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EVALUATING THE INFECTION POTENTIAL OF FIELD PREVAILING NEWCASTLE DISEASE VIRUS AND INFECTIOUS BRONCHITIS VIRUS ALONGWITH ASSOCIATED MICROSCOPIC CHANGES IN COMMERCIAL POULTRY

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ABSTRACT

Newcastle disease virus (NDV) and Infectious bronchitis virus (IBV) are endemic to Pakistan. Though vaccine is being used to protect the birds from infection, outbreaks even in vaccinates are not uncommon throughout the year. Here, we assessed infectious potential of field prevailing NDV and IBV strains in broiler birds and determined a varying clinical exhibition and tissue tropism upon exposure to each of the virus either alone or in combination. At the age three week, the broiler chickens (n = 210) were divided into six groups (35 birds per group). These were group A (NDV-alone challenged), B (IBV-alone challenged), C (NDV+IBV-sequentially challenged), D (IBV+NDV-sequentially challenged), E (NDV and IBV-simultaneously challenged) and F (negative control). The birds were challenged with $10^{-5.76}$ and $10^{-6.03}$ EID_{50} dose of NDV and IBV, respectively. Tissue samples were taken from the infected birds after 2nd, 4th and 6th day post inoculation (dpi) for histopathology and immunohistochemistry (IHC). Clinical examination revealed a more severe form of respiratory infection in birds exposed with IBV, whereas, both respiratory and nervous signs were evident among birds infected with NDV. However, compared to birds infected with each of virus, severity of infection was much more in birds co-infected with both viruses. A variable degree of microscopic changes such as congestion and haemorrhages were observed in the trachea, lungs, proventriculus, caecal tonsil and kidney tissues. Evidenced through immunohistochemistry, the presence of antigen across various tissues of the birds indicated a wide spectrum of host tissue tropism for each of the virus where it was maximum in co-infected groups (C, D and E) than individual virus (groups A and B). The study concludes the currently prevailing strains are much pathogenic and that the presence of one virus facilitates further enhancement infection-associated tissue damage as a result of co-infection.

Key words: Newcastle disease virus, Infectious Bronchitis virus, Co-infection, Histopathology, Immunohistochemistry.

INTRODUCTION

The respiratory diseases, caused by Newcastle disease viruses (NDVs) (Shabbir *et al*., 2013), Infectious bronchitis viruses (IBVs) (Rafique *et al*., 2018) and avian influenza viruses (AIVs) (Kausar *et al*., 2018), are frequent in commercial poultry in Pakistan. These viruses have abundant economic concerns due to their ability to cause infection either independently or in association with other viruses such as avian influenza virus, infectious bronchitis virus (Alexander and Senne, 2008; Roussan *et al*., 2008) or with other bacterial pathogens such as *E.coli* (Qamar-un-Nisa *et al*., 2018; Umar *et al*., 2018).

Newcastle disease virus (NDV) is a non-segmented, single stranded negative sense RNA virus which is classified within family *Paramyxoviridae* (Alexander and Senne, 2008). The whole genome (15198 bp) of the virus usually follows that "rule-of-six", 5′-NP-P-M-F-HN-L-3′. Based upon pathogenicity, NDVs are categorized into three pathotypes named as velogenic, mesogenic and lentogenic (avirulent) (Alexander and Senne, 2008). On the other hand, IBV is a single stranded RNA virus that has positive-sense genome surrounded by an envelope of a lipid coat (Deming and Baric, 2008). The whole genome (27.6 kb nucleotide) encodes different structural and functional proteins in an order of 5′-S-M-E-N-3′ and, on the basis of molecular epidemiology, lineage VAR-II is continuously reported from chickens. It predominantly affects the respiratory tract (tracheal rales, gasping, sneezing and coughing), urinary tract (acute nephritis, stones development in the kidney microtubules and deposition of urates in the kidney) and the reproductive system (decline in egg production with inferior quality of eggs both internal and external) of the birds (Bande *et al*., 2016; Kiss *et al*., 2016).

Both diseases are endemic in Pakistan and, apart from this fact, co-infection with NDV and IBV consequences may induce severe morbidity and mortality rates in commercial poultry due to synergistic relationship among both viruses for tissue tropism (Nili and Asasi, 2002). However, scanty information regarding the alterations in histopathological changes during the course of co-infection caused by NDV and IBV is available in the country. Therefore, the current study was designed to investigate the infectious potential of each of

the individual viruses as well as co-infection of NDV and IBV in broiler birds. These study outcomes are expected to further elucidate baseline information towards pathogenesis of both viruses either in alone or in case of co-infection in commercial poultry.

MATERIALS AND METHODS

Management of birds and preparation of viruses: A total of one hundred and twenty (n = 210) day-old broiler chicks were procured from local well reputed ISO certified hatchery (AW breeder farms and hatcheries (pvt) Ltd., Kasur road, Lahore). Standard management conditions were provided to birds with *ad libitum* feeding and watering and to avoid any possibility of cross contamination. The birds received 16 hours of artificial light per day as per requirement of brooding of chicks. All birds were housed for three weeks for attaining maturity. Both NDV and IBV strains were isolated during outbreaks in broiler birds of poultry farms in Lahore (unpublished data) and found velogenic/pathogenic in nature. Virus isolation was performed using 9-d-old embryonated chicken eggs and confirmed by Haemagglutination Inhibition (HI) assay using NDV- and IBV-specific antisera as per described previously (OIE, 2012) and PCR assays (Shabbir *et al*., 2013). The positive-harvested allantoic fluid of NDV and IBV were diluted in PBS containing gentamycin (250µg/mL) and penicillin (200U/mL) antibiotics to finally obtain a titer of $10^{5.76}$ and $10^{6.03}$ EID_{50}, respectively. The EID_{50} of each of the virus was calculated independently as per method described previously (Reed and Muench, 1938).

