

## Research Article

# Immunological and Molecular Evaluation of Newcastle Disease Virus in Tissue Specimens from Free-living Birds

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**Abstract**

Newcastle disease (ND) is a viral disease that affects domestic and wild birds, highly contagious and can cause acute mortality in some species. Little is known about transmission and behaviour of the Newcastle Disease Virus (NDV) in avian species, particularly in free-living species and with the exception of commercial birds, which is extremely important owing to the possible ease of contact between free-living species and commercial birds. The purpose of ND diagnosis is to guide the decisions to control the disease and thus prevent the spread of the disease. The aim of the present study was to evaluate immunological (immunohistochemical) and molecular techniques Real Time RT PCR (rRT-PCR) in the diagnosis of Newcastle disease in free-living bird tissue samples. A total of 150 birds belonging to 14 orders and 46 avian species were evaluated. Positive immunoblotting for NDV in at least one of the evaluated tissues was found in 43 (28.6%) of the 150 birds tested and 110 (71.4%) were negative by Immunohistochemistry (IHC) for NDV. Regarding the results of Real Time RT PCR (rRT-PCR), only one positive sample was recorded for the class 2 NDV from the trachea of a specimen of striped owl (*Asioclamator*). Therefore, it is essential to carry out epidemiological monitoring, with a constant characterization of circulating viral samples in free-living birds, especially in regions of high poultry production, to identify possible biosecurity measures that could prevent outbreaks in commercial poultry.

**INTRODUCTION**

Newcastle disease (ND) is a viral disease that affects domestic and wild birds, highly contagious and can cause acute mortality in some species. It is caused by the Newcastle Disease Virus (NDV) which is an Avian Paramyxovirus Type 1 (APMV1) that is endemic in many countries, a member of the genus *Avulavirus* of the family *Paramyxoviridae* [1].

Viruses belonging to the *Paramyxoviridae* family are single-stranded RNA viruses, with non-segmented envelope and genome [2]. The NDV can be classified as class I and II. The class I is characterized by lentogenic samples [3] and the class II contains the vast majority of pathogenic samples circulating in the various avian species [4]. Similar to other RNA viruses, the NDV has been constantly evolving. At least 18 genomes and subgenomes have been described and this genetic diversity of NDV is a factor increasing the difficulty of monitoring the viral circulation [3,5,6]. Although all NDVs belong to the same serotype, this large genetic variability results in high viral diversity in field samples [7].

Based on pathogenicity studies, ND is categorized into three groups: lentogenic, mesogenic, and velogenic (low, moderate, and high virulence, respectively). The velogenic strain may be viscerotropic or neurotropic, depending on its predilection site [8]. The majority of isolated samples of free-living bird have lentogenic characteristics, and these lentogenic samples are usually described in specimens of the orders *Anseriformes* and *Charadriiformes* [4,5,9,10].

NDV can infect about 241 bird species, distributed among 27 of the 50 avian orders [11,12]. Most countries with a developed poultry industry conduct NDV monitoring in commercial and free-living bird populations [13]. To maintain the virus in a particular avian population, there is a need for reservoirs and/or disseminators whose participation and identification in the pathogenesis of the disease has still been poorly understood.

Numerous wild bird species have been described as susceptible to NDV infection and there is a possibility that the virus remains dormant in these birds. Under favorable

conditions, mutations can occur, which can lead to the formation of virulent strains and, consequently, cause clinical presentation of the disease. Thus, this proximity of free-living and commercial birds should be monitored [14-16]

Factors that are probably involved in the maintenance of infection are presence of carriers, introduction of susceptible birds, multiplicity of avian species (commercial birds x wild birds), and heterogeneity of NDV strains [17,8,18]. With the exception of commercial birds, little is known about the transmission and behaviour of NDV (vaccine samples and pathogenic samples) compared to other avian species, particularly those of free-living, which is extremely important due to the ease of contact that those birds might have with commercial birds [5,16,19].

According to some authors, ND remains endemic in Brazil, as well as throughout South America, thus serving as a constant source of virus spread, mainly through the illegal trade of wild birds [20,21].

ND presents variable clinical signs, which are closely related to the viral strain involved, age, health status and host susceptibility [22-25]. In addition to the influence of the viral strain involved, the host is of crucial importance in the dissemination of the agent. NDV strains were used to infect several species, although some species tended to show few signs of disease, even when infected with the most virulent NDV strains. On this aspect, it has been observed that some strains cause 100% mortality in chickens and they produce only mild disease in other species [26]. Such interspecific viral behaviour must be thoroughly studied [27].

Free-living birds can be held responsible for being the primary source of disease introduction. Therefore, comparative studies of NDV strains isolated from birds and strains used in the preparation of live vaccines have become crucially important [12,18,19].

The purpose of ND diagnosis is to guide the decisions to control the disease and thus avoid the spread of the disease [8]. Therefore, a reliable, safe, and easy-to-perform diagnosis should be considered because the faster it is performed, the more effective is the implemented disease control measures. Consequently, greater losses and the spread of the disease can be avoided [24].

Studies involving the use of tissue samples are extremely important to better understand the distribution of viral agents in infected organs, as well as the tissue protection capacity of a vaccine or the pathogenicity of sample [28- 32].

When we evaluated the tissue samples to be collected, 33 and 34 indicate that the spleen is one of the main organs to be harvested. In agreement, pigeons experimentally infected with PPMV-1 strains had the highest detection rate of NDV in spleen when evaluated by RT-PCR [35]. Thus, the authors recommend that spleen, liver, lung, and trachea should be considered as the choice for NDV detection. Interestingly, some authors [36,28,37] observed that RT-PCR was able to detect the viral genome in brain, lung, and spleen samples at the same frequency.

Molecular techniques are an alternative method to viral isolation, and they are being widely used. There are numerous protocols described. However, these techniques have a limiting factor that there must be a constant updating of the primers

and probes so that the technique can detect samples with high mutation rate and/or recombination [38].

Some researches [39] were successful to identify the presence of NDV in the tissues analysed (spleen, liver, lung, and trachea) of experimentally infected birds using the RT-PCR. However, in some tissue samples from experimentally infected birds, detection of viral RNA was not possible.

