Universidad de los Andes
Facultad de Ingeniería

Antibody responses of healthy poultry vaccinated against Newcastle Disease to lentogenic and velogenic isolates of the virus in Colombia

por

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201110711

Presentado para optar al título de
Magister en Ingeniería Biomédica
Facultad de Ingeniería
Universidad de los Andes, Colombia
Enero 2021

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Antibody responses of healthy poultry vaccinated against Newcastle Disease to lentogenic and velogenic isolates of the virus in Colombia

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Abstract

Newcastle disease virus (NDV) is an avian pathogen known for its significant economic repercussions on the worldwide poultry industry. This avian *Avulavirus* belongs to the Paramyxoviridae family serotype 1 (APMV-1), and it has been identified in more than 236 species of birds. Despite vaccination plans in Colombia, there has been an increase in the NDV reports in several departments of the territory in recent decades. In order to evaluate serum reactivity from healthy poultry with NVD vaccination schemes against reference strains, lentogenic and velogenic NDV isolates; first serum samples were collected and selected. Secondly, the pathogenicity of NDV isolates from the Colombian territory was confirmed by the molecular characterization of amino acid motifs from the Fusion gene cleavage site. Four distinct vaccination programs were evaluated and differences in serum reactivity were found against lentogenic and velogenic NDV isolates. Results evidenced the presence of circulating antigenic variability in NDV isolates from the territory. Our findings contribute to increase the knowledge about the effectiveness of the vaccines used in common immunization schemes nationwide and motivate future studies for the development of control and prevention strategies.

Key words: *Newcastle disease virus, pathogenicity, poultry, serology, vaccine*

1. Introduction

Newcastle disease virus (NDV) is an avian pathogen known for its significant economic repercussions in the worldwide poultry industry (Diel et al., 2012; Jordan et al., 2018). This avian Avulavirus belongs to the Paramyxoviridae family serotype 1 (APMV-1), and it has been identified in at least 250 species of wild and domestic birds (Dey et al., 2019). NDV's genome is composed of negatively charged single-stranded RNA that encodes eight proteins. Six of these are related to structural function: matrix protein (M), fusion protein (F), Hemagglutinin-Neuraminidase protein (HN), nucleocapsid protein (NP), phosphoprotein (P) and polymerase protein (L). Additionally, proteins V and W, are involved in editing processes during the transcription of the P gene (Peeters & Koch, 2019). F and HN proteins are located on the viral surface and mediate host interaction and infection (Miller et al., 2009; Peeters & Koch, 2019). NDV's infection process begins with the recognition of sialic acid on host cell receptors by the HN protein, followed by the fusion of viral and host membranes mediated by the F protein. However, to initiate viral fusion, the F protein precursor (F0) must be proteolytically cleaved at aminoacidic position 117 to produce disulfide-linked F1 and F2 polypeptides derived from amino-terminal and carboxyl-terminal domains of F0 (Dey et al., 2019; Gravel & Morrison, 2003).

Three categories of virulence have been described, which are associated with symptoms and clinical signs of the disease in birds. Lentogenic strains have low virulence and tend to be asymptomatic or with mild respiratory and digestive symptoms, mesogenic strains have moderate virulence and are associated with respiratory, digestive, reproductive symptoms with a low mortality rate, and velogenic strains are highly virulent and generate fatal neurological symptoms that eventually cause death (Cattoli et al., 2011; Peeters & Koch, 2019).

Severity of NDV strains can be determined by the intracerebral pathogenicity index (ICPI), or by differential amino acid motifs in the F protein cleavage site. The motif ^{112}G/E-K/R-Q-G/E-R-↓L^{117} is

characteristic of lentogenic strains and allows cleavage by trypsin-like enzymes restricted mainly to the respiratory and intestinal tracts. On the other hand, mesogenic and velogenic strains present the motif ^{112}R/K-R-Q/K/R-K/R-R-↓F^{117}, which is cleaved by furin-convertase receptors widely distributed in host cells, producing lethal and systemic infections (Collins et al., 1993; Peeters & Koch, 2019). Considering its virulence and economic impact, NDV is of mandatory report by the World Animal Health Information System (OIE) meaning that strains with ICPI>0.7 or virulent must be notified (OIE World Animal Health Information System, 2017).

