Full Length Research Paper

Use of immunohistochemistry (IHC) in the detection of Newcastle disease virus (NDV) in experimentally and naturally infected birds

Adriano de Oliveira Torres Carrasco[1]*, Meire Christina Seki[1], Tiago Wilson Patriarca Mineo[2], Jayme Augusto Peres[1], Laura Marques Knychala[2], Mario Henrique Alves[1], Jyan Lucas Benevenute[1], Priscila Ikeda[1] and Aramis Augusto Pinto[3]

[1]Departamento de Medicina Veterinária, Universidade Estadual do Centro-Oeste (UNICENTRO), Guarapuava, PR, Brazil.
[2]Laboratório de Imunoparasitologia, Instituto de Biociências, Universidade Federal de Uberlândia (UFU), Uberlândia, MG, Brazil.
[3]Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista (FCAV/UNESP), Jaboticabal, SP, Brazil.

The objective of the present study was to standardize an immunohistochemical reaction (IHC) for the diagnosis of Newcastle disease virus (NDV) in tissue samples. In order to achieve this aim, pigeons (*Columba livia)* and chicken (*Gallus* gallus) were experimentally infected with a pathogenic sample of NDV (São Joao do Meriti strain). The IHC reaction was standardized and tested in samples of lung, liver, spleen and trachea of the experimentally infected birds, and the results showed that from the 20 infected birds, 17 were positive for Newcastle disease antigen immunomarking in the trachea and 11 were positive in the spleen. NDV detection was not achieved in samples of lung and liver, and these tissues are not indicated in NDV detection in the present study. After that, the standardized technique was used in the detection of NDV in samples of trachea and spleen of 46 free-living birds which belonged to 11 different bird orders. The virus was detected in 24 individuals. The order Caprimulgiforme was the only one that did not show any positive animal. These NDV strains circulate in bird populations, generally without causing the disease, in a parasite vs. host balance. Outbreaks may occur when these free-living birds get in contact with commercial birds, with considerable losses to countries that raise and export poultry and poultry products. In conclusion, IHC is a diagnostic tool that may be used in locations that do not have biosafety levels for virus isolation, or that are even not able to perform molecular assays.

Key words: *Columba livia*, diagnostic, experimental infection, G*allus gallus,* wild birds.

INTRODUCTION

Newcastle disease (ND) is an acute, highly contagious and widespread viral disease of birds that can cause high mortality (up to 100%) and severe economic losses in poultry, the most important natural host of the disease. The virus can also affect a wide variety of avian species causing severe disease (Alexander, 2003). ND is regarded

as endemic in many countries, and is caused by an avian Paramyxovirus type 1 (APMV 1), a member of the genus *Avulavirus,* from the *Paramyxoviridae* family (Mayo, 2002). There are five pathotypes of ND in chickens: viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and the asymptomatic enteric form (Alexander, 1995). As demonstrated in intensive surveys, nearly 236 free-living species from 27 of the 50 orders of birds have been reported to be susceptible to either natural or experimental infection with ND (Wan et al., 2004). On several occasions, Newcastle disease virus (NDV) was isolated from wildlife birds (Hoque et al., 2012), and most outbreaks of NDV has involved unvaccinated susceptible birds. Contact with infected exotic birds or free-living birds, such as ducks, geese, pigeons, pheasants, partridges, crows, sparrows, mynas and martins, may transmit ND virus to poultry(Alexander, 2003), and ND outbreaks may occur under field conditions, if a pathogenic strain is introduced in a previously unchallenged area (Alexander, 1995).In some cases, the source of the virus in poultry has been presumed to be wild birds, because avirulent NDVs have commonly been found in them (Hoque et al., 2012). The presence of virulent strains in poultry or free-living birds requires screening and control measures even in countries where the virus is endemic, because the existence of the agent severely affects commercial productivity and international trade of poultry and poultry products (Miller et al., 2010). This fact shows the importance of wild birds in the maintenance and transmission of NDV (Jindal et al., 2010).

