12). Arch. Virol. 2013;158:2233–43.

5. Diel G, da Silva LHA, Liu H, Wang Z, Miller PJ, Afonso CL. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of

Newcastle disease virus genotypes. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 2012;12:1770–9.

6. Lee D-H, Park J-K, Yuk S-S, Erdene-Ochir T-O, Kwon J-H, Lee J-B, et al. Complete genome sequence of a natural reassortant H9N2 avian influenza virus found in bean goose (Anser fabalis): direct evidence for virus exchange between Korea and China via wild birds. Infect. Genet. Evol. J. Mol. Epidemiol. Evol. Genet. Infect. Dis. 2014;26:250–4.

7. Capua I, Alexander DJ. Avian influenza: recent developments. Avian Pathol. J. WVPA. 2004;33:393–404.

8. Dormitorio TV, Giambrone JJ, Guo K, Hepp GR. Detection and characterization of avian influenza and other avian paramyxoviruses from wild waterfowl in parts of the southeastern United States. Poult. Sci. 2009;88:851–5.

9. El Zowalaty ME, Chander Y, Redig PT, Abd El Latif HK, El Sayed MA, Goyal SM. Selective isolation of Avian influenza virus (AIV) from cloacal samples containing AIV and Newcastle disease virus. J. Vet. Diagn. Investig. Off. Publ. Am. Assoc. Vet. Lab. Diagn. Inc. 2011;23:330–2.

10. Goekjian VH, Smith JT, Howell DL, Senne DA, Swayne DE, Stallknecht DE. Avian influenza viruses and avian paramyxoviruses in wintering and breeding waterfowl populations in North Carolina, USA. J. Wildl. Dis. 2011;47:240–5.

11. Roussan DA, Haddad R, Khawaldeh G. Molecular survey of avian respiratory pathogens in commercial broiler chicken flocks with respiratory diseases in Jordan. Poult. Sci. 2008;87:444–8.

12. Jakhesara SJ, Prasad VVSP, Pal JK, Jhala MK, Prajapati KS, Joshi CG. Pathotypic and Sequence Characterization of Newcastle Disease Viruses from Vaccinated Chickens Reveals Circulation of Genotype II, IV and XIII and in India. Transbound. Emerg. Dis. 2014;

13. Khorajiya JH, Sunanda Pandey, Ghodasara PD, Joshi BP, Prajapati KS, Ghodasara DJ, et al. Patho-epidemiological study on Genotype-XIII Newcastle disease virus infection in commercial vaccinated layer farms. Vet. World. 2015;8:372–81.

14. Siddique N, Naeem K, Abbas MA, Ali Malik A, Rashid F, Rafique S, et al. Sequence and phylogenetic analysis of virulent Newcastle disease virus isolates from Pakistan during 2009-2013 reveals circulation of new sub genotype. Virology. 2013;444:37–40.

15. Gowthaman V. Etio-Pathology and Differential Diagnosis of Low Pathogenic Avian Influenza (LPAI) in Poultry. PhD Thesis. Indian Vet. Reserach Inst. 2011;

16. Luna L. Manual on Histologic Staining Methods of the Armed Force Institute of Pathology. 3rd Ed. McGraw-Hill Book Co USA. 1968;32–46.

17. Ottiger H-P. Development, standardization and assessment of PCR systems for purity testing of avian viral vaccines. Biol. J. Int. Assoc. Biol. Stand. 2010;38:381–8.

18. Toyoda T, Sakaguchi T, Hirota H, Gotoh B, Kuma K, Miyata T, et al. Newcastle disease virus evolution. II. Lack of gene recombination in generating virulent and avirulent strains. Virology. 1989;169:273–82.

19. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch. Virol. 2001;146:2275–89.

20. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 2010;59:307–21.

21. Keane TM, Creevey CJ, Pentony MM, Naughton TJ, Mclnerney JO. Assessment of methods for amino acid matrix selection and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol. Biol. 2006;6:29.

22. Glisson J, Jackwood M, Pearson J, Reed, W, Swayne D, Woolcock P. Isolation, Identification, and Characterization of Avian Pathogens. Am Assoc Avian Pathol 5th Ed. 2008;

23. Mendoza-Espinoza A, Koga Y, Zavaleta AI. Amplified 16S ribosomal DNA restriction analysis for identification of Avibacterium paragallinarum. Avian Dis. 2008;52:54–8.

24. OIE. Avian Mycoplasmosis. Man. Diagn. Tests Vaccines Terr. Anim. 2008;482–96.

25. Ahmed Z, Pandurang G, Acharya RS, Parihar NS. A report on outbreaks of respiratory disease in chicken in andhra pradesh with particular reference to infectious laryngotracheitis. Indian Vet. J. 1969;46:646–50.

26. Bradbury JM. Avian mycoplasma infections: prototype of mixed infections with mycoplasmas, bacteria and viruses. Ann. Microbiol. (Paris). 1984;135A:83–9.

27. Malik BS, Verma KC. Coexistence of antibodies against chronic respiratory disease, infectious laryngotracheitis, and infectious bronchitis on poultry farms of Uttar Pradesh, Andhra Pradesh, and Madras. Avian Dis. 1969;13:695–9.