Despite vaccination measures for control and prevention, the Colombian poultry sector has been affected by NDV outbreaks in recent decades (Berhane et al., 2017; Chumbe et al., 2017). In 2018, a sanitary alert was declared by the Colombian Agricultural Institute (ICA) generating significant economic repercussions in the Colombian poultry industry (Sosa, 2018). Generally, intense vaccination programs are used, with at least three doses of inactivated, live, and recombinant vaccines and up to five doses where the virus incidence is high. Despite government mandatory request for vaccination with the administration of lentogenic strains such as LaSota, B1, Clone 30, and VG/GA belonging to the class II genotype II; NDV outbreaks persist and have been partly attributed to antigenic variability in circulating NDV in Colombia and worldwide (Absalón et al., 2019; Berhane et al., 2017; Miller et al., 2007; Yang et al., 2017). Previous research has demonstrated that F and HN protein sequence homology from LaSota reference strain, and strains isolated in the United States, Peru, Mexico, and Malaysia vary in between 87.7% to 88.6% and 86.7% to 89.0% (Miller et al., 2013).

This study aims to examine antibody responses of healthy poultry vaccinated against NDV to circulating lentogenic and velogenic isolates of the virus. Our findings seek to contribute to the control and prevention of the disease across Colombia.

2. Materials and methods

2.2 NDV strains

NDV strains were obtained from the Collection of Microorganisms of interest in Animal Health of the Colombian Corporation for Agricultural Research (AGROSAVIA). Two reference strains (B1, LaSota) and eight strains isolated from birds in the department of Cundinamarca since 1995 were selected for the study, including four lentogenic (NC001, NC002, NC018, NC042) and four velogenic strains (NC007, NC038, NC039, NC056). Strains were previously characterized by hemagglutination activity (HA) and hemagglutination inhibition (HI) assay. Strain pathogenicity was previously characterized by (Bustos, 1999), based on MDT (mean death time) which measures the time (hours) when embryonic mortality is achieved after inoculation of the strain in SPF embryos, IVPI (intravenous pathogenicity index), and ICPI (intracerebral pathogenicity index) that evaluates clinical symptoms in hatched chicks after inoculation of the strain intravenously and intracerebrally.

2.3 RNA extraction, RT-PCR and nucleotide sequencing

Purification of virions was achieved from infected Vero cells culture by applying three freeze-thaw cycles followed by supernatant recovery with the virions after centrifugation at 2000 rpm for 10min (Grupo de Investigación de Bioprospección de Biomoléculas y Microorganismos con Aplicaciones Agropecuarias de Agrosavia, 2019). Purified virions were used for total RNA extraction by following MagMax-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems-Thermo Fisher Scientific, Massachusetts, USA) instructions. Briefly, 50 uL of NDV virions were lysed followed by RNA-binding to magnetic beads.

Three washing cycles were performed to obtain RNA in 20 uL of elution buffer and stored at -80ºC. RNA quality and quantification were verified using Nanodrop 2000. Amplification of the 700 bp fragment corresponding to the F protein cleavage site (^{112}G/E-K/R-Q-G/E-R-↓L^{117} or ^{112}R/K-R-Q/K/R-K/R-R-↓F^{117}) was assessed using previously reported forward 5'-GACCGCTGACCACGAGGTTA-3' and reverse primers 5'-AGTCGGAGGATYTTGGCAGC-3' (Perozo et al., 2008). Reverse transcription polymerase chain reaction (RT-PCR) was performed using SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase Kit (Invitrogen-Thermo Fisher Scientific Massachusetts, USA), with melting temperatures of: 50ºC for 30 min, 94 ºC for 2 min; 40 cycles at 94ºC for 15 s, 56ºC for 30 s, 68ºC for 1 min; and a final extension at 68ºC for 5 min. Products from NDV strains were visualized in a 1,3% agarose gel and further sequenced by the Sanger method. Sequence analysis was done in MegAlign™ by DNASTAR Lasergene (Wisconsin, USA).

Serum sample collection

Serum samples from healthy layers vaccinated against NDV were collected in poultry flocks in the municipalities of Fomeque, Ubaque and Fosca from the department of Cundinamarca, Colombia.

20-24 Birds between 12 to 54 weeks were randomly selected by making an "X" across poultry sheds containing approximately 6000 birds.Blood samples were taken under aseptic conditions from the wing brachial vein and were refrigerated during transport to the laboratory. Samples were centrifuged at 1600 x g for 10 min followed by serum extraction and storage at -20ºC. Individual samples for each flock were evaluated by Enzyme-linked immunosorbent assay (ELISA) using Biochek NDV antibody test kit. Samples from each flock were pooled for further WesternBlot analysis.

2.2. Antibody analysis

Samples with previous reports of sanitary events or symptoms related to diseases during the raising of the poultry flocks or at the moment of sample collection were not considered for the study. Flocks were numbered from 1-23 and reference strains included on each vaccination scheme were identified, e.g., LaS= LaSota; B1= Hitchner B1; CL30=Clone 30; CL79=CL/79 clon.