The objective of ND diagnosis is to guide decision-making in the control of the disease in a way to prevent the spread of the disease (Alexander, 2003). Therefore, a reliable, safe and easy-to-perform method for the diagnosis should be considered, as the quicker the diagnosis, the more efficient the disease control measures to be put in place, preventing greater losses and the spread of the disease (Kho et al., 2000). Studies involving the use of tissue samples are extremely important for better understanding of the distribution of the viral agent in infected organs, the protection ability of a vaccine, or the pathogenicity of a strain (Barbezange and Jestin, 2005; Nakamura et al., 2008, 2014; Ezema et al., 2009; Cattoli et al., 2011).

Conventionally, ND was characterized by isolation of the virus in embryonated chicken eggs or cell culture, followed by *in vivo* tests to determine the virulence of the strain, such as intracerebral pathogenicity index (ICPI), intravenous pathogenicity index (IVPI) and mean death time (MDT) in SPF chicken embryos/birds. These tests are cumbersome, labor-intensive, inhumane and, sometimes, inconclusive (Tiwari et al., 2004). Virological,

pathologic and immunohistochemical analysis of chickens with ND in field conditions have been useful in the investigation of the mechanisms of ND outbreaks in vaccinated chickens (Nakamura et al., 2008; Bwala et al., 2012). IHC is performed on formalin-fixed, paraffin-embedded tissues and, in many cases, the viral antigen can be detected in autholyzed tissues. This technique has been valuable in determining virus distribution in samples in NDV pathogenesis studies. The advantage of this method is that diagnosis is still possible when fresh sera or tissues are unavailable (Lockaby et al., 1993; Wakamatsu et al., 2007; Bwala et al., 2012). IHQ is not in routine use as a diagnostic assay, but the information from experimental studies can supply a roadmap in understanding the clinicopathological picture presented with the various pathotypes (Cattoli et al., 2011).

As ND does not cause pathognomonic histopathological lesions, immunological techniques, such as IHC have become important tools in diagnosis and may, therefore, detect the agent in tissue samples (Ojok and Brown, 1996; Oldoni et al., 2005; Piacenti et al., 2006; Wakamatsu et al., 2006; Nakamura et al., 2008). Besides, this technique may replace virus isolation, or even molecular tests, in places where there are no laboratories of adequate biosafety level for this procedure (Brown et al., 1999a, b; Wakamatsu et al., 2006).

Based on these observations, the objective of the present study was to standardize an immuno-histochemical reaction for the diagnosis of NDV in samples of experimentally infected birds, followed by the use of the technique in the diagnosis of NDV in free-living birds.

MATERIALS AND METHODS

Birds

In order to standardize the IHC technique, a total of 15 pigeons (*Columba livia*) and 15 chickens (*Gallus gallus*), adults, were used for the experimental infection with a pathogenic strain of NDV. Ten birds of both species (pigeons and chickens) were experimentally infected,and the five remaining birds of both species were inoculated with PBS pH 7.2 to be used as negative controls. Experimental infection was performed by oral route. These ten birds were kept in separate facilities. On the day of experimental infection, pigeons and chickens, divided into two experimental groups, were moved to Negative Pressure Isolators (Alesco®, Brazil), under biosafety conditions.

After the standardization of the IHC technique, samples from 46 free-living birds, that were identified as adults and died of unknown causes were analyzed. Birds belonged to the following orders: Passeriformes (N=18); Strigiformes (N=9); Ciconiiformes (N=5); Charadriiformes (N=3); Psitaciformes(N=2); Accipitriformes (N=2); Piciformes (N=2); Nyctibiiformes (N=2); Caprimulgiformes (N=1);

*Corresponding author. E-mail: adriano.carrasco@gmail.com

Trogoniformes (N=1) and Columbiformes (N=1). None of thesebirds presented clinical signs compatible with Newcastle disease. Samples from experimental infectioninoculated with pathogenic NDV strain and those inoculated with sterile phosphate buffered saline (PBS) were used as positive and negative controls, respectively.

All procedures were performed according to the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation, and to the 2000 Report of the AVMA Panel on Euthanasia (AVMA, 2001) and were approved by Ethics and Animal Welfare Commission of the Biomedical Sciences Institute, University of Sao Paulo (N 093; P.16; B.2).

Viruses

Experimental infection was performed using the Sao Joao do Meriti strain (Gene Bank Number: EF534701), a highly pathogenic NDV (VVNDV– Velogenic Viscerotropic Newcastle Disease Virus) strain for chickens (mean death time in chicken embryos = 48 h; ICPI in day-old chicken = 1.78). This virus stock solution titer was $10^{9.0}$ median embryo lethal dose/mL (ELD_{50} /0.1 mL). All virus dilutions were carried out with sterile PBS, pH 7.2.