Immunohistochemistry (IHC) can be used to explore the pathogenesis of ND. The primary antibody makes it possible to locate the viral antigen in the affected tissue which increases the diagnostic accuracy [40]. Trachea is the main site of sampling for IHC, presents a good index of diagnostic sensitivity, and it is even possible to use it for clinical samples evaluation [41]. In the same way, the efficiency of IHC in research using naturally and experimentally infected birds by the NDV was proven. In the aforementioned study, immunostaining occurred efficiently in spleen and trachea. However, in the lung and liver, which were also analysed, the result was not satisfactory [39]. The major advantage is that, in some cases, IHC can be used in degraded tissues, allowing the diagnosis in samples that could not be used in other techniques such as RT-PCR or viral isolation [32].

As the ND does not cause pathognomonic histopathological lesions, immunological techniques such as IHC are important diagnostic tools, and can detect the agent in tissue samples [30,33,42-44]. Therefore, such a technique can replace viral isolation at sites that do not have laboratories with biosafety levels for this procedure [45,46], present a good level of sensitivity, be comparable to the viral isolation, and have a fast execution time [32].

Vaccination, in addition to biosecurity measures, is the best measure to prevent ND [3]. However, even successful vaccination programs prevent only the development of clinical signs and are not effective in preventing viral replication and stopping viral elimination in the environment. Thus, even with these programs, viral circulation remains in vaccinated animals [47,48]. In addition, there are reports of the detection of NDV vaccine samples in free-living birds [16,19].

The combination of diagnostic techniques can be decisive in the diagnosis of ND. Studies comparing IHC with RT-PCR for the diagnosis of ND, observed that no technique was 100% sensitive and a combination of immunological and molecular techniques was recommended [33,49,50]. In addition, use of molecular techniques can fill the gap related to the pathogenicity of the agent, mainly based on the gene sequencing in positive samples by RT-PCR [51].

The aim of the present study was to evaluate immunological (immunohistochemical) and molecular techniques (rRT-PCR) to diagnose Newcastle disease in free-living bird tissue samples.

## MATERIALS AND METHODS

### Tissue samples

Spleen and trachea samples were collected for IHC and PCR. These samples came from 150 birds (Table 1) that died at the Wild Animals Service - SAAS, Unicentro, Brazil from January 2015 to August 2017. The birds died without any clinical signs compatible with ND. Samples from 150 birds were submitted to the Real-Time PCR (rRT-PCR) and IHC. In 13 birds, harvest was

not possible, and consequently, spleen samples were used due to autolysis of the organ during necropsy. Samples of bird tissues experimentally infected with NDV and sterile saline solution were used as positive and negative controls, respectively.

After the collection, the samples were fixed in 10% formaldehyde for 24 hours and then maintained in 70% alcohol until histological slides were performed. For the rRT-PCR technique, aliquots of each tissue were stored in 1.5 mL plastic microtubes DNA/RNA-free identified, and maintained at a temperature of -20°C until RNA extraction was performed. After extraction of RNA, the samples were conditioned in 2.0 mL plastic cryotubes DNA/RNA-free and stored in liquid nitrogen canisters at a temperature of -196°C until the molecular tests were performed.

### Immunohistochemical (IHC) reaction

For the IHC, the protocol described by [39] was used. NDV polyclonal anti-HN protein, produced in rabbits (rabbit IgG), was used at its optimum reactivity dose (1:800) as the primary antibody. In sum, the slides were incubated with the primary antibodies for 60 minutes at 25°C after deparaffinization and endogenous peroxidase blocking the process. For amplification of the immunological signal, the Easy Link One® (Immunobioscience Corp., USA, imported by Erviegas®, Brazil) was used, according to the manufacturer's recommendation. The slides were stained with Harris hematoxylin and subsequently observed in the optical microscope, in a 40 × magnification.

### RNA extraction from tissues

For the extraction of viral RNA from the tissue samples, the Total RNA Extraction Kit (Mini) from Real Genomics® was used following the manufacturer's instructions. The product of the extraction was maintained in liquid nitrogen (-196°C) until its use.

### Polymerase chain reaction - real-time RT-PCR (rRT-PCR)

Real-time amplification was performed on a 96-well PCR plate (Applied Biosystems), in which 5µL of RNA was diluted in 20 mM Tris-HCl buffer [pH 8.4] / 50 mM KCl / 2 mM MgCl<sub>2</sub> / 0.2 mM of each dNTP (Ambion), 10 pmol of each primer (primed for Newcastle class1 [52] and class 2 [53], 10 pmol of probes, 1µL of Enzyme (AgPath 1-step Ambion Kit), and Ultra Pure water qsp 25µL. The plates were amplified in the Real Time 3300 PCR System (Applied Biosystems) thermocycler. Amplification was performed from a reverse transcription step at 45°C for 15 minutes, 95°C denaturation for 10 minutes, followed by 45 cycles of 95°C for 15 seconds for denaturation of DNA strands; 56°C for 1 minute for primer pairing and extension of the DNA strands (phase at which fluorescence data were collected). As a positive control, a culture sample of the vaccine strain was included in embryonic chicken egg (La Sota - Fort Dodge) and as a negative control, sterile DNA/RNase-free water (Gibco) was used.

## RESULTS AND DISCUSSION

Table 2 shows the animals that were tested positive for IHC. A total of 150 birds belonging to 14 orders and 46 species were evaluated, and positive immunostaining for the DNV in at least one of the evaluated tissues was found in 43 one of them (28.6%). Immunostains in the trachea occurred in 28/150 (18.6%) of the

tissue samples tested, whereas 18.9% (26/137) of the spleen samples were positive (Figure 1). It should be emphasized that the number of spleen samples evaluated by IHC was low because in some animals it was impossible to sample this tissue due to the autolysis. Only ten animals (6.6%) presented immunostains in both tissues. Of the total of 150 birds tested, 107 (71.3%) were negative by IHC for NDV.