Seropositivity in antibodies against NDV from serum samples collected was evaluated by the ELISA using Biochek NDV antibody test kit. Positive controls for ELISA test were reference strains commonly used in the manufacture of NDV vaccines (La sota and B1 strains) and pre-immune serum was obtained from unvaccinated backyard poultry birds implementing the same sampling protocol mentioned above. For each 100% seropositive sampled flock, three parameters were examined: the geometric mean titer of NDV antibodies (GMT), which provides information related to the intensity of NDV antibody response after vaccination (10000-25000 Biochek Reference Units) (Girón, 2014), the coefficient of variation (%CV), which indicates NDV antibodies uniformity in the poultry flock through titers distribution (20-60 %) and the vaccination index (VI), which gives a vaccination score that allows the interpretation of the serological monitoring (300-2000 Biochek Reference Units).

2.4 Western blot analysis

NDV virions were first concentrated with 3kDa Amicon Ultra-0.5 Centrifugal Filter Unit (Millipore-Merck, New Jersey, USA) and quantified by Bradford method (Bio-Rad Laboratories California, USA). Samples from reference strains and NDV isolates (5 ug) were subjected to denaturation at 100ºC for 5 min in 1:1 volume of loading buffer (100 uL 0.5 M Tris-base pH 8.5, 60 uL 20 % SDS, 300 uL glycerol, 0.018 mg bromophenol blue, 176.5 uL B-mercaptoethanol). Two negative controls were included: Vero cells

that underwent the same procedure for obtaining purified virions (supernatant), as well as fetal bovine serum (FBS) present in cell culture media. NDV samples were run in sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) (12,5 %). Resolving gel was made with 2.15 mL ultra-pure water, 1.25 mL 1.5 M Tris pH 8.8, 1.55 mL 40 % bis-acrylamide, 50 uL 10 % SDS, 30 uL 10 % APS and 3.5 uL TEMED. The stacking gel was made with 1.25 mL ultra-pure water, 500 uL 0.5 mM, 175 uL 40 % bis-acrylamide, 15 uL 10 % APS and 2.5 uL TEMED. Rainbow protein marker (Amersham™ ECL™-Merck, New Jersey, USA) was used, and samples were run at 140 V for 2 hours. Gels were visualized with Coomassie brilliant blue stain (R250, Thermo Fisher Scientific, Massachusetts, USA).

SDS-PAGE gels were transferred to a nitrocellulose membrane (Amersham™ Protran™ 0.2 um NC, GE Healthcare Life Science, Illinois, USA) at 100 V for 1 hour. The membranes were blocked with 5 % (Phosphate-Buffered Saline, 0.1% Tween 20) PBST/milk at slow speed at 4ºC overnight and were washed 4 times, 15min each in PBST. Primary antibody incubation was done with: i) pooled serums from healthy poultry vaccinated against NDV (1:500), ii) positive monoclonal Ab control from Biochek diagnostic kit (1:500), or iii) pre-immune serum from unvaccinated backyard poultry (1:500). All antibodies were diluted in PBST/milk for 2 h 30 min at room temperature and further washed 4 times, 15min each in PBST. Goat anti-Chicken IgY (H+L) that was used as a secondary antibody conjugated with peroxidase (HRP) (Novus Biologicals, Colorado, USA), diluted in PBST (1:5000) for 2 h 30 min at room temperature and washed 4 times, 15min each in PBST. The membranes were developed using Opti-4CN™ kit substrate solution from Bio-Rad Laboratories (California, USA). Following the manufacturer instructions, colored bands were detected after 1 min of exposure to the substrate. The reaction was stopped with distilled waterandmembranes were dried to determine the molecular weight of reactive NDV proteins to serum samples.

3. Results

3.2 Molecular characterization of the pathogenicity of NDV strains

Products of 700bp of the F gene from NDV strains are shown in **Figure 1**. Amino acid motifs from the F gene confirmed four lentogenic isolates (NC001, NC002, NC018, NC042) with the motif ^{112}GRQGR-L^{117}, and four velogenic isolates (NC007, NC038, NC039, NC056) with the motif ^{112}RRQKR-F^{117} **(Table 1)**, which corroborates previous classification done by Bustos and collaborators (Bustos, 1999).