Experimental design and clinical observation: All birds (n = 210) were divided into six different groups (35 birds per group) as group A (NDV-challenge), B (IBV-challenge), C (first NDV then IBV challenge after 24 hrs), D (first IBV then NDV challenge after 24 hrs), E (NDV and IBV challenge simultaneously) and F (negative control) before one week of start of experiment for acclimatization in environment. One day before, all birds were screened for presence of NDV, IBV and H9N2 viruses and specific antibodies using haemagglutination inhibition assay (HI) and reverse transcriptase polymerase chain reaction (RT-PCR) (Shabbir *et al*., 2013). Birds were monitored for clinical signs twice a day up to 6 dpi with special monitoring of respiratory and nervous disorders. A scoring system for evaluation of degree of severity of infection was used by following scales as no sign (0), slight or mild signs (1), moderate signs (2) and severe signs (3). The mean score of clinical signs was measured as sum of clinical scores for each sign divided by the number of birds showing signs in each group as previously described (Jirjis *et al*., 2004).

Necropsy findings and histopathological examination: From each group, a total of 05 chickens each were randomly slaughtered at day 0 dpi and thereafter, 10 chickens from each group (A, B, C, D, E and F) were sacrificed at 2nd, 4th and 6th days post infection. The necropsy was performed during the post-mortem of birds. The presence of pathologic lesions of trachea, lung, proventriculus, kidney and caecal tonsils were observed and scored as follow no lesion (0), mild lesions (1), moderate lesions (2) and severe lesions (3). The sum of lesions score in one group was used for statistical comparison to investigate the severity of infection between groups. Approximately 5 mm^3 tissue samples were collected from each aforementioned organ. For this purpose, tissue sections of corresponding organ preserved in 10% formalin were fixed on microscopic glass and stained with Hematoxylin and Eosin (HE) as per method described previously (Subtain *et al*., 2011). The paraffin-embedded tissues were sectioned, mounted, stained with and examined under light microscope for histological changes under 100X magnification and 3X view field repetition. During histopathological examination, lesions or any pathological change was graded as follows no lesion (-), slight or mild lesions (+), moderate lesions (++) and marked or severe lesions (+++) as described previously (Jirjis *et al*., 2004).

Immunohistochemistry: The process was conducted as per commercially available IHC kit following manufacturer's instruction (ab64264 – Mouse and Rabbit Specific HRP/DAB (ABC) detection Kit, abcam®, Austria). Using 1.0% solution of Poly-L-lysine and/or trypsin in PBS for 20 minutes at 37°C, the tissue sections were deparaffinised from the microscopic slide and antigen was retrieved using 10X target retrieval solution at pH 6.0 inside a microwave in 100°C for 15 minutes followed by 20 minutes' incubation at room temperature. A 3.0% solution of H_2O_2 was employed to block the endogenous enzyme activity for peroxidase. The target tissue was incubated with the highly specific primary mouse monoclonal antibodies for 60 minutes in a humidifier chamber. The secondary antibodies (biotinylated anti-rabbit antibody) were labeled as peroxidase polymer conjugated to anti-mouse and anti-rabbit immunoglobulins and incubated for 30 minutes. Following that, a solution containing peroxidase-conjugated streptavidin was added and tissue section was incubated for 10-15 minutes. Finally, counterstaining was done with a bath of Mayer's Hematoxylin for 2-3 min and sections were examined under phase contrast microscope using 400X magnification (Pantin *et al*., 2003). The tissue sections collected from group F (negative control) were incubated without specific primary antibodies.

RESULTS

Clinical presentation of experimentally infected commercial broiler chickens upon exposure of field isolates of ND and IB viruses: Upon experimental infection with NDV and IBV, varying degrees of clinical infections were observed in different groups (A, B, C, D and E). Overall, the birds of group E gained high score for the exhibition of clinical signs following group C, D, A and B (Fig. 1A). Additionally, the birds infected with IBV (group B) gained high score for the exhibition of respiratory signs as compared to those birds infected with NDV (group A). Whereas, the birds of group A gained high score for the presentation of nervous signs as compared to those birds infected with IBV (group B) (Fig. 1B). Birds of the infected groups C, D and E showed mild to high level of gasping signs at 2nd dpi. These signs were severe after 4th dpi. Respiratory clinical signs were predominant in all of the inoculated groups and were intense and more severe until 6th dpi, with no clear differences in the pathogenicity caused by NDV or IBV strains. The severity of nervous signs was variable according to the groups such as high severe signs were observed in group E and gained maximum score. While, birds of the infected groups A, C and D showed moderate level of nervous signs at 3rd dpi. These signs were severe after 5th dpi. These clinical signs were predominant in all of the inoculated groups and were more severe until 6thdpi. No abnormal clinical signs either respiratory or nervous were observed in the negative control birds (group F) (Fig. 1A, B).