Tissue collection

Post-mortem examination was carried out in birds that died (natural infection) or were euthanized (experimental infection) during the experimental period (20 days), and their tissues were submitted to microscopic analysis. All chickens died until the 5th day post-inoculation (D.P.I.). As the pigeons did not show any clinical sign, they were euthanized on day 20 D.P.I.Tissue samples were collected and fixed by immersion in 10% neutral buffered formalin for approximately 60 h. Then, samples were embedded in paraffin by routine methods.

Samples from free-living birds were collected throughout a period of one year. They were processed soon after the birds died and stored in paraffin blocks until the moment of IHC analysis. Samples of liver, spleen, lungs and trachea were collected from the birds submitted to experimental infection (chickens and pigeons), including the negative controls. From the free-living birds, only the trachea and spleen were collected, once these tissues presented better results in the evaluation of experimental infection.

Positive and negative controls were made up by samples of trachea and spleen that came from animals that were experimentally infected with NDV and in which positivity and negativity were confirmed by RT-PCR.

Immunohistochemistry

Immunohistochemical (IHC) assays were performed in paraffin-embedded tissues according to a previously published protocol (Mineo et al., 2009), with some modifications. Briefly, slides were assayed with polyclonal antibodies against Newcastle disease virus (1:800) obtained from experimentally infected rabbits, as primary antibodies. For the production of primary antibodies, specific-pathogen free (SPF) rabbits were inoculated with 1mL of a purified NDV suspension by intramuscular route. This suspension was produced in embryonated eggs and later on, 1 mL of Freund's complete adjuvant was added. The mixture (NDV + adjuvant) was administered three times at 21-day intervals. Later on, blood was collected from the rabbits, followed by titration of polyclonal antibodies.

When the reaction was carried out, primary antibodies were incubated for 60 min at 25°C, after removal of paraffin and blockage of endogenous peroxidase (hydrogen peroxide 3% solution in methanol). For signal amplification, the *EasyLink One*® (Immunobioscience Corp. – USA, imported by Erviegas®, Brazil) kit was used according to the manufacturer's instructions. Counter staining was performed with Harris hematoxylin (10%), and slides were covered with coverslips after dehydration. The reaction was read under a light microscope (Nikon, Japan).

RESULTS

Results of the experimental infection showed that from the 20 infected birds, 17 were positive for Newcastle disease antigen immunomarking in the trachea, 11 were positive in the spleen, with immunomarking mainly observed in the cytoplasm (Figure 1). None of the other tissues (lungs and liver) were positive. A large number of unspecific reactions were observed. Negative controls did not show immunomarking in any of the samples.

Results for free-living birds are shown in Table 1. From the 46 samples analyzed, 24 presented positive immuno-marking in at least one of the tissues (trachea or spleen). From these positive samples, 23 showed positive reaction in trachea, and only one sample was positive only in the spleen. From the 24 positive birds, only 5 presented positive results in both tissue samples.

DISCUSSION

This is the first study in Brazil using the standardization of immunohistochemistry for NDV diagnosis in samples of free-living birds. The use of polyclonal antibodies showed was effective in the standardization of the technique, enabling NDV diagnosis in experimentally and naturally infected birds. Unsatisfactory results of IHC for NDV have already been described in some cases, as when monoclonal antibodies are used (Bhaiyat et al., 2004).

The results of this study were partially similar to those presented elsewhere (Nakamura et al., 2008),with the use of immunohistochemistry after an outbreak in the detection of NDV antigens of the disease in different samples of tissues from 25 broilers that had been previously vaccinated. This study also showed immunomarking in the epithelium of the trachea, as well as other organs, such as the lungs. Experimental infection carried out in the present study showed that lung and liver were not efficient for IHC: none of the experimentally infected birds presented positive reactions in these tissues, and they also showed a large number of unspecific reactions, making it difficult to interpret IHC results. Because of this, the evaluation of free-living birds was only carried out in trachea and spleen samples. In contrast, Cattoli et al. (2011),showed that birds infected with VVNDV presented intense distribution of the virus, in various tissues (bursa, spleen and thymus), detected by IHC.