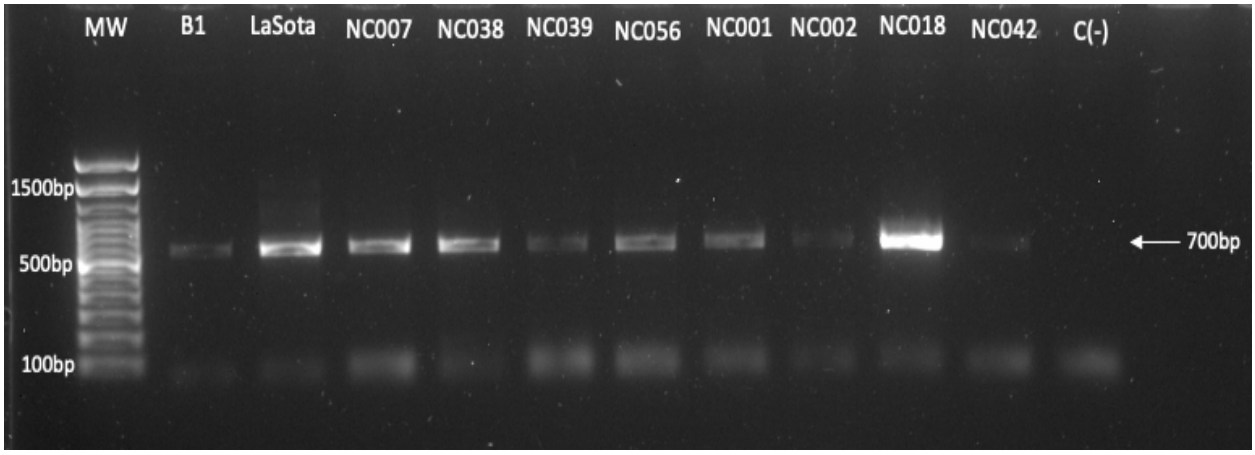


Figure 1. Agarose gel with amplified 700bp products from the F gene of the lentogenic and velogenic NDV isolates

Table 1. Physiological and molecular characterization of NDV isolates pathogenicity in the department of Cundinamarca

NDV accession number	**Location Municipality**	**Physiological characterization** by Bustos and collaborators			**Molecular characterization**	**Pathotype**
		MDT	**ICPI**	**IVPI**	**F_0 cleavage site sequence motif**	
NC001	La Mesa	72	1.3	0	^{112}GRQGR-L^{117}	Lentogenic

NC002	Guayabal	0.2*	2	0	^{112}GRQGR-L^{117}	Lentogenic
NC007	Guayabal	36	2	2.4	^{112}RRQKR-F^{117}	Velogenic
NC018	Sasaima	96	1.5	0.9	^{112}GRQGR-L^{117}	Lentogenic
NC038	Guayabal	50	2	3	^{112}RRQKR-F^{117}	Velogenic
NC039	Silvania	48	2	3	^{112}RRQKR-F^{117}	Velogenic
NC042	Sasaima	72	0.6	0	^{112}GRQGR-L^{117}	Lentogenic
NC056	Villeta	48	2	3	^{112}RRQKR-F^{117}	Velogenic

*The MDT value was reported by Bustos and collaborators but must be reviewed.

3.1 Serum sample collection and selection

A total of 476 serum samples in 23 flocks were collected in Fomeque, Ubaque and Fosca in the department of Cundinamarca (**Table 2**). Pools from 13 flocks were removed from the study due to the presence of respiratory symptoms associated with *Escherichia coli, Mycoplasma sp*. and *Gallibacterium anatis* (flock 1, flock 4, flock 7, flock 8), poor vaccination coverage (flock 17), suspected field challenges or immunosupression (flock 3, flock 9, flock 11, flock 12, flock 13, flock 15, flock 21, flock 23) **(Supplementary Table S1)**.

Table 2. Selected serum samples collected from healthy poultry vaccinated against NDV in the department of Cundinamarca.

Flock ID	Reference strains included	Municipality	Age	Vaccination program and reference strains included*	Number of doses	ELISA parameters
2	LaS+B1	Fomeque	33 weeks	Day 15 Live (LaSota, B1) + Inactivated (LaSota) Week 8: Live (LaSota, B1) + Inactivated (LaSota) Week 15: Live (LaSota, B1) + Inactivated (LaSota) Week 24: Live (LaSota, B1)	4	GMT: 18962 %CV: 20 VI: 969

6	LaS	Fomeque	17 weeks	Day 8: Live (LaSota) Day 12: Inactivated (LaSota) Day 25: Live (LaSota) Week 8: Live (LaSota) + Inactivated (LaSota) Week 15: Live (LaSota) + Inactivated (LaSota)	5	GMT: 15702 %CV: 24 VI: 672
18	LaS+CL79	Ubaque	18 weeks	Day 12: Live (CL/79 clon) Day 18: Live (CL/79 clon) Week 7: Live (CL/79 clon) + Inactivated (LaSota) Week 15: Live (CL/79 clon) + Inactivated (LaSota)	4	GMT: 20088 %CV: 31 VI: 675
22	LaS+CL79	Fosca	20 weeks	Day 13: Live (CL/79 clon) Day 19: Live (CL/79 clon) Week 8: Live (CL/79 clon) + Inactivated (LaSota) Week 15: Live (CL/79 clon) + Inactivated (LaSota)	4	GMT: 11883 %CV: 35 VI: 358