Gross pathological and histopathological findings: At necropsy most of the birds from group A-E showed variable intensity (mild, moderate and severe) of pathological lesions suggestive of NDV and IBV infection. Lesions on the parenchymatous organs/tissues particularly trachea, proventriculus, lungs, kidneys and caecal tonsils were recorded. Generally, haemorrhagic tracheitis in trachea, hyperaemia in lungs, enlargement of kidneys, haemorrhagic and swollen proventriculus and caecal tonsils were observed. There were observed the severe haemorrhagic tracheitis, mucosal congestion and catarrhal exudates in the trachea in all birds of group A, B, C, D and E. Many of the necropsies showed mild, moderate and severe congestion and haemorrhages in the trachea and lungs collected from the birds of group A and B. The Proventriculus was found swollen in many necropsies of birds of group A, C, D and E with the presence of haemorrhages in submucosa and mild hemorrhagic papillae. The kidneys and caecal tonsils collected from the birds of group C and E showed mild to severe congestion and mild to moderate haemorrhages along with inflammation (Fig 2).

At the histopathological examination, moderate to very severe pathogenesis of NDV and IBV infection was observed in trachea, lungs, proventriculus, kidneys and caecal tonsils collected at 0, 2nd, 4thand 6th dpi. With the fact of high level of clinical presentation between 4-6 dpi, maximum evidences were observed at the tissue collected at 6th dpi. The infiltration of lymphocytes, prominent blood vessels, haemorrhages of blood vessels with oedematous fluid in surrounding area were observed in the proventriculus, kidneys and caecal tonsils tissues collected from group A. While, similar type of lesions was also observed in the trachea and lung tissues collected from group B. These findings were also recorded in trachea, kidneys, proventriculus and caecal tonsils of group C and D. Comparably, these all histopathological findings with sloughing of ciliary cells of trachea were observed in all tissue collected from group E. As compared to all groups, no histopathological changes were observed in any tissue collected from group F (Fig. 3).

Immunohistochemical evidences of the viral antigens in different collected tissues: Tissues collected at 6th dpi were used for the evidences of viral antigen distribution through immunohistochemistry. Overall, strong immune-labelling was evident in the tissue of co-infected birds of group C, D and E as compared to individual virus challenged group A and B. The viral antigen was present in several organs of birds inoculated with NDV and IBV. Mostly, the presence of IBV antigens was highly observed in hyperplastic epithelial cells and submucosa of the trachea in the group E and B, moderate to low existence in group C and D. Likewise, the presence of IBV antigens was highly observed in epithelium of air capillaries and necrotic debris in lungs in the group E and B, moderate to low existence in group C and D. Whereas, NDV antigen positive cells were highly detected in the proventriculus, kidney and caecal tonsils of group E and moderate in group C, D and A but not in group B and F. In the kidney, antigen was detected in tubular epithelial cells in most of the groups with varying degree. Antigen positive tubular epithelial cells or glandular caecal tonsil were more intensely labelled in group E and A compared with the other groups (C and D). The expression of viral antigens was absent in group F (Fig. 4).

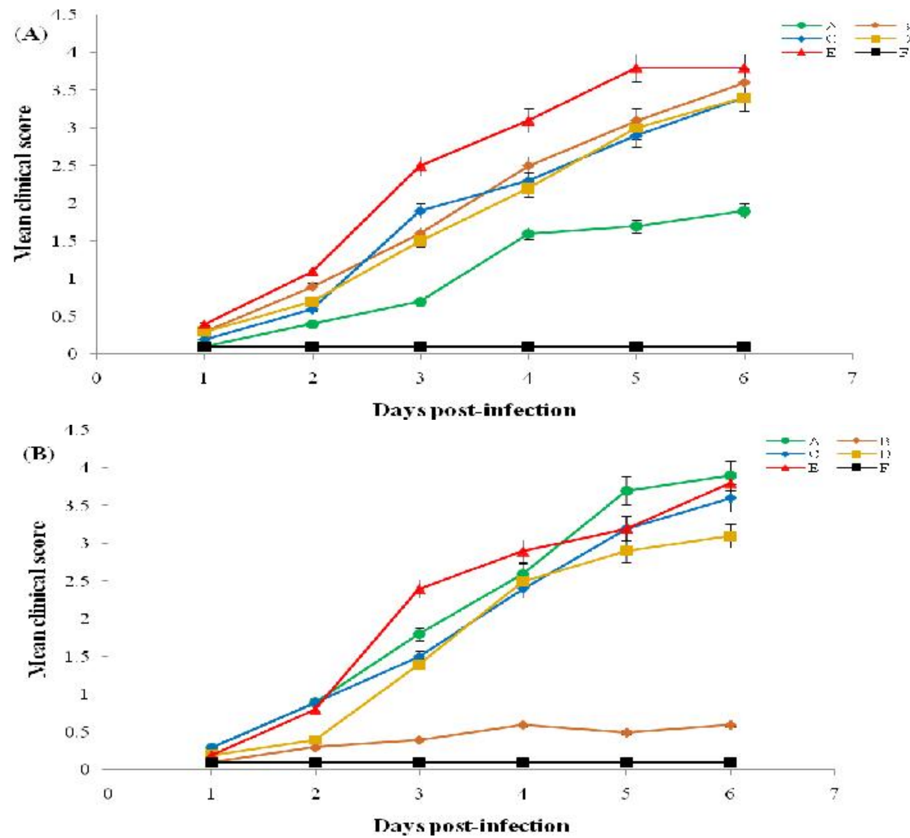

Fig. 1. Clinical score of respiratory signs (A) and nervous signs (B) in all groups post-challenge with NDV and IBV alone, simultaneously and in association.