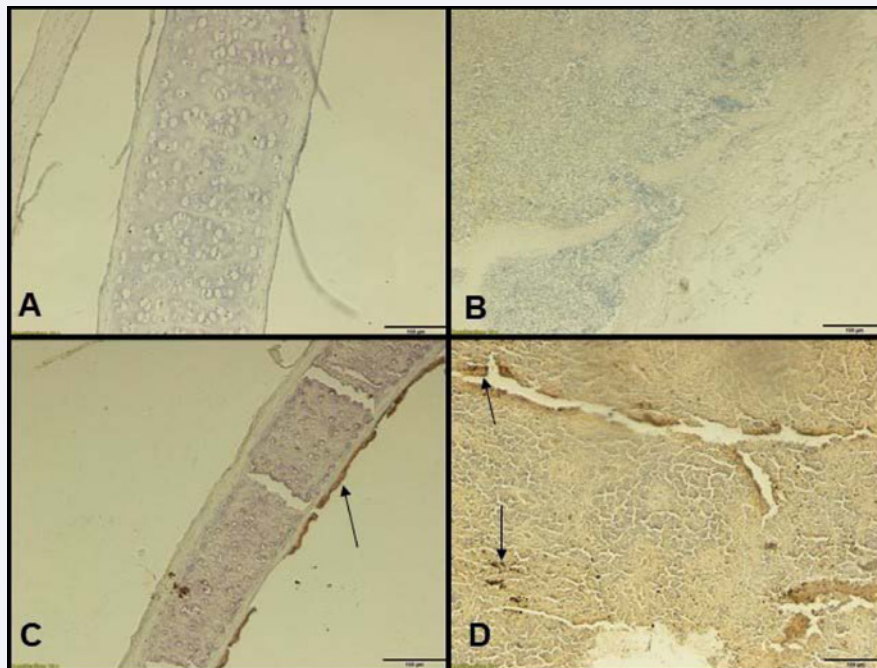
Regarding the results of rRT-PCR, only one positive sample was recorded for the class 2 NDV from the trachea of a specimen of striped owl (*Asio clamator*). This specimen was negative in a tracheal sample by the IHC. This animal had sepsis as a cause of death and due to tissue degradation, it was impossible to collect samples from the spleen. Due to a low concentration of genetic material, it was not possible to perform the gene sequencing.

Studies involving the use of tissue samples using immunological and/or molecular techniques are of extreme importance to better understand the distribution of the viral agent in the infected organs, as well as the tissue protection capacity of a vaccine or the pathogenicity of the sample [28,29].

In 23 out of 46 birds (50%), NDV was present in trachea and spleen samples evaluated by IHC technique [39]. Therefore, there was a higher percentage than the present study, in which it was 26.6% (40/150). However, there was a big difference in the number of analysed samples. Also observed that the orders Passeriformes and Strigiformes had greater number of positives, agreeing with our results. In the present study, in addition to the orders cited above, the order *Pelecaniformes* had a considerable number of positive (n=8) results [39]. However, in this study, no samples of *Pelecaniformes* were evaluated.

This fact indicates the importance of reaching the highest species diversity in studies with free-living birds. Therefore, a better characterization of the disease occurrence can be obtained in studied populations. In addition, this difference in the percentage of positivity reflects the moment of sample evaluations because contact and viral circulation do not occur in a linear and constant way in free-living birds. Moreover, innumerable factors such as climate change and infections due to other pathogens may directly affect the NDV circulation. It is important to note that, the individuals evaluated in the present study did not present clinical signs compatible with ND. The circulation of NDV vaccine samples in wild birds cannot be neglected. The presence of vaccine samples (LaSota and B1) in free-living birds has been described. Although there are still no descriptions of viral recombination between vaccine samples and high pathogenicity, the presence of viral samples in wild birds may allow a selection pressure on the NDV and increase virulence. However, the participation of free-living birds in the ND cycle should be thoroughly studied [19,16]. The LaSota and B1 samples are widely used for commercial bird vaccination against the NDV in Brazil.

Other authors [34] evaluated the distribution of the NDV in lymphoid tissues (spleen, Bursa of Fabricius and thymus) of experimentally infected geese. It was observed that around the 4<sup>th</sup> post-infection day (PID), there was the peak of tissue injury and viral detection by IHC and after the 10<sup>th</sup> PID, lesions were concentrated in the spleen and viral detection by IHC was possible only in the spleen and thymus, but with a smaller amount of immunostaining. In this sense, it confirms the choice of



**Figure 1** Samples of tissues submitted to immunohistochemistry: A- Negative trachea sample from *Vanellus chilensis*; B- Negative spleen sample from *Ciccaba virgata*; C- Positive immunoblot trachea sample (arrow) from *Zenaida auriculata*; D- Sample of spleen with positive immunoblot (arrow) from *Rhamphastos dicolorus*.

the spleen as one of the sampling sites to detect the NDV by IHC.

Some studies [41], sought to verify the presence of APMV-1 in tracheas of 26 experimentally and naturally infected chickens using the IHC technique. Only 30.76% (8/26) of the tracheas were positive for IHC, in the other tracheas, there was no evidence of the APMV-1 antigen, although they had lesions suggestive of tracheitis. The NDV may have a lesser role as a respiratory pathogen, allowing other respiratory pathogens to infect and worsen the clinical situation. In contrast, some authors [6] suggest the use of trachea, lungs, kidneys, proventriculus, cecal tonsil and brain as tissues of choice to detect the NDV by IHC.

In a study in Brazil [54], domestic pigeons (*Columba livia*) were evaluated, after a high mortality of these birds, with signs compatible with the ND. The dead birds were necropsied, and samples were taken for the IHC test. The positivity in the IHC test was 37.5% (9/24), a similar result to the present study. However, in that study, other tissue samples such as brain, pancreas, liver, and kidney were used, and these animals have been evaluated for clinical signs compatible with a NDV infection.

In contrast, [55] evaluated two NDV strains in an experimental infection using the IHC technique and found that a large amount of viral antigen in the epithelium of the digestive tract and respiratory tract.

IHC is a technique that can be used in the diagnosis of the Newcastle disease thanks to its accuracy, low cost, and it enables a better understanding of the pathogenesis of the agent through viral detection in tissues [6,56]. In addition, IHC does not require laboratories with a high level of biosafety because it is not carried out with live infectious agents [6].