*Commercial names of the vaccines were kept confidential

Selected flock samples had vaccination schedules that combined live and inactivated vaccines from various commercial manufacturers and employed three to five doses **(Supplementary Table S1)**. Samples representing differential vaccine types were selected for the evaluation of antibody responses against reference strains and NDV isolates **(Table 2)**: Flock 2, vaccinated against LaSota and Hitchner B1 strains (LaS+B1, GMT: 18962), flock 6, vaccinated against LaSota strain (LaS, GMT: 15702), flocks 18 and 22, vaccinated against LaSota and CL/79 clon strains (LaS+CL79, GMT: 20088 and GMT: 11883 respectively).

3.3 Antibody responses to lentogenic and velogenic NDV isolates

Protein profile of NDV strains revealed several bands within the size range previously documented for the structural proteins HN, F_0, P, F_1, NP, M **(Figure 2a)** (Hemmatzadeh & Kazemimanesh, 2017; Umino et al., 1990). In agreement with previous studies, the L protein was not detected around 180-220 kDa (Ren et al., 2012). No antibody response against NDV strains was observed with the pre-immune serum beyond non-specific reactions that generated background **(Figure 2b)**. Apart from the reference strains LaSota and B1, the reactivity of specific monoclonal antibodies against NDV was only seen with the F_0 protein for the lentogenic NC018 and velogenic NC039 isolates **(Figure 2c)**.