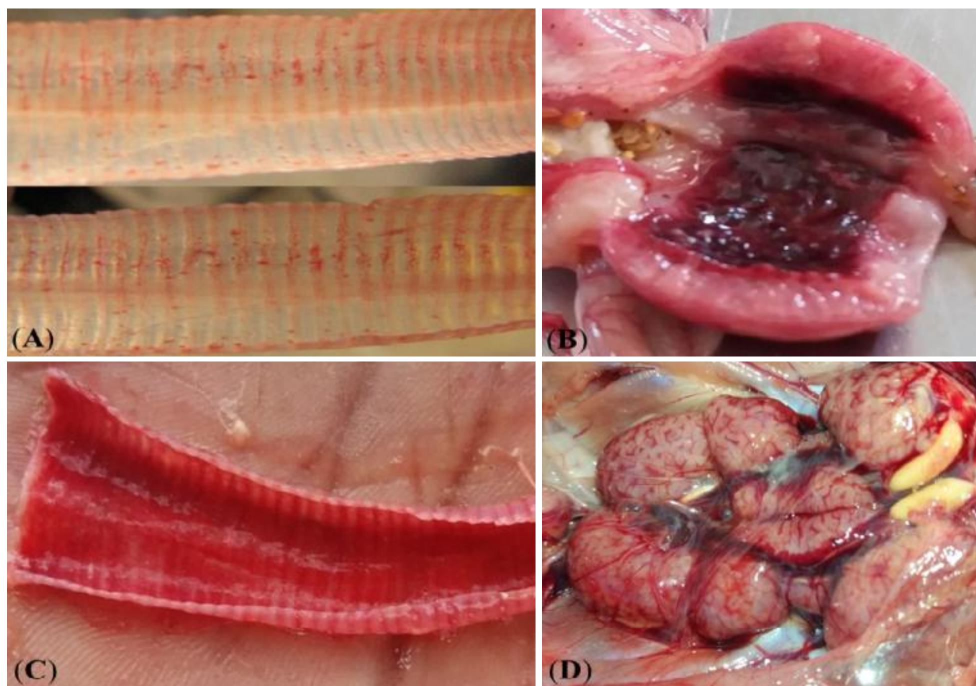

Fig. 2. Necropsy lesions in different organs representing the clinical infection of NDV and IBV. These observations includes haemorrhage in trachea (A), pinpoint haemorrhagic lesions in proventriculus (B), haemorrhagic trachitis (C) and congested and haemorrhagic kidneys (D).