On the other hand, Wakamatsu et al. (2007), who studied 10 broilers experimentally inoculated with different NDV strains, a low virulent (LaSota strain) and a
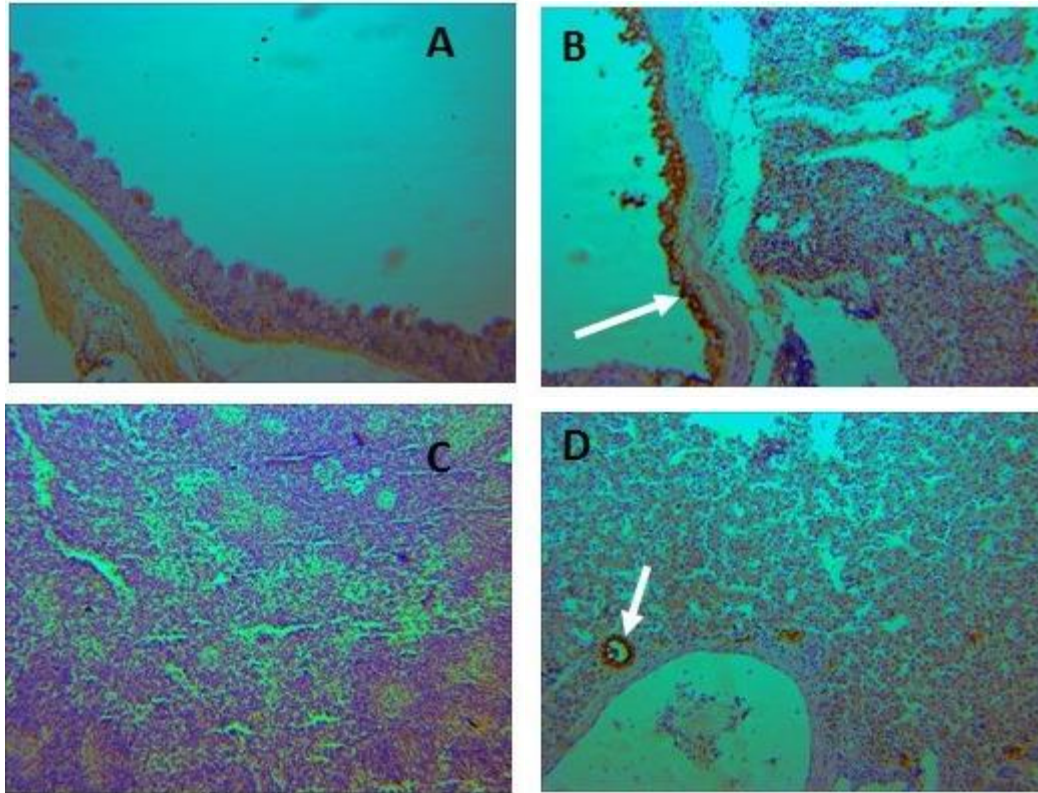
Figure 1. Samples of pigeons experimentally infected with positive and negative immunomarking. A-Negative trachea; B- Positive trachea (arrow); C- Negative spleen; D-Positive spleen (arrow).

Table 1. Immunohistochemistry results of samples of trachea and spleen of 46 free-living birds.

Avian order	Animal number	Avian species	Samples	
			Trachea	Spleen
	1	*Carduelis magellanica*	+	-
	2	*Cyanocompsa brissonii*	+	+
	3	*Cyanocompsa brissonii*	+	-
	4	*Cyanocompsa brissonii*	-	-
	5	*Cyanocompsa brissonii*	+	-
	6	*Cyanocompsa brissonii*	-	-
	7	*Cyanocorax chrysops*	+	-
	8	*Furnarius rufus*	-	-
Passeriformes	9	*Pitangus sulphuratus*	+	-
(N =18)	10	*Saltator similis*	-	-
	11	*Saltator similis*	-	-
	12	*Sicalis flaveola*	+	+
	13	*Sicalis flaveola*	+	-
	14	*Sicalis flaveola*	+	-
	15	*Sporophila angolensis*	+	+
	16	*Turdus rufiventris*	-	-
	17	*Turdus rufiventris*	-	-
	18	*Tyrannus savanna*	+	+
	19	*Asio stygius*	+	-
	20	*Athene cunicularia*	-	-

Table 1. Contd.