Regarding the results of rRT-PCR, only one positive sample was recorded for the class II NDV from the trachea of a specimen of striped owl (*Asioclamator*). This specimen was negative in the IHC. Molecular techniques have a certain limitation in the diagnosis of NDV in free-living birds and some studies recommend the accomplishment of a viral isolation to increase the possibility of detection and, later, application of molecular techniques. In this context, another necessary assessment is that a positive sample by RT-PCR may not present viability because this technique can detect defective and/or dead viruses. In this way, this positive bird may not be able to contribute to the transmission cycle of the disease. Therefore, in principle, rRT-PCR uses small but high specificity gene sequences. However, when evaluating the NDV, it is generally agreed that this agent may exhibit a high rate of mutation and/or recombination, especially in free-living bird samples, which will not be detected by the standard primers used by these molecular techniques [3,13,38]. Studies highlight evidence of a great genetic diversity of the NDV, and this evidence has not yet fully understood, including the interaction of these genetic variants and their respective hosts, which are mostly free-living birds. However, there is no evidence that this diversity contributes to increased pathogenicity for farmed birds [57].

Thus, the occurrence of negative animals by the rRT-PCR, but positive by the IHC, is justified as presented in our study. It is important to point out that the IHC technique used in the present study is based on the use of anti-HN antibodies with polyclonal characteristics. That is, these antibodies have an affinity to a large number of NDV samples, including those that present genotypic alterations and those that might not present phenotypic alterations can be differentiated by immunological parameters such as IHC. However, this characterization of NDV samples of

**Table 1:** Total number of animals, in descending order, with their respective avian order, scientific name and common name, submitted to necropsy for spleen and tracheal harvesting and IHC and RNA extraction for real-time RT-PCR.

Order	Scientific name (No.of animals)	Common Name
Passeriformes (N=38)	<i>Pitangussulphuratus</i> (12)	Great kiskadee
	<i>Passer domesticus</i> (9)	House sparrow
	<i>Turdusrufiventris</i> (4)	Rufous-bellied thrush
	<i>Cacicushaemorrhous</i> (2)	Red-rumped cacique
	<i>Saltator fuliginosus</i> (1)	Black-throated grosbeak
	<i>Sporophilacaerulescens</i> (1)	Double-collared seedeater
	<i>Saltator similis</i> (1)	Green-winged saltator
	<i>Sporophilabouvreuil</i> (1)	Copper seedeater
	<i>Turdusrufiventris</i> (1)	Rufous-bellied thrush
	<i>Molothrusbonariensis</i> (1)	Shiny cowbird
	<i>Furnariusrufus</i> (1)	Rufous hornero
	<i>Turdus fumigatus</i> (1)	Cocoa thrush
	<i>Sicalisflaveola</i> (1)	Saffron finch
	<i>Cyanocompsabrissoni</i> (1)	Glaucous-blue grosbeak
	<i>Tyrannus savana</i> (1)	Fork-tailed flycatcher
Strigiformes (N=23)	<i>Ciccabavirgata</i> (6)	Mottled owl
	<i>Tyto alba</i> (6)	Barn owl
	<i>Athene cunicularia</i> (6)	Burrowing owl
	<i>Asioclamar</i> (4)	Striped Owl
	<i>Asiostygus</i> (1)	Stygian owl
Columbiformes (N=19)	<i>Zenaida auriculata</i> (14)	Eared dove
	<i>Columba livia</i> (2)	Rock dove
	<i>Columbinatalpacoti</i> (2)	Ruddy ground dove
	<i>Patagioenaspicazuro</i> (1)	Picazuro pigeon
Pelecaniformes (N=15)	<i>Theristicuscaudatus</i> (14)	Buff-necked ibis
	<i>Ardea alba</i> (1)	Great egret
Psittaciformes (N=14)	<i>Melopsittacusundulatus</i> (4)	Budgerigar
	<i>Pyrrhura frontalis</i> (3)	Maroon-bellied parakeet
	<i>Amazonavinacea</i> (3)	Vinaceous-breasted amazon
	<i>Nymphichushollandicus</i> (3)	Cockatiel
	<i>Amazonaestiva</i> (1)	Turquoise-fronted amazon
Piciformes (N=13)	<i>Ramphastosdicolorus</i> (10)	Green-billed toucan
	<i>Colaptesmelanochloros</i> (2)	Green-barred woodpecker
	<i>Ramphastostoco</i> (1)	Toco toucan
Charadriiformes (N=8)	<i>Vanellus chilensis</i> (8)	Southern lapwing
Falconiformes (N=4)	<i>Falco sparverius</i> (3)	American kestrel
	<i>Caracara plancus</i> (1)	Southern crested caracara
Caprimulgiformes (N=4)	<i>Leucochlorisalbicollis</i> (2)	White-throated hummingbird
	<i>Nyctibius griseus</i> (2)	Common potoo
Galliformes (N=3)	<i>Penelope obscura</i> (2)	Dusky-legged guan
	<i>Penelope superciliaris</i> (1)	Rusty-margined guan
Gruiformes (N=3)	<i>Gallinulagaleata</i> (2)	Common gallinule
	<i>Aramidessaracura</i> (1)	Slaty-breasted wood rail
Accipitriformes (N=3)	<i>Rupornismagnirostris</i> (3)	Roadside hawk
Trogoniformes (N=2)	<i>Trogon surrucura</i> (2)	Surucua trogon
Cuculiformes (N=1)	<i>Guiraguira</i> (1)	Guira cuckoo

the same serotype, displaying genetic differences between them, was previously described [7].

Some authors observed that the detection of NDV in tissue samples by molecular techniques should not be the main focus of a diagnostic attempt, due to the fact that the tissue tropism does not follow a pattern, compared to the different viral strains [58]. Thus, in the evaluation of clinical specimens, cloacal and

tracheal swabs are possibly the best option, although the possible presence of some PCR inhibitors should be considered in faecal samples and it may be necessary to perform a viral isolation prior to the test [58]. In addition, the difference between the results obtained by the IHC and rRT-PCR is due to the fact that we used different techniques (immunological and molecular) and should be evaluated as complementary and not as exclusive.