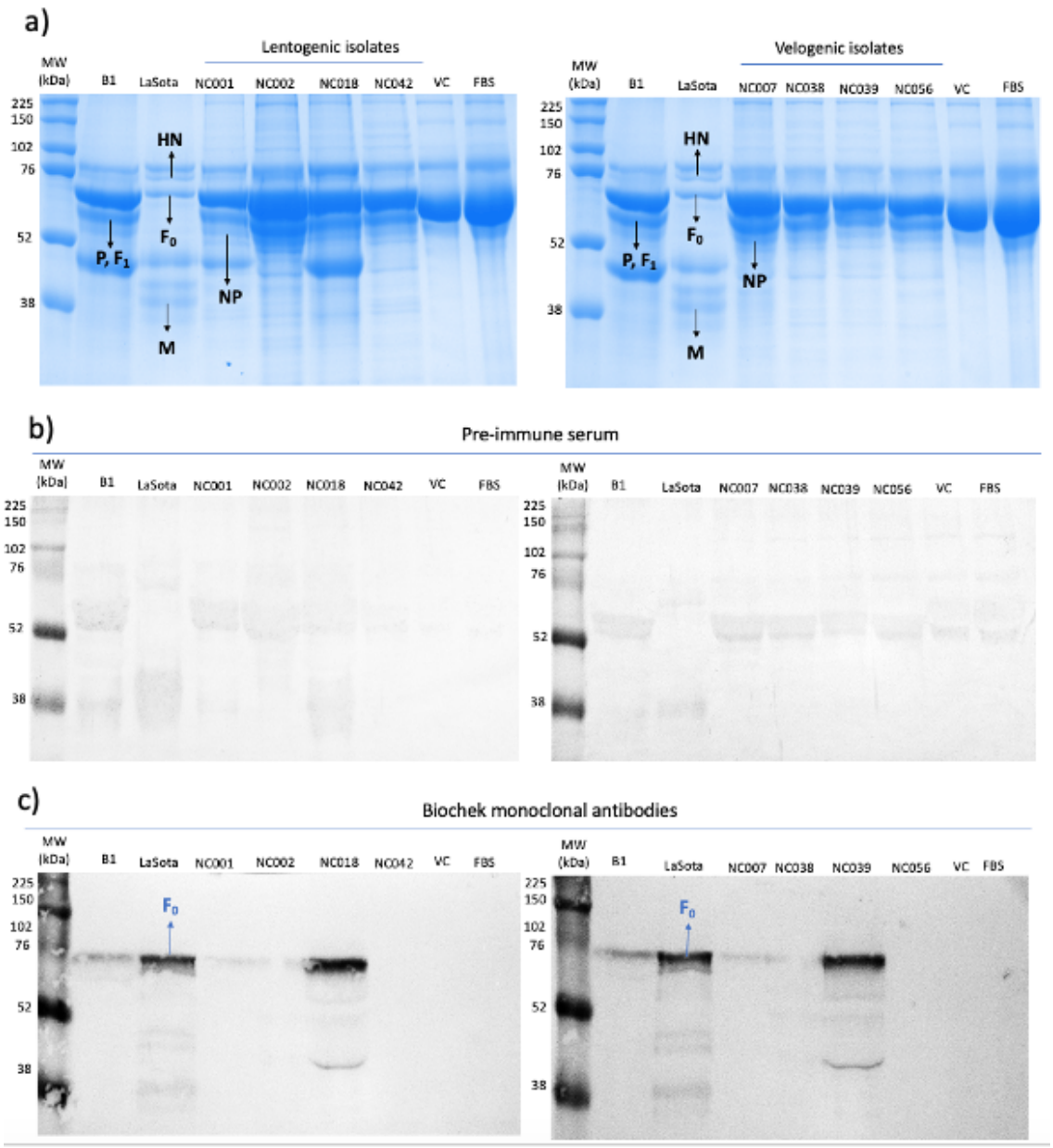
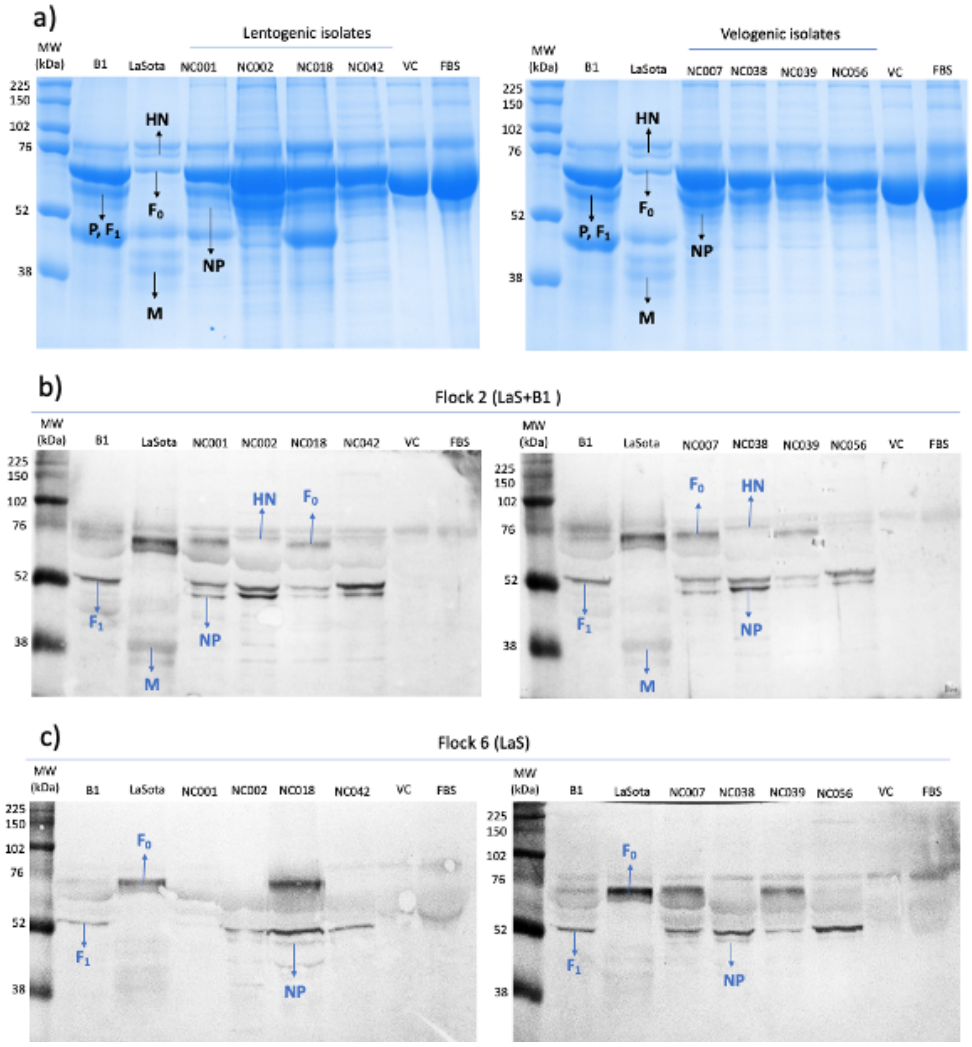


Figure 2. a) SDS PAGE protein profile of purified lentogenic and velogenic NDV virions. VC: Non-infected Vero cells with the same purification procedure of the virions. FBS: Fetal Bovine Serum. Antibody responses of **b)** pre-immune serum from unvaccinated backyard poultry birds, c) NDV monoclonal antibodies from Biochek to lentogenic and velogenic isolates of NDV.