Strigiformes (N = 9)	21	*Athene cunicularia*	-	-
	22	*Otus choliba*	+	-
	23	*Otus choliba*	+	-
	24	*Tyto alba*	-	-
	25	*Tyto alba*	-	-
	26	*Tyto alba*	-	-
	27	*Tyto alba*	-	-
Ciconiiformes (N = 5)	28	*Butorides striatus*	-	-
	29	*Theristicus caudatus*	-	-
	30	*Theristicus caudatus*	-	-
	31	*Theristicus caudatus*	-	-
	32	*Tigrisoma fasciatum*	+	-
Charadriiformes (N = 3)	33	*Vanellus chilensis*	+	-
	34	*Vanellus chilensis*	+	-
	35	*Vanellus chilensis*	-	-
Psittaciformes (N = 2)	36	*Pionus maximiliani*	+	-
	37	*Pionus maximilini*	+	-
Accipitriformes (N = 2)	38	*Buteo magnirostris*	+	-
	39	*Elanus leucurus*	-	-
Piciformes (N = 2)	40	*Ramphastos dicolorus*	+	-
	41	*Ramphastos dicolorus*	-	-
Nyctibiiformes (N = 2)	42	*Nyctibius griseus*	-	-
	43	*Nyctibius griseus*	-	+
Caprimulgiformes	44	*Chordeiles pusillus*	-	-
Trogoniformes	45	*Trogon surrucura*	+	-
Columbiformes	46	*Columba livia*	+	+

+: Positive immunomarking; -: negative immunomarking.

virulent one (CA END strain), observed immunomarking in four samples (infected with the virulent strain), collected 3 to 5 days after inoculation, both in the spleen and lungs. Oldoni et al. (2005), also observed in embryos, 72 h after NDV infection, immunomarking in the lungs, as well as in muscles, skin, kidneys and corioalantoid membrane, when eggs were inoculated with mesogenic and velogenic strains. This finding demonstrated greater infectivity to other organs when infection was caused by virulent strains, as well as the presence of clinical signs. Finding of the viral antigen is directly related to the lesions and clinical signs presented by the birds (Nakamura et al., 2008). Because of this, as the trachea is the primary site of replication of NDV no matter the strain involved, it is the main tissue used in IHC (Alexander, 2003).

In another investigation, Bwala et al.(2012) used immunohistochemistry to determine the distribution of NDV in the oviduct of chickens vaccinated with LaSota strain and experimentally infected with the virulent strain. Immunomarking was observed in epithelial cells and lymphocytes in the interstitium of the oviduct, corroborating greater infectivity to different organs, when virulent samples are considered.

In free-living birds, from the 11 avian orders analyzed, only one (Caprimulgiforms) did not show any positive individual (Table 1). It should be emphasized that, as these were dead birds that were sent to our facilities, the number of individuals analyzed was not standardized. But it could be observed that the Passeriform order was the one with the greatest number of individuals analyzed and, consequently, the greatest number of positive birds (N =11). From the 24 positive free-living birds, 19 presented immunomarking exclusively in the trachea, 5 showed immunostaining in trachea and spleen, and only one presented immunostaining exclusively in the spleen. It is possible that the amount of circulating virus that goes through the spleen is too small to be detected by IHC, as

already described (Kumar et al., 2010). This finding may be even more pronounced in the case of the present study, as birds did not show clinical signs compatible with NDV, and died of other causes. Birds analyzed in the present study were sent to the wildlife center by environmental authorities (Environmental Police and Environmental Institute of Paraná). These birds were undernourished, had traumas and fractures because they were apprehended from wild animal traffic, and died suddenly. Post-mortem descriptive analysis was carried out, and none of the birds showed macro- or microscopic lesions compatible with ND.

There are nine serotypes of Avian Paramyxo virus (APMV), and virulence varies according to the virus strain and host species. The possible occurrence of cross-reactions between APMV-1, which causes Newcastle disease, and APMV-3, whose pathogenicity is little known, has already been reported, and these cross-reactions may affect diagnosis (Kumar et al., 2010). As the present study was based on polyclonal antibodies, cross-reactivity with other APMV may have occurred, once a high number of positive birds was found. This finding does not invalidate the present study, but demonstrates both APMV circulation and the need for typing studies to assess which virus strains may be found in free-living bird populations.