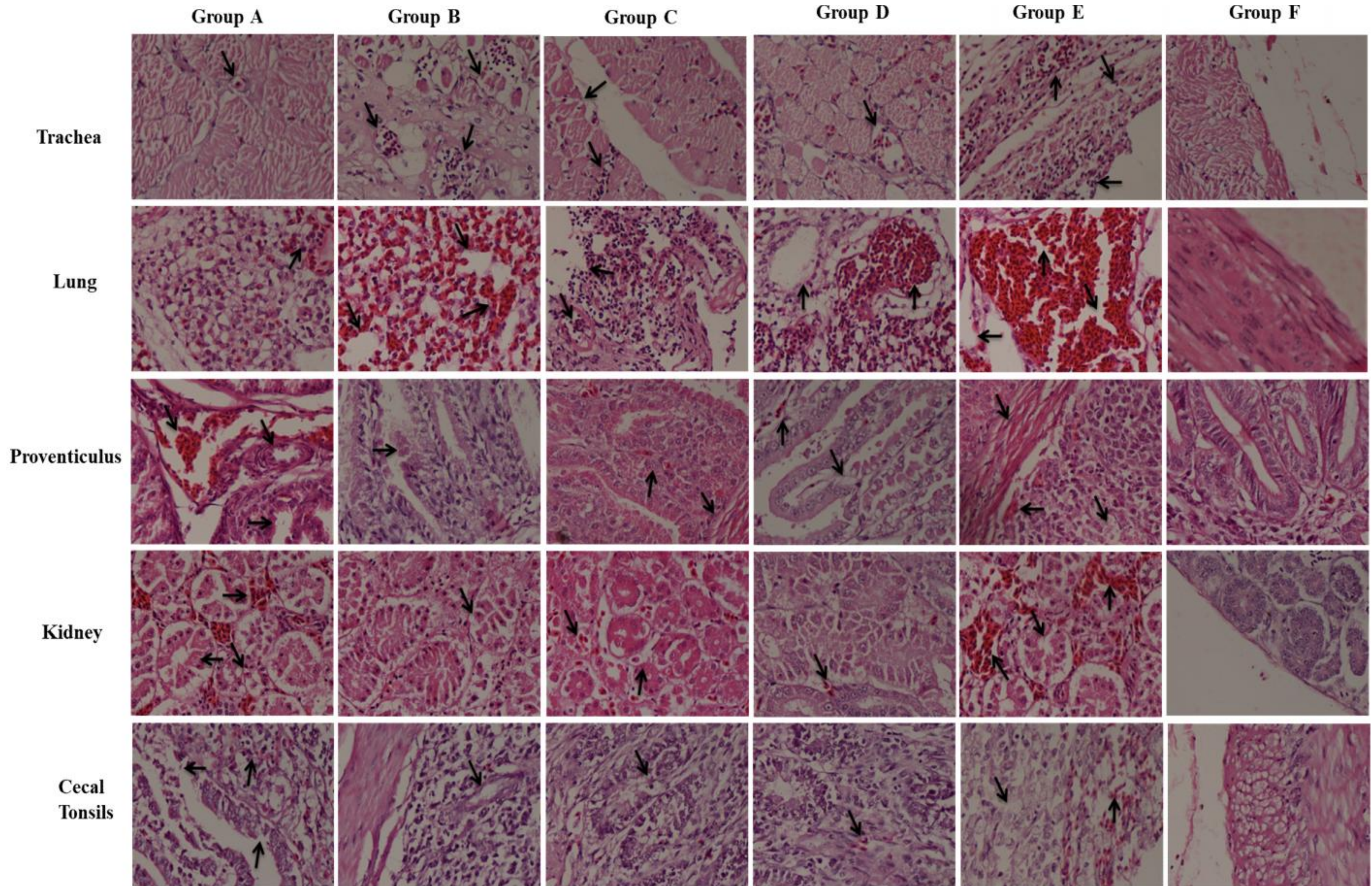

Fig. 3. Histopathological changes in different tissues collected from group A-F.

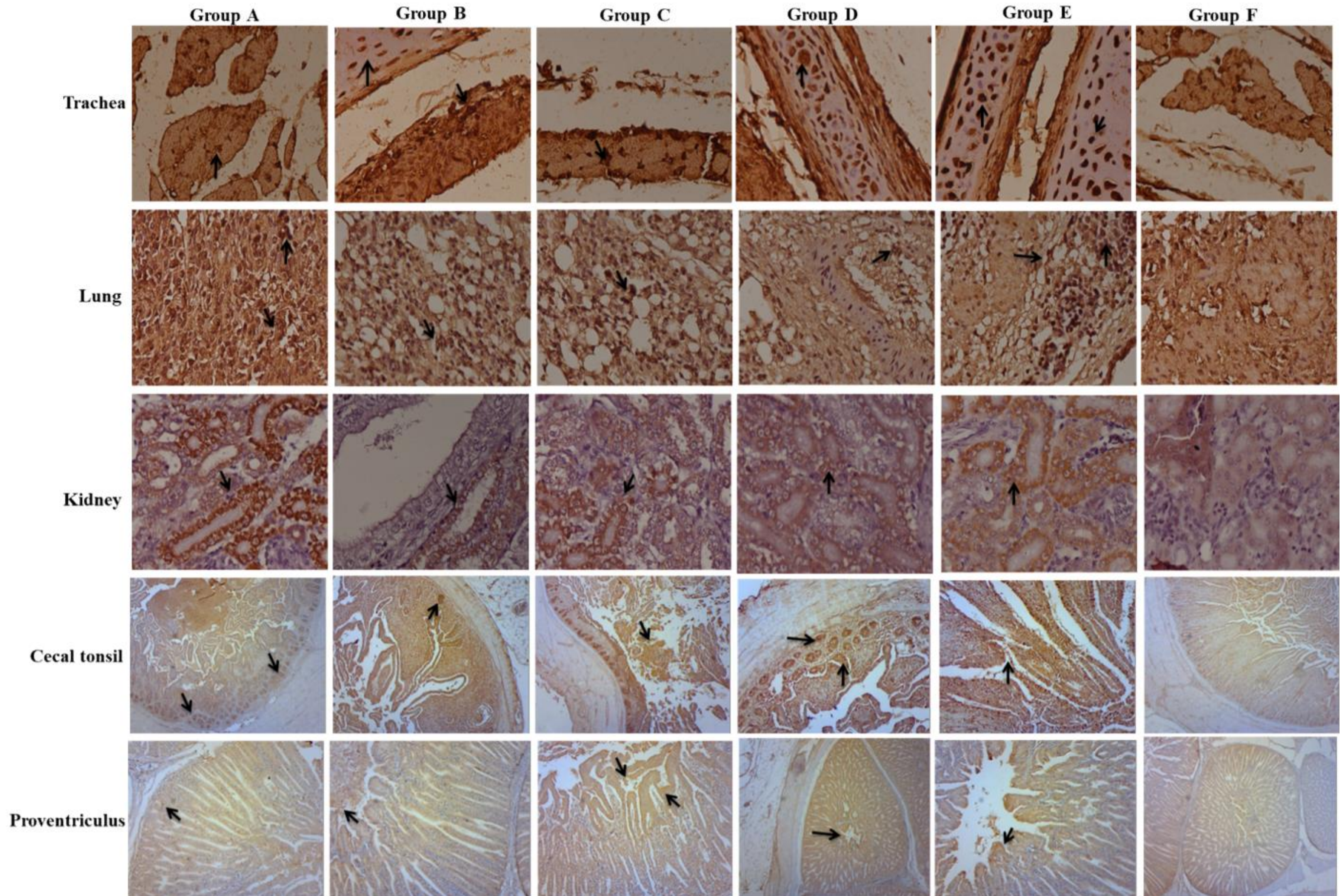

Fig. 4. The distribution of NDV and IBV antigens in different tissues collected from groups (A-F).