**Table 2:** Birds presenting positive reactions to the Newcastle Disease Virus by direct immunohistochemistry test in at least one tissue sample.

Order	Common Name	Scientific name	Spleen	Trachea
Passeriformes (N=8)	House Sparrow	<i>Passer domesticus</i>	+	-
	House Sparrow	<i>Passer domesticus</i>	+	-
	House Sparrow	<i>Passer domesticus</i>	-	+
	House Sparrow	<i>Passer domesticus</i>	+	+
	House Sparrow	<i>Passer domesticus</i>	-	+
	Great kiskadee	<i>Pitangus sulphuratus</i>	-	+
	Great kiskadee	<i>Pitangus sulphuratus</i>	+	+
	Fork-tailed flycatcher	<i>Tyrannus savana</i>	-	+
Strigiformes (N=8)	Barn owl	<i>Tyto alba</i>	-	+
	Barn owl	<i>Tyto alba</i>	+	-
	Barn owl	<i>Tyto alba</i>	+	+
	Burrowing owl	<i>Athene cunicularia</i>	-	+
	Burrowing owl	<i>Athene cunicularia</i>	+	-
	Mottled owl	<i>Ciccaba virgata</i>	+	-
	Mottled owl	<i>Ciccaba virgata</i>	-	+
	Striped owl	<i>Asio clamator</i>	-	+
Pelecaniformes (N=7)	Buff-necked ibis	<i>Theristicus caudatus</i>	+	-
	Buff-necked ibis	<i>Theristicus caudatus</i>	+	+
	Buff-necked ibis	<i>Theristicus caudatus</i>	+	-
	Buff-necked ibis	<i>Theristicus caudatus</i>	-	+
	Buff-necked ibis	<i>Theristicus caudatus</i>	-	+
	Buff-necked ibis	<i>Theristicus caudatus</i>	+	-
	Buff-necked ibis	<i>Theristicus caudatus</i>	+	+
Columbiformes (N=5)	Eared dove	<i>Zenaida auriculata</i>	-	+
	Eared dove	<i>Zenaida auriculata</i>	-	+
	Eared dove	<i>Zenaida auriculata</i>	+	+
	Eared dove	<i>Zenaida auriculata</i>	+	-
	Ruddy ground dove	<i>Columbina talpacoti</i>	+	-
Piciformes (N=5)	Toco toucan	<i>Ramphastos toco</i>	+	+
	Green-billed toucan	<i>Ramphastos dicolorus</i>	+	+
	Green-billed toucan	<i>Ramphastos dicolorus</i>	-	+
	Green-billed toucan	<i>Ramphastos dicolorus</i>	+	-
	Green-billed toucan	<i>Ramphastos dicolorus</i>	-	+
Charadriiformes (N=4)	Southern lapwing	<i>Vanellus chilensis</i>	-	+
	Southern lapwing	<i>Vanellus chilensis</i>	-	+
	Southern lapwing	<i>Vanellus chilensis</i>	+	-
	Southern lapwing	<i>Vanellus chilensis</i>	+	-
Psittaciformes (N=3)	Turquoise-fronted amazon	<i>Amazona aestiva</i>	+	-
	Cockatiel	<i>Nymphicus hollandicus</i>	+	+
	Cockatiel	<i>Nymphicus hollandicus</i>	+	-
Galliformes	Dusky-legged guan	<i>Penelope obscura</i>	+	+
Caprimulgiformes	White-throated hummingbird	<i>Leucochloris albicollis</i>	-	+
Gruiformes	Common gallinule	<i>Gallinula galeata</i>	+	+
<b>Total Positive Birds</b>			<b>Total Positive Spleens</b>	<b>Total Positive Trachea</b>
43			26	28

Results of viral isolation of cloacal swab specimens from wild birds, followed by molecular evaluation were presented. Of the samples collected from 6735 free-living birds, the highest detection rate occurred in the order *Charadriiformes*, *Passeriformes* and *Anseriformes*. *Charadriiformes* and *Passeriformes* also presented a high occurrence of positivity in the present study, however, when using the IHC [13]. This viral isolation is an interesting measure to increase viral load and, therefore, facilitate the detection of positive animals.

In a study where the brain tissue of 5608 dead birds was harvested, representing 21 avian orders, only 15 samples of the NDV were collected from birds of the order *Columbiformes* and were positive by the RT-PCR technique [59]. Based on genomic sequencing, these viruses were from the class 2, as found in the present study. This fact corroborates with the results of the present study, ratifying the hypothesis of the absence of positive animals due to the fact that we included relatively few samples compared to the study [59].

In a study that detected and characterized the NDV by the RT-PCR technique in 60 birds of prey from rehabilitation centres in the USA. Swabs of cloaca and oropharynx were used. Of all the 60 birds, three were positive by the technique, two species of eagles (order *Accipitriformes*) and one of owl (order *Strigiformes*) [60]. In this study, the only positive copy by rRT-PCR was also a specimen of the order *Strigiformes*. According to the phylogenetic analysis, all three isolates were classified as the class 2, just as in the present study and once again exemplifying, being the most comprehensive the class of APMV-1 strains.

An investigation of four specimens of *Columba livia* affected by neurological signs compatible with those caused by the NDV (ataxia, torticollis) performed the histological, immunohistochemical and molecular evaluation to evaluate the presence of the NDV in samples of liver, trachea, spleen, kidneys, heart, lung, duodenum, pancreas, among other tissues [56]. In this study, although in the histological sections showed that tracheitis occurred, immunostaining occurred only in renal tissue. Moreover, in the renal tissue of a bird, molecular detection was possible by the RT-PCR. In contrast, in the present study, it was possible to detect in trachea and spleen samples by the IHC, and in a tracheal sample by the rRT-PCR. The possibility of using renal tissue as a site of viral multiplication and, consequently, diagnostic viability should be studied.