Another possibility that supports the large number of positive results is the wide use of live vaccines against ND in Brazil (La Sota and Ulster strains). These live vaccines behave as pathogenic strains in the host, including primary replication in the trachea. Besides, the contact between vaccinated and unvaccinated birds may transmit the vaccine virus, leading to antibody levels similar to those of vaccinated birds (Carrasco et al., 2009). Therefore, it is possible that positive birds had contact with vaccinated birds, although Wakamatsu et al. (2007) demonstrated that vaccinated birds did not show positive IHC reaction in lung and spleen samples. It should be emphasized that, although IHC provides important information on the distribution of NDV in tissues, molecular techniques should be also used, adding to the diagnostic sensitivity.

Several differences have been observed in virus strains isolated from different species of birds, mainly free-living birds in different locations throughout the planet. It is very important to devise more accurate methods to evaluate the virulence of NDV isolates, especially in hosts other than chickens. Further studies are also needed to investigate the determinant factors in interspecies transmission (Guo et al., 2014). These NDV strains circulate in bird populations, generally without causing the disease, in a parasite vs. host balance. Outbreaks may occur when these free-living birds get in contact with commercial birds, with considerable losses to countries that raise and export poultry and poultry products.

In conclusion, IHC, using polyclonal antibodies, is a diagnostic tool that may be used in locations that do not have biosafety levels for virus isolation, or that are even not able to perform molecular assays. IHC is applicable to free living birds, and brings important contributions to the knowledge on the circulation of both NDV and the other APMV, adapting, for example, the use of monoclonal antibodies.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

Alexander DJ (1995). The epidemiological and control of Avian Influenza and Newcastle Disease. J. Comp. Pathol. 112(2):105-126.
Alexander DJ (2003). Newcastle Diseases and other avian paramyxoviridade infections. In: Saif YM, Barnes HJ, Glisson JR, Fadly AM, McDougal LR and Swayne DE. Diseases of poultry 11 ed. Ames.Iowa State University Press. pp. 64-87.
AVMA Panel on Euthanasia American Veterinary Medical Association (2001). 2000 Report of the AVMA panel on Euthanasia. J. Am. Vet. Med. Assoc. 218(5):669-696.
Barbezange C, Jestin V (2005). Molecular study of the quasispecies evolution of a typical pigeon paramyxovirus type 1 after serial passages in pigeons by contact. Avian Pathol. 34(2):111-122.
Bhaiyat MI, Ochiai K, Itakura C, Islam MA, Kida H (1994). Brain lesions in young broiler chickens naturally infected with a mesogenic strain of Newcastle disease virus. Avian Pathol. 23(4):693-708.
Brown C, King DJ, Seal BS (1999b). Pathogenesis of Newcastle Disease in Chickens Experimentally Infected with Viruses of Different Virulence. Vet. Pathol. 36(2):125-132.
Brown CC, King DJ, Seal BS (1999a). Comparison of Pathology-based techniques for detection of viscerotropic velogenic newcastle disease virus in chickens. J. Comp. Pathol. 120(4):383-389.
Bwala DG, Clift S, Duncan NM, Bisschop SPR, Oludayo FF (2012). Determination of the distribution of lentogenic vaccine and virulent Newcastle disease virus antigen in the oviduct of SPF and commercial hen using immunohistochemistry. Res. Vet. Sci. 93(1):520-524.
Carrasco AOT, Seki MC, Sousa RLM, Raso TF, Pinto AA (2009). Protection levels of vaccinated pigeons (Columba livia) against a highly pathogenic Newcastle Disease Virus strain. Trop. Anim. Health Prod. 41(7):1325-33.
Cattoli, GL, Susta, CT, Brown C (2011). Newcastle disease: a review of field recognition and current methods of laboratory detection. J. Vet. Diagn. Investig. 23(4):637-656.
Ezema WS, Okoye JOA, Nwanta JA (2009). LaSota vaccination may not protect against the lesions of velogenic Newcastle disease in chickens. Trop. Anim. Health Prod. 41(4):477-484.
Guo H, Liu X, Xu Y Han Z, Shao Y, Kong X, Liu S (2014). A comparative study of pigeons and chicken experimentally infected with PPMV-1 to determine antigenic relationships between PPMV-1 and NDV strains. Vet. Microbiol. 168(1):88-97.
Hoque MA, Burgess GW, Karo-Karo D, Cheam AL, Skerratt LF (2012). Monitoring of wild birds for Newcastle disease vírus in north Queensland, Australia. Prev. Vet. Med. 103(1):49-62.