28. Smietanka K, Minta Z, Swiętoń E, Olszewska M, Jóźwiak M, Domańska-Blicharz K, et al. Avian influenza H9N2 subtype in Poland--characterization of the isolates and evidence of concomitant infections. Avian Pathol. J. WVPA. 2014;43:427–36.

29. Al-Mohana A, Kadhimv H, Al-Charrakh A, Al-Habubi Z, Nasir F, Al-Hilali A, et al. Molecular diagnosis of avian respiratory diseases in commercial broiler chicken flocks in province of Najaf,Iraq. Sci Res Essays. 2013;8:1191–5.

30. Seififi S, Asasi K, Ali Mohammadi. Natural co-infection caused by avian inflfl uenza H9 subtype and infectious bronchitis viruses in broiler chicken farms. Vet. Arh. 2010;80:269–81.

31. Spickler A., Roth J. Emerging and Exotic Diseases of Animals. CFSPH Iowa State Univ. 2008;203–4.

32. Turpin EA, Perkins LEL, Swayne DE. Experimental infection of turkeys with avian pneumovirus and either Newcastle disease virus or Escherichia coli. Avian Dis. 2002;46:412–22.

33. Nili H, Asasi K. Avian influenza (H9N2) outbreak in Iran. Avian Dis. 2003;47:828–31.

34. Pan Q, Liu A, Zhang F, Ling Y, Ou C, Hou N, et al. Co-infection of broilers with Ornithobacterium rhinotracheale and H9N2 avian influenza virus. BMC Vet. Res. 2012;8:104.

35. Costa-Hurtado M, Afonso CL, Miller PJ, Spackman E, Kapczynski DR, Swayne DE, et al. Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic Newcastle disease virus in experimental co-infections in chickens and turkeys. Vet. Res. 2014;45:1.

36. França M, Howerth EW, Carter D, Byas A, Poulson R, Afonso CL, et al. Co-infection of mallards with low-virulence Newcastle disease virus and low-pathogenic avian influenza virus. Avian Pathol. J. WVPA. 2014;43:96–104.

37. Haghighat-Jahromi M, Asasi K, Nili H, Dadras H, Shooshtari AH. Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Arch. Virol. 2008;153:651–5.

38. Mo IP, Brugh M, Fletcher OJ, Rowland GN, Swayne DE. Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. Avian Dis. 1997;41:125–36.

39. Shalaby AA, Slemons RD, Swayne DE. Pathological studies of A/chicken/Alabama/7395/75 (H4N8) influenza virus in specific-pathogen-free laying hens. Avian Dis. 1994;38:22–32.

40. Bano S, Naeem K, Malik SA. Evaluation of pathogenic potential of avian influenza virus serotype H9N2 in chickens. Avian Dis. 2003;47:817–22.

41. Kishida N, Sakoda Y, Eto M, Sunaga Y, Kida H. Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. Arch. Virol. 2004;149:2095–104.

42. Tashiro M, Ciborowski P, Klenk HD, Pulverer G, Rott R. Role of Staphylococcus protease in the development of influenza pneumonia. Nature. 1987;325:536–7.

43. Kotani T, Odagiri Y, Nakamura J, Horiuchi T. Pathological changes of tracheal mucosa in chickens infected with lentogenic Newcastle disease virus. Avian Dis. 1987;31:491–7.

44. Mast J, Nanbru C, van den Berg T, Meulemans G. Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus. Vet. Pathol. 2005;42:559–65.

Legends for figures

Figure 1. A: Phylogenetic analysis of the partial F gene sequences representing different genotypes. Sequences analyzed in this study are marked with arrow. B: A higher resolution clustering patterns of the NDV strains studied here and available in public domains. Sequences presented here are marked with black filled circle. Boostraps lower than 80% are not shown.

Figure 2. Maximum likelihood consensus tree derived from H9 sequences from 178 H9 Influenza A virus hemagglutinin nucleotide sequences. Computations were realized with the GTR+I+G evolutionary model (I = 0.42 ; α = 1.13). Blue branches highlight viruses isolated in India. Sequences generated in this study and corresponding accession numbers are indicated in bold. Red dots indicated bootstrap values higher than 80. UAE: United Arab Emirates.

Figure 3. Maximum likelihood consensus tree derived from 160 N2 Influenza A virus neuraminidase nucleotide sequences. Computations were realized with the GTR+I+G evolutionary model (I = 0.29 ; α = 0.72). Blue branches highlight viruses isolated in India. Sequences generated in this study and corresponding accession numbers are indicated in bold. Red dots indicated bootstrap values higher than 80. UAE: United Arab Emirates.

Figure 4**.** Clinical picture of the virus infected birds. A: Birds exhibiting depression and somnalescence, B: Birds exhibiting uneven growth in the affected flock, C: The affected bird showing facial edema, lacrimal discharge, closed eye lids, severe respiratory distress with open mouth breathing, D: Affected bird displays torticollis, E: Lungs showing congestion, edema and frothy exudate, F: Dry, wrinkled, and cyanotic appearance of the comb, G: Multiple petechiae on the surface of gizzard fat, H: Moderate multifocal petechiae at the tips of proventricular papillae,

I: Acute follicular haemorrhage results in purplish/tarry and enlarged follicles with liver-like appearance, J: Thickened and opaque abdominal air sacs.