Serum from vaccinated flock 2 against LaS+B1 reacted against the reference strains and NDV isolates, particularly, to F_1 and NP proteins from the lentogenic and velogenic NDV isolates. The 74kDa HN protein from the lentogenic NC002, NC042 and velogenic NC038 NDV isolates could be identified, as

well as the 68kDa F_0 protein from the lentogenic NC001, NC018 and velogenic NC007, NC039 NDV isolates **(Figure 3b)**.



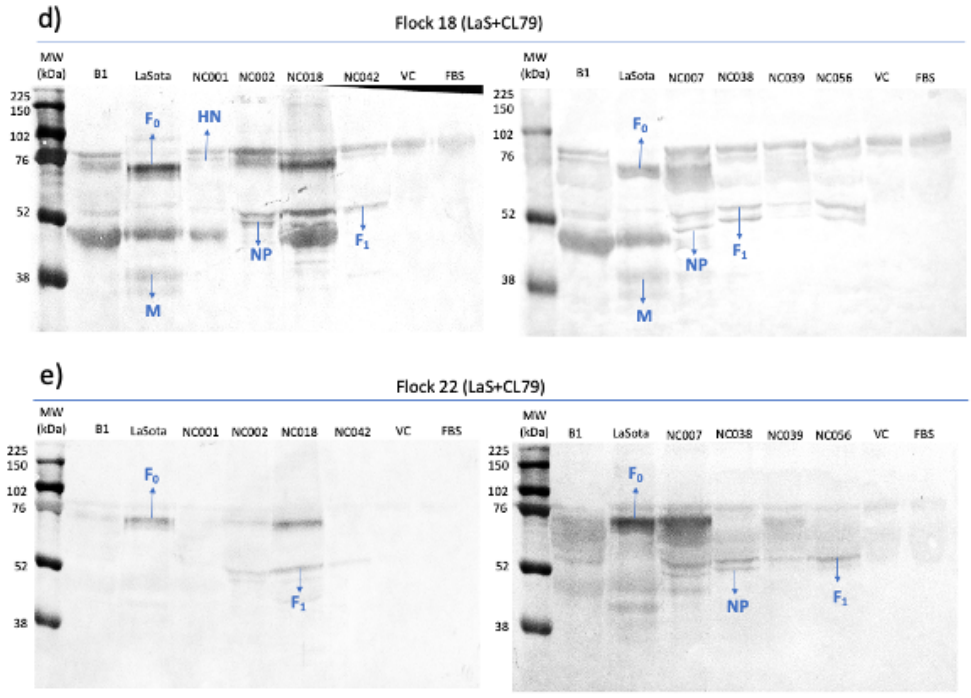


Figure 3. a) SDS PAGE protein profile of purified lentogenic and velogenic NDV virions. VC: Non-infected Vero cells with the same purification procedure of the virions. FBS: Fetal Bovine Serum. Antibody responses of **b)** serum from flock 2 vaccinated against LaS+B1, **c)** serum from flock 6 vaccinated against LaS, **d)** serum from flock 18 vaccinated against LaS+CL79, **e)** serum from flock 22 vaccinated against LaS+CL79 to lentogenic and velogenic isolates of NDV.

For, the serum from vaccinated flock 6 against LaS, antibody reactivity was seen for the reference strains and NDV isolates except for the lentogenic NC001, which didn't react with the serum **(Figure 3c)**. In addition, the F_1 protein was detected for the remaining NDV isolates and the F_0 protein only for the lentogenic NC018 and velogenic NC007 and NC039. Besides, the NP protein reacted for lentogenic NC002, NC018 and velogenic NC007, NC038, NC056 NDV isolates**.**

In the same way, specific reactions were evidenced with the serum from vaccinated flock 18 against LaS+CL79 against the reference strains, the lentogenic and velogenic NDV isolates **(Figure 3d)**. The F_1 protein reacted inmost the NDV isolates except for the lentogenic NC001 and velogenic NC039. Yet, a

74kDa band was evidenced for the lentogenic NC001, NC042 matching with the molecular weight of the HN protein. The F_0 protein reacted for the lentogenic NC002, NC018 and velogenic NC007 NDV isolates, whereas that the NP protein reacted for all the NDV isolates except for the lentogenic NC042 and velogenic NC039

Interestingly, with the serum from vaccinated flock 22 against LaS+CL79, no antibody reactivity was observed against the Hitchner B1 reference strain, and the lentogenic NC001 NDV isolate **(Figure 3e)**. Regarding the lentogenic NDV isolates, specific reactions were only seen with the F_0 protein from NC002 and NC018, and F_1 protein was evidenced for NC002, NC018 and NC042. Concerning the velogenic NDV isolates, antibody reactivity was notified for the F_0 protein from NC007and NC039; the F_1 protein from NC007, NC038 and NC056, and NP protein from NC007 and NC038 isolates.