RT-PCR test were performed to detect the presence of Paramyxovirus type I in dead pigeons harvested in squares in the city of Porto Alegre, with clinical signs compatible with those caused by the NDV. In these samples, it was possible to detect the viral genome in 25% (6/24) of the samples submitted to the test. Viral detection may be justified by the selection of animals that showed clinical signs similar to those of the NDV [54]. In opposition to the present study, in which the harvest of the material under analysis occurred independently of the causa mortis, and that, in our study, no bird died with compatible ND signals.

Another study carried out with 28 isolates obtained from wild free-living birds, wild captive birds, and commercial farm birds between 2008 and 2011 in different regions of Mexico [4]. These samples were submitted to inoculation in embryonated eggs and, later, the rRT-PCR technique was performed, in which

the virus genome was detected. This inoculation of the samples into embryonated eggs may increase the sensitivity threshold because it allows an increase in viral load and then be subjected to molecular tests.

With the use of live vaccines to combat the circulation of the NDV, the dissemination of vaccine particles in free-living bird populations is described as evidenced by the isolation of vaccine samples (LaSota) in wild birds [4,19]. In addition, the maintenance, evolution, and dissemination of NDV is relevant to the health of both free-living and commercial birds [57]. Possible occurrences of NDV subpopulations are described, characterized by the avian species involved, as well as the geographic distance of their hosts. These subpopulations may pose a potential risk to the poultry industry and this monitoring in free-living birds should be carried out in a constant manner to avoid the entry of new NDV variants [13,16,19].

Free-living birds are usually exposed to the NDV but active viral elimination in clinically healthy birds is relatively uncommon. Thus, a dissemination of these viral samples to commercial birds is an unlikely fact but epidemiological studies should not be ruled out because it is extremely difficult to predict the behavior of NDV in both free-living birds as accurately as in poultry [10,16]. In addition, high-virulence samples that cause outbreaks in commercial birds usually have their origin in poultry, not in free-living birds [61].

## CONCLUSION

In view of the above, it can be concluded that the IHC technique proved to be efficient for the detection of NDV in free-living samples. The large number of birds positive for the IHC reinforces the need to carry out epidemiological studies in free-living birds and search for molecular techniques of higher efficiency in order to type the collected samples.

Therefore, conducting epidemiological monitoring with the constant detection and characterization of circulating viral samples in free-living birds, especially in regions of high poultry production, is essential to identify possible biosecurity measures to prevent outbreaks in commercial birds.

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## REFERENCES

1. Mayo MA. Virus taxonomy-Houston. Arch Virol. 2002; 147: 1071-1076.
2. International Committee on Taxonomy of Viruses ICTVdb Index of Viruses: Family 01.048. Paramyxoviridae. 2002. December, 2008.
3. Dimitrov KM, Afonso CL, Yu Q, Miller PJ. Newcastle Disease Vaccines-A Solved Problem or a Continuous Challenge? Vet Microbiol. 2017; 206: 126-136.
4. Garcia SC, Lopez RN, Morales R, Olivera MA, Marquez MA, Merino R, et al. Molecular Epidemiology of Newcastle Disease in Mexico: Potential Spillover of Viruses from Poultry into Wild Bird Species. Appl Environ Microbiol. 2013; 79: 4985-4992.
5. Snoeck CJ, Adeyanju AT, Owoade AA, Couacy-Hymann E, Alkali BR, Ottosson U, et al. Genetic Diversity of Newcastle Disease Virus in Wild