DISCUSSION

The respiratory diseases caused by viruses have a marked influence on the poultry production and, in a disease endemic setting such as Pakistan, may further enhance economic losses (Yashpal *et al*., 2004). A co-infection in poultry not only may cause difficulty in disease diagnosis by an exhibition of varying clinical manifestation but, limited information regarding interaction between co-infecting viruses such NDV and IBV, may make situation further worse than before (Costa-Hurtado *et al*., 2014). Over the last few decades, NDV and IBV infections has accounted for a significant losses to the poultry sector in Pakistan (Ahmed *et al*., 2007; Shabbir *et al*., 2012). Although, natural or experimental infectious potential of NDV and IBV in commercial poultry are well established when studied alone (Qamar-un-Nisa *et al*., 2018; Kanwal *et al*., 2018; Aziz-ul-Rahman *et al*., 2018, 2019) however, information related to pathobiology of these viruses as a result of co-infection is scarce. This is important because co-infection may cause huge economic losses to the farmers in the form of disease progression and condemnation of the morbid bird meat. Therefore, it is imperative to elucidate aspects of disease pathogenesis among birds upon exposure to either alone or in combination of both NDV and IBV (co-infection) under the field circumstances. Certain control measures are adopted to control individual pathogen in commercial poultry farms; however, such strategies may not be equally applicable in case of infection caused by two or more than two closely related pathogens. With this background, the current study was designed to investigate individual as well as co-infection of NDV and IBV in broiler chickens.

In the present study, clinical exhibition was not limited to severe respiratory signs but mild to moderate nervous signs were also observed in case of birds challenged with both NDV and IBV which is in agreement to observation reported elsewhere (Kouakou *et al*., 2015). Indeed, similar to finding reported in the previous studies, the clinical signs and lesions were severe in co-infected birds than those infected with each of the individual virus (Kouakou *et al*., 2015; Shirvani *et al*., 2018) possibly due to exaggeration of inflammatory responses (Dwars *et al*., 2009). This is important because the infection caused by one virus in a host may have similar tissue tropism and, therefore, is very likely to provoke or enhance an ongoing infection upon exposure to another virus (Kimura *et al*., 1976).Contrary to this, there are multiple mechanisms that could be involved in resultant interference among the viruses. Hence, the replication of new virus might be interfered by an earlier replication of a previous virus at the same location in the body due to activation of the immune system and subsequent immune responses to earlier viral infection at the same site (Pantin-Jackwood *et al*., 2015).Considering the ongoing situation in the country, an exposure to IBV is almost unavoidable for commercial poultry setting where there is every possibility of occurrence of co-infections with NDV simply due to the fact that chances of viral infection sharing similar tissue tropism (e.g., NDV, IBV and AIV) get further enhanced in a disease endemic setting (Naguib *et al*., 2017).

The observed microscopic changes or histopathological lesions were suggestive of classical lesions due to infection of NDV and IBV in commercial poultry (Qamar-un-Nisa *et al*., 2018; Kanwal *et al*., 2018; Aziz-ul-Rahman *et al*., 2018, 2019). The observed lesions were not only closely related to those reported previously during field outbreak (Miller *et al*., 2013; Kanwal *et al*., 2018) but also those where they were observed while performing a challenge-protection related experiments (Shabbir *et al*., 2016; Amarasinghe *et al*., 2018). For instance, the findings of haemorrhages in trachea and lungs in infected birds of present study are in agreement to evidence observed in experimental poultry (Purcell *et al*., 1976). Subsequent to co-infection of NDV and IBV in birds of group C, D and E, severe haemorrhages in submucosa of trachea that progressed to congestion and tracheitis were observed constantly. Such findings further support the hypothesis that NDV and/or IBV play a vital role in resultant severity of clinical sings in respiratory system, possibly *via* impairment of other viral infection in the air way tract of bird facilitating the entry of other pathogen (Haghighat-Jahromi *et al*., 2008). Additionally, owing to high degree of infection, the congestion in the different tissues collected from co-infected birds indicates the deciliation and leukocytic infiltration which is in agreement to previously reported observations (Nili and Asasi, 2002). The lungs, kidneys, proventriculus and caecal tonsil tissues had remarkable congestion followed by atrophy. Such findings indicate that, while dissemination throughout the body *via* blood or lymph vessels, the virus had infected immune cells (Kwon *et al*., 2008). Here it is important to indicate that we excluded brain tissue from histopathological processing simply because we studied co-infection of both viruses where IB does not have potential to affect brain tissues but respiratory, GIT and urinary tract. On the basis of the antigen-antibody reactions, NDV and IBV antigens were successfully detected in different tissues using IHC technique (Igwe *et al*., 2018). Not only that it indicate a wide spectrum of tissue tropism of each of the virus individually but also the detection of high level of NDV and IBV antigens in trachea, lungs and proventriculus collected from birds of group C, D and E indicate the analogous tissue tropism of both viruses in a pattern reported previously by others (Naguib *et al*., 2017).

Conclusions: The current study concluded that co-infection of NDV and IBV cause severe infection in the commercial poultry. The clinical, histopathological and

immunohistochemical evidences revealed a high degree of infection in those birds challenged with both NDV and IBV simultaneously and sequentially as compared to those birds challenged with individual virus.