Jindal N, Chander Y, Primus A, Redig PT, Goyal SM (2010). Isolation and molecular characterization of Newcastle disease viruses from raptors. Avian Pathol. 39(6):441-445.

Kho CL, Mohd-Azmi ML, Arshad SS, Yusoff K (2000).Performance of an RT-Nested PCR Elisa for detection of Newcastle disease virus. J. Virol. Methods 86(1):71-83.

Kumar S, Dias FM, Nayak B, Collins PL, Samal SK (2010).Experimental avian paramyxovirus serotype-3 infection in chickens and turkeys. Vet. Res. 41(5):72-74.

Lockaby SB, Hoerr FJ, Ellis AC, Yu MS, Roberts CS (1993). Immunohistochemical detection of Newcastle Disease virus in chickens. Avian. Dis. 37(2):433-437.

Mayo MA (2002). Virus taxonomy. Arch. Virol. 147(5):1071-1076.

Miller PJ, Decanini EL, Afonso C (2010). Newcastle disease: Evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. 10(1):26-35.

Mineo TWP, Carrasco AOT, Marciano JA, Werther K, Pinto AA, Machado RZ (2009). Pigeons (Columba livia) are a suitable experimental model for Neosporacaninum infection in birds. Vet. Parasitol. 159(2):149-153.

Nakamura K, Ito M, Nakamura T, Yamamoto Y, Yamada M, Mase M, Imai K (2014). Pathogenesis of Newcastle Disease in vaccinated chickens: Pathogenicity of isolated virus and vaccine effect on challenge of its virus. J. Vet. Med. Sci. 76(1):31-36.

Nakamura K, Ohtsu N, Nakamura T, Yamamoto Y, Yamada M, Mase M, Imai K (2008). Pathologic and Immunohistochemical Studies of Newcastle Disease (ND) in Broiler Chickens Vaccinated with ND: Severe Nonpurulent Encephalitis and Necrotizing Pancreatitis. Vet. Pathol. 45(6):928-933.

Ojok L, Brown C (1996). An Immunohistochemical Study of the Pathogenesis of Virulent Viscerotropic Newcastle Disease in Chickens. J. Comp. Pathol. 115(3):221-227.

Oldoni I, Brown CC, King DJ, Samal S, Seal BS (2005). The use of hybridization and immunohistochemistry to study the pathogenesis of various Newcastle disease virus strains and recombinants in ebrionated chicken eggs. Microb. Pathog. 39(3):69-75.

Piacenti AM, King DJ, Seal BS, Zhang J, Brown CC (2006). Pathogenesis of Newcastle Disease in commercial and specific pathogen-free turkeys experimentally infected with isolates of different virulence. Vet. Pathol. 43(2):168-178.

Tiwari AK, Kataria RS, Nanthakumar T, Dash BB, Desai G (2004). Diffrential detection of Newcastle Disease virus strains by degenerate primers based RT-PCR. Comp. Immunol. Microbiol. Infect. Dis. 27(3):163-169.

Wakamatsu N, King DJ, Kapczynski DR, Seal BS, Brounw CC (2006). Experimental pathogenisis for chicken, turkeys and pigeons of exotic Newcastle disease virus from a outbreak in California during 2002-2003. Vet. Pathol. 43(6):925-933.

Wakamatsu N, King DJ, Seal BS, Brown CC (2007). Detection of Newcastle Disease Virus RNA by reverse transcription-polymerase chain reaction using formalin-fixed, paraffin-embedded and comparision with immunohistochemistry and in situ hybridization. J. Vet. Diagn. Invest. 19(4):396-400.

Wan H, Chen L, Wu L, Liu X (2004). Newcastle disease in geese: Natural occurrence and experimental infection. Avian Pathol. 33(2):216-221.