- Birds and Pigeons in West Africa. *Appl Environ Microbiol.* 2013; 79: 7867-7874.
6. Etriwati, Ratih D, Handharyani E, Setiyaningsih S. Pathology and Immunohistochemistry Study of Newcastle Disease Field Case in Chicken. *Indonesia. Vet World.* 2017; 10: 1066-1071.
  7. Taylor TL, Miller PJ, Olivier TL, Montiel E, Garcia SC, Dimitrov KM, et al. Repeated Challenge with Virulent Newcastle Disease Virus does not Decrease the Efficacy of Vaccines. *Avian Dis.* 2017; 61: 245-249.
  8. Alexander DJ. Newcastle Disease and other Avian Paramyxoviridae infections in: Calnek BW, Barnes HJ, Beard CW, McDougald LR. (Eds.), *Diseases of Poultry.* 10<sup>th</sup> edn Ames: Iowa State University Press. 1997; 541-569.
  9. Kim LM, Jing DJ, Curry PE, Suarez DL, Swayne DE, Stallknecht DE, et al. Phylogenetic Diversity among Low-Virulence Newcastle Disease Viruses from Waterfowl and Shorebirds and Comparison of Genotype Distributions to Those of Poultry-Origin Isolates. *J Virol.* 2007; 81: 12641-12653.
  10. Pedersen K, Marks DR, Afonso CL, Stopak SR, Williams-Coplin D, Dimitrov KM, et al. Identification of Avian Paramyxovirus Serotype-1 in Wild Birds in the USA. *J Wildl Dis.* 2016; 52: 657-662.
  11. Kaleta EF, Baldauf C. Newcastle Disease in Free-Living and Pets Birds. In: Alexander DJ. (Ed). *Newcastle disease.* Boston: Kluwer. 1988; 197-246.
  12. Panshin A, Shihmanter E, Weisman Y, Örvell C, Lipkind M. Antigenic Heterogeneity among the Fields Isolates of Newcastle Disease Virus (NDV) in Relation to the Vaccine Strain. 1. Studies on Viruses Isolated from Wild Birds in Israel. *Comp Immunol Microbiol Infect Dis.* 2002; 25: 173-185.
  13. Muzyka D, Jackwood MP, Stegnyl, Ruka O, Bolotin V, Stegnyl A, et al. Wild Bird Surveillance for Avian Paramyxoviruses in the Azov-Black Sea Region of Ukraine (2006 to 2011) Reveals Epidemiological Connections with Europe and Africa. *Appl Environ Microbiol.* 2014; 80: 5427-5438.
  14. Alexander DJ, Russel PH, Parson G, Elzein EM, Ballouh A, Cernik K, et al. Antigenic and Biological Characterization of Avian Paramyxovirus Type 1 Isolates from Pigeon -- an International Collaborative Study. *Avian Pathol.* 1985; 14: 365-376.
  15. Monne I, Beato MS, Capua I, Mandola ML. Pigeon Paramyxovirus Isolated from a Robin in Italy. *The Vet Record.* 2006; 158: 384.
  16. Brown VR, Bevins SN. A Review of Virulent Newcastle Disease Viruses in the United States and the Role of Wild Birds in Viral Persistence and Spread. *Vet Res.* 2017; 48: 1-15.
  17. Awan MA, Otte MJ, James AD. The Epidemiology of Newcastle Disease in Rural Poultry: A Review. *Avian Pathol.* 1994; 23: 405-423.
  18. Kapczynski DR, Wise MG, King DJ. Susceptibility and Protection of Naïve and Vaccinated Racing Pigeons (*Columba Livia*) against Exotic Newcastle Disease Virus from California 2002-2003 Outbreak. *Avian Dis.* 2006; 50: 336-341.
  19. Ayala AJ, Dimitrov KM, Becker CR, Goraichuk IV, Arns CW, Bolotin VI, et al. Presence of Vaccine-Derived Newcastle Disease Virus in Wild Birds. *PLoS One.* 2016; 11.
  20. Seal BS, King DJ, Locke DP, Senne DA, Jackwood MW. Phylogenetic Relationship among high Virulent NDV Isolates Obtained from Exotic Birds and Poultry from 1989 to 1996. *J Clin Microbiol.* 1998; 36: 1141-1145.
  21. Clavijo A, Robinson Y, Booth T, Munroe F. Velogenic Newcastle Disease in Imported Caged Birds. *Can Vet J.* 2000; 41: 404-406.
  22. Brugh M, Beard CW. Atypical Disease Produced in Chickens by Newcastle Disease Virus Isolated from Exotic Birds. *Avian Dis.* 1984; 28: 482-488.
  23. Ballagi-Pordány A, Wehmann E, Herczeg J, Belák S, Lomniczi B. Identification and Grouping of Newcastle Disease Virus Strains by Restriction Site Analysis of a Region from the F Gene. *Arch Virol.* 1996; 141: 243-261.
  24. Kho CL, Mohd-Azmi ML, Arshad SS, Yusoff K. Performance of an RT-Nested PCR Elisa for Detection of Newcastle Disease Virus. *J Virol Methods.* 2000; 86: 71-83.
  25. Aldous EW, Alexander DJ. Detection and differentiation of Newcastle disease virus (avian paramyxovirus type 1). *Avian Pathol.* 2001; 30: 117-128.
  26. Carrasco AOT, Sseki MC, Raso TF, Paulillo AC, Pinto AA. Experimental Infection of Newcastle Disease Virus in Pigeons (*Columba Livia*): Humoral Antibody Response, Contact Transmission and Viral Genome Shedding. *Vet Microbiol.* 2008; 129: 89-96.
  27. Beard W, Brugh M. Immunity to Newcastle Disease. *American Journal of Veterinary Research.* 1975; 36: 509-512.
  28. Barbezange C, Jestin V. Development of a RT-Nested PCR test Detecting Pigeon Paramyxovirus-1 Directly from organs of Infected Animals. *J Virol Methods.* 2002; 106: 197-207.
  29. Barbezange C, Jestin V. Quasispecies Nature of an Usual Avian Paramyxovirus Type-1 Isolated from Pigeons. *Virus Genes.* 2005; 30: 363-370.
  30. Nakamura K, Ohtsu N, Nakamura T, Yamamoto Y, Yamada M, Mase M, et al. Pathologic and Immunohistochemical Studies of Newcastle Disease (ND) in Broiler Chickens Vaccinated with ND: Severe Nonpurulent Encephalitis and Necrotizing Pancreatitis. *Vet Pathol.* 2008; 45: 928-933.
  31. Ezema WS, Okoye JOA, Nwanta JA. LaSota Vaccination may not protect Against the Lesions of Velogenic Newcastle Disease in Chickens. *Trop Anim Health Prod.* 2008; 41: 477-484.
  32. Bwala DG, Clift S, Duncan NM, Bisschop SP, Oludayo FF. 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.* 2012; 93: 520-528.
  33. Wakamatsu N, King DJ, Seal BS, Brown CC. Detection of Newcastle Disease Virus RNA by Reverse Transcription-Polymerase Chain Reaction using Formalin-Fixed, Paraffin-Embedded Tissue and Comparison with Immunohistochemistry and in Situ Hybridization. *J Vet Diagn Invest.* 2007; 19: 396-400.
  34. Lu A, Diao Y, Chen H, Wang J, Ge P, Sun X, et al. Evaluation of Histopathological Changes, Viral Load and Immune Function of Domestic Geese Infected with Newcastle Disease Virus. *Avian Pathol.* 2014; 43: 325-332.
  35. Barbezange C, Jestin V. Monitoring of Pigeon Paramyxovirus Type-1 in Organs of Pigeons Naturally Infected with *Salmonella Typhimurium*. *Avian Pathol.* 2003; 32: 277-283.
  36. Kant A, Koch G, Van Roozelaar DJ, Balk F, Huurne AT. Differentiation of virulent and non-virulent strains of Newcastle Disease virus within 24 hours by polymerase chain reaction. *Avian Pathol.* 1997; 26: 837-849.
  37. Wambura P, Meers J, Spradbrow P. Determination of Organ Tropism of Newcastle Disease Virus (Strain I-2) by Virus Isolation and Reverse Transcription-Polymerase Chain Reaction. *Vet Res Commun.* 2006; 30: 697-706.
  38. Sabra M, Dimitrov KM, Goraichuk IV, Wajid A, Sharma P, Williams-Coplin D, et al. Phylogenetic Assessment Reveals Continuous Evolution