Conflict of interest: All authors declared no competing interest regarding this article

REFERENCES

Ahmed, Z., K. Naeem and A. Hameed (2007). Detection and seroprevalence of infectious bronchitis virus strains in commercial poultry in Pakistan. Poult Sci., 86(7):1329-1335.

Alexander, D. J. and D. A Senne (2008). Newcastle disease. In: Diseases of poultry, 12th edition (eds.Y.M. Saif, J.R. Glisson, L.R. McDougald, L.K. Nolan and D.E. Swayne). Blackwell Publishing, Ames, Iowa, USA, pp. 75–100.

Amarasinghe, A., U. D.S. Senapathi, M.S. Abdul-Cader, S. Popowich, F. Marshall, S.C. Cork, F. van der Meer, S. Gomis and M.F. Abdul-Careem (2018). Comparative features of infections of two Massachusetts (Mass) infectious bronchitis virus (IBV) variants isolated from Western Canadian layer flocks. BMC Vet Res,14(1): 391.

Aziz-ul-Rahman., T. Yaqub, M. Imran, M. Habib, T. Sohail, M. S. Furqan, M. Munir and M.Z. Shabbir (2018). 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 Dis, 62(4): 404-415.

Aziz-ul-Rahman., M.A. Rohaim, R.F. El Naggar, G. Mustafa, U. Chaudhry and M.Z. Shabbir (2019). Comparative clinico-pathological assessment of velogenic (sub-genotype VIIi) and mesogenic (sub-genotype VIm) Avian avulavirus 1 in chickens and pigeons. Avian Pathol, 1-12.

Bande, F., S.S. Arshad, A.R. Omar, M.H. Bejo, M.S. Abubakar and Y. Abba (2016). Pathogenesis and diagnostic approaches of avian infectious bronchitis. Adv Virol,*2016*.

Costa-Hurtado, M., C.L. Afonso, P.J. Miller, E. Spackman, D.R. Kapczynski, D.E. Swayne, E. Shepherd, D. Smith, A. Zsak and M. Pantin-Jackwood (2014). Virus interference between H7N2 low pathogenic avian influenza virus and lentogenic Newcastle disease virus in experimental co-infections in chickens and turkeys. Vet Res,45:1.

Deming, D.J. and R.S. Baric (2008). Genetics and reverse genetics of nidoviruses. In Nidoviruses (pp. 47-64). American Society of Microbiology.

Dwars, R.M., M.G.R. Matthijs, A.J.J.M. Daemen, J.H.H. van Eck, L. Vervelde and W.J.M. Landman (2009). Progression of lesions in the respiratory tract of broilers after single infection with *Escherichia coli* compared to superinfection with *E. coli* after infection with infectious bronchitis virus. Vet Immunol Immunopathol,127: 65-76. https://doi.org/10.1016/j.vetimm.2008.09.019

Haghighat-Jahromi, M., K. Asasi, H. Nili, H. Dadras and A.H. Shooshtari (2008). Coinfection of avian influenza virus (H9N2 subtype) with infectious bronchitis live vaccine. Arch Virol,153(4): 651-655.

Igwe, A.O., C.L. Afonso, W.S. Ezema, C.C. Brown and J.O.A. Okoye (2018). Pathology and Distribution of Velogenic Viscerotropic Newcastle Disease Virus in the Reproductive System of Vaccinated and Unvaccinated Laying Hens (*Gallus gallus domesticus*) by Immunohistochemical Labelling. J Comp Pathol,159: 36-48.

Jirjis, F.F., S.L. Noll, D.A. Halvorson, K.V. Nagaraja, F. Martin and D.P. Shaw (2004). Effects of bacterial coinfection on the pathogenesis of avian pneumovirus infection in turkeys. Avian Dis,48(1): 34-49.

Kanwal, B., A. A. Channo, N.H. Kalhoro, H. Soomro, N.A. Korejo and S. Tauseef (2018). Prevalence and clinical pathology caused by infectious bronchitis virus in poultry birds at Sindh, Pakistan. J Vet Med Ani Health,10(9): 231-236.

Kausar, A., S. Anwar, N. Siddique, S. Ahmed and J. I. Dasti (2018). Prevalence of Avian influenza H9N2 Virus among Wild and Domesticated Bird Species across Pakistan. Pakistan J Zool,50(4): 1347-1347.

Kimura, Y., E. Norrby, I. Nagata, Y. Ito, K. Shimokata and Y. Nishiyama (1976). Homologous interference induced by a temperature-sensitive mutant derived from an HVJ (Sendai virus) carrier culture. J Gen Virol,33(2): 333-343.

Kiss, I., T. Mató, Z. Homonnay, T. Tatár-Kis and V. Palya (2016). Successive occurrence of recombinant infectious bronchitis virus strains in restricted area of Middle East. Virus Evol,2(2).

Kouakou, A.V., V. Kouakou,C. Kouakou, P. Godji, A.L. Kouassi, H.A. Krou, Q. Langeois, R.J. Webby, M.F. Ducatez and E. Couacy-Hymann (2015). Prevalence of Newcastle disease virus and infectious bronchitis virus in avian influenza negative birds from live bird markets and backyard and commercial farms in Ivory-Coast. Res Vet Sci,102: 83-88.

Kwon, J.S., H.J. Lee, D.H. Lee, Y.J. Lee, I.P. Mo, S.S. Nahm, M.J. Kim, J.B. Lee, S.Y. Park, I.S. Choi and C.S. Song (2008). Immune responses and pathogenesis in immunocompromised chickens

in response to infection with the H9N2 low pathogenic avian influenza virus. Virus Res,133(2): 187-194.

Miller, P.J., C.L. Afonso, J. El Attrache, K.M. Dorsey, S.C. Courtney, Z. Guo and D.R. Kapczynski (2013). Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. Dev Comp Immunol,41: 505-513.

Naguib, M.M., M.F. El-Kady, D. Lüschow, K.E. Hassan, A.S. Arafa, A. El-Zanaty, M.K. Hassan, H.M. Hafez, C. Grund and T.C. Harder (2017). New real time and conventional RT-PCRs for updated molecular diagnosis of infectious bronchitis virus infection (IBV) in chickens in Egypt associated with frequent co-infections with avian influenza and Newcastle disease viruses. J Virol Methods,245: 19-27.

Nili, H. and K. Asasi (2002). Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. Avian Pathol,31(3): 247-252.

OIE, 2012. Manual of diagnostic tests and vaccines for terrestrial animals. [Internet]. World Organisation for Animal Health, pp. Available from. http://www.oie.int/international-standard-setting/terrestrial-manual/accessonline/.

Pantin, H., J.D. Coatsworth, D.J. Feaster, F.L. Newman, E. Briones, G. Prado, S.J. Schwartz and J. Szapocznik (2003). Familias Unidas: The efficacy of an intervention to promote parental investment in Hispanic immigrant families. Prev Sci., 4(3): 189-201.

Pantin-Jackwood, M.J., M. Costa-Hurtado, P.J. Miller, C.L. Afonso, E. Spackman, D.R. Kapczynski, E. Shepherd, D. Smith and D.E. Swayne (2015). Experimental co-infections of domestic ducks with a virulent Newcastle disease virus and low or highly pathogenic avian influenza viruses. Vet Microbiol, 177(1-2): 7-17.

Purcell, D. A., V. L. Tham and P. G. Surman (1976). The histopathology of infectious bronchitis in fowls infected with a nephrotropic “T” strain of virus. Australian Vet J, 52(2): 85-91.

Qamar-un-Nisa, M. Younus, A. Maqbool and S. Umar (2018). Pathological alterations during co-infection of Newcastle disease virus with Escherichia coli in broiler chicken. Pakistan J. Zool,50(2): 495-495.

Rafique, S., K. Naeem, N. Siddique, M.A. Abbas, A.A. Shah, A. Ali, A. Rahim and F. Rashid (2018). Determination of genetic variability in Avian Infectious Bronchitis Virus (AIBV) isolated from Pakistan. Pak J Zool,50(2): 695-695.

Reed, L.J. and H. Muench (1938). A simple method of estimating fifty per cent endpoints. American J of Epidemiol,27(3): 493-497.

Roussan, D.A., W.S. Totanji and G.Y. Khawaldeh (2008). Molecular subtype of infectious bronchitis virus in broiler flocks in Jordan. Poul Sci,87(4): 661-664.

Shabbir, M.Z., S. Akhtar, Y. Tang, T. Yaqub, A. Ahmad, G. Mustafa, M.A. Alam, D. Santhakumar, V.Nair and M. Munir (2016). Infectivity of wild bird-origin avian paramyxovirus serotype 1 and vaccine effectiveness in chickens. J Gen Virol,97(12): 3161-3173.

Shabbir, M. Z., A. N. Malik, A. Wajid, S. F. Rehmani and M. Munir (2012). Newcastle disease virus: disease appraisal with global and Pakistan perspectives. J. Infect. Molecul. Biol.,1: 52-57.

Shabbir, M.Z., S. Zohari, T. Yaqub, J. Nazir, M.A.B. Shabbir, N. Mukhtar, M. Shafee, M. Sajid, M. Anees, M. Abbas and M.T. Khan (2013). Genetic diversity of Newcastle disease virus in Pakistan: a countrywide perspective. Virol J,10(1): 170.

Shirvani, E., A. Paldurai, V.K. Manoharan, B.P. Varghese and S.K. Samal (2018). A Recombinant Newcastle Disease Virus (NDV) Expressing S Protein of Infectious Bronchitis Virus (IBV) Protects Chickens against IBV and NDV. Sci Rep, 8(1): 11951.

Subtain, S. M., Z. I. Chaudhry, A. A. Anjum, A. Maqbool and U. Sadique (2011). Study on pathogenesis of low pathogenic avian influenza virus H9 in broiler chickens. Pakistan J. Zool,43(5).

Umar, S., M. Delverdier, M. Delpont, S.F. Belkasmi, A. Teillaud, C. Bleuart, I. Pardo, M. EL Houadfi, J.L. Guérin and M.F. Ducatez (2018). Co-infection of turkeys with *Escherichia coli* (O78) and H6N1 avian influenza virus. Avian Pathol,47(3): 314-324.

Yashpal, S. M., P. P. Devi and M. G. Sagar (2004). Detection of three avian respiratory viruses by single-tube multiplex reverse transcription-polymerase chain reaction assay. J Vet Diagn Invest,16: 244–248.