- and Circulation of Pigeon-Derived Virulent Avian Avulaviruses 1 in Eastern Europe, Asia and Africa. *BMC Vet Res.* 2017; 13: 1-13.
39. Carrasco AOT, Seki MC, Mineo TWP, Peres JA, Knychala LM, Alves MH, et al. Use of Immunohistochemistry (IHC) in the Detection of Newcastle Disease Virus (NDV) in Experimentally and Naturally Infected Birds. *Afr J Microbiol Res.* 2015; 9: 2225-2231.
40. Ramos-Vara JA, Miller MA. When Tissue Antigens and Antibodies get along: Revisiting the Technical Aspects of Immunohistochemistry – the Red, Brown and Blue Technique. *Vet Pathol.* 2014; 51: 42-87.
41. Brown CC, Sullivan L, Dufour-Zavala L, Kulkarni A, Williams S, Zhang J, et al. Comparing Presence of Avian Paramyxovirus-1 Through Immunohistochemistry in Tracheas of Experimentally and Naturally Infected Chickens. *Avian Dis.* 2013; 57: 36-40.
42. Ojok L, Brown C. An Immunohistochemical Study of the Pathogenesis of Virulent Viscerotropic Newcastle Disease in Chickens. *J Comp Pathol.* 1996; 115: 221-227.
43. Oldoni I, Brown CC, King DJ, Samal S, Seal BS. The Use of in situ Hybridization and Immunohistochemistry to Study the Pathogenesis of Various Newcastle Disease Virus Strains and Recombinants in Embryonated Chicken Eggs. *Microb Pathog.* 2005; 39: 69-75.
44. Piacenti AM, King DJ, Seal BS, Zhang J, Brown CC. Pathogenesis of Newcastle Disease in Commercial and Specific Pathogen-Free Turkeys Experimentally Infected with Isolates of Different Virulence. *Vet Pathol.* 2006; 43: 168-178.
45. Brown CC, King DJ, Seal BS. Comparison of Pathology-Based Techniques for Detection of Viscerotropic Velogenic Newcastle Disease Virus in Chickens. *J Comp Pathol.* 1999; 120: 383-389.
46. Brown C, King DJ, Seal BS. Pathogenesis of Newcastle Disease in Chickens Experimentally Infected with Viruses of Different Virulence. *Vet Pathol.* 1999; 36: 125-132.
47. Miller PJ, King DJ, Afonso CL, Suarez DL. Antigenic Differences among Newcastle Disease Virus Strains of Different Genotypes used in Vaccine Formulation Affect Viral Shedding after a Virulent Challenge. *Vaccine.* 2007; 25: 7238-7246.
48. Carrasco AO, Seki MC, Benevenuto JL, Ikeda P, Pinto AA. Experimental Infection With Brazilian Newcastle Disease Virus Strain In Pigeons And Chickens. *Braz J Microbiol.* 2016; 47: 231-242.
49. Kuiken T, Leighton FA, Wobeser G, Danesik KL, Riva J, Heckert RA. An Epidemic of Newcastle Disease in Double-Crested Cormorants from Saskatchewan. *J Wildl Dis.* 2013; 34: 457-471.
50. Adi AA, Astawa NM, Putra KS, Hayashi Y, Matsumoto Y. Isolation and Characterization of a Pathogenic Newcastle Disease Virus from a 4 Natural Case in Indonesia. *J Vet Med Sci.* 2010; 72: 313-319.
51. Carrasco AOT, Rodrigues JNM, Seki MC, Moraes FE, Silva JR, Durigon EL et al. Use of Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) in Molecular Screening of Newcastle Disease Virus in Poultry and Free-Living Bird Populations. *Trop Anim Health Prod.* 2013; 45: 569-576.
52. Kim LM, Suarez DL, Afonso CL. Detection of a Broad Range of Class I and II Newcastle Disease Viruses Using a Multiplex Real-Time Reverse Transcription Polymerase Chain Reaction Assay. *J Vet Diagn Invest.* 2008; 20: 414-425.
53. Wise MG, Suarez DI, Seal BS, Pedersen JC, Senne DA, King DJ, et al. Development of A Real-Time Reverse-Transcription PCR for Detection of Newcastle Disease Virus RNA in Clinical Samples. *J Clin Microbiol.* 2004; 42: 329-338.
54. Souza SO. Infecção por Paramyxovírustipo 1 empombos (Columba livia) no sul do Brasil. Dissertação (Ciências Veterinárias). Universidade Federal do Rio Grande do Sul, Porto Alegre, BR-RS. 2016. 51f.
55. Kumar S, Dias FM, Nayak B, Collins PL, Samal SK. Experimental Avian Paramyxovirus Serotype-3 Infection in Chickens and Turkeys. *Vet Res.* 2010; 41: 1-14.
56. Nakamura K, Fujimori H, Koyama A, Dai TQ, Imai K, Ikezawa M, et al. Immunohistochemistry and Molecular Epidemiology of Avian Paramyxovirus 1 From Formalin-Fixed and Paraffin-Embedded Sections of Japanese Dove (Columba Livia) Affected with Neurological Signs. *J Vet Med Sci.* 2015; 77: 837-841.
57. Ramey AM, Goraichuchuk IV, Hicks JT, Dimitrov KM, Poulson RL, Stallknecht DE, et al. Assessment of Contemporary Genetic Diversity and Inter-Taxa/Inter-Region Exchange of Avian Paramyxovirus Serotype 1 in Wild Birds Sampled in North America. *Virology.* 2017; 14: 1-12.
58. Creelan JL, Graham DA, McCullough SJ. Detection and Differentiation of Pathogenicity of Avian Paramyxovirus Serotype 1 from Field Cases Using One-Step Reverse Transcriptase-Polymerase Chain Reaction. *Avian Pathol.* 2002; 31: 493-499.
59. Kim LM, King DJ, Guzman H, Tesh RB, Da Rosa APT, Bueno R Jr, et al. Biological and Phylogenetic Characterization of Pigeon Paramyxovirus Serotype 1 Circulating in Wild North American Pigeons and Doves. *J Clin Microbiol.* 2008; 46: 3303-3310.
60. Jindall N, Chander Y, Primus A, Redig PT, Goyall SM. Isolation and Molecular Characterization of Newcastle Disease Viruses from Raptors. *Avian Pathol.* 2010; 39: 441-445.
61. Ramey AM, Reeves AB, Ogawa H, Ip HS, Imai K, Bui VN, et al. Genetic Diversity and Mutation of Avian Paramyxovirus Serotype 1 (Newcastle Disease Virus) in Wild Birds and Evidence for Intercontinental Spread. *Arch Virol.* 2013; 158: 2495-2503.

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