Discussion

Validation of the pathogenicity of the NDV isolates by the identification of the amino acid motifs in the F gene cleavage site allowed to confirm that the protocols used by Bustos and collaborators for the previous physiological characterization were successful **(Table 1)**; to adopt rapid and less invasive molecular tools; and to ensure the evaluation of antibodies reactivity to lentogenic versus velogenic isolates with certainty (Bustos, 1999).

NDV vaccination schedules can vary between poultry flocks depending on the epidemiological record of the farm concerning NDV episodes (Farmers personal communication). Commercial vaccines administered include commonly the reference strain LaSota and require up to seven doses of live or

inactivated vaccines **(Table 2 and Supplementary Table S1).** In general, vaccination strategies observed in the field are similar to the ones reported for other Latin American countries such as Mexico, which also employs extensive vaccination programs in poultry layers (Absalón et al., 2019; Berhane et al., 2017).

Regarding the LaSota reference strain, the M protein showed reaction with the serums from vaccinated flock 2 against LaS+B1 and flock 18 against LaS+CL79 **(Figure 3a, d),** which is consistent with previous studies that have already described the presence of linear B cell epitopes of the M protein of LaSota strain. It is known that the M protein is conserved, but no significant reactivity was observed with all the evaluated sera against LaSota strain (Bi et al., 2019).

On the other hand, the serum from vaccinated flock 2 against LaS+B1 was the only one that generated a uniform reactivity against the F_1 and NP proteins for both lentogenic and velogenic NDV isolates, suggesting that the inclusion of the Hitchner B1 strain could be providing a greater coverage in terms of antibody responses to linear epitopes **(Figure 3b)**. Notably, the serum from vaccinated flock 6 against LaS that only included LaSota strain in the vaccination schedule reacted with all the F1 proteins from the NDV isolates except for the lentogenic NC001. Indeed, serums from vaccinated flock 6 against LaS and flock 22 against LaS+CL79 did not react to lentogenic NC001 NDV isolate, as well as the flock 18 against LaS+CL79 to the velogenic NC039 **(Figure 3c)** indicating first clues about existent antigenic variability in circulating NDV. In fact, the antibody reactivity was different even within the reference strains B1 and LaSota **(Figures 3a,b c)** , suggesting the existence of antigenic variability not only between circulating strains. To better understand, it could be of great interest to corroborate these results by comparing the

genetic similarities within the lentogenic and velogenic isolates and correlating nucleotide sequences with the reference strains LaSota and Hitchner B1.

Equally important, it was noticed that the reactivity of the serums from vaccinated flocks 18 and 22 against LaS+CL79 to both lentogenic and velogenic isolates **(Figure 3 d, e)** was different despite the inclusion of the same reference strains LaSota and CL/79 clon on each vaccination schedule. Nevertheless, the reactivity of the serum from vaccinated flock 22 against the reference strain LaSota was not as expected in comparison to the rest of the evaluated serums. The absence of reactivity could be attributed to NDV antibody titers obtained in the ELISA test that were close to the accepted lower limit for the serological monitoring (GMT:11883) in comparison to the flock 18, which reached almost twice the antibody titer (GMT: 20088) **(Table 2**).

The reactivity against HN protein was hardly seen and could be identified for the lentogenic NC001, NC002, NC042 and velogenic NC038 NDV isolates with different serums from vaccinated flocks 2 and 18 **(Figure 3 b, d)**. The observed HN reactivity is congruent with previous findings in which continuous epitopes were evidenced in the HN protein at positions 242-256aa and 341-355aa and could be related with the ones seen in the Western Blots (R M Iorio et al., 1991; Jin et al., 2021). Yet, the majority of HN antibodies with neutralizing activity have been attributed to conformational epitopes (Ronald M. Iorio et al., 1989; Umino et al., 1990).

Concerning the F protein, conformational epitopes have been documented (Yusoff et al., 1989). Nevertheless, linear epitopes were evidenced in the reference strains and NDV isolates **(Figure 3.)**. Differences in antibody reactivity between NDV isolates with each evaluated sample suggest antigenic variability. This is consistent with the monoclonal antibody reactivity results that were only evidenced for

the lentogenic NC018 and velogenic NC039 isolates **(Figure 2c)**. This differential reactivity against linear epitopes of the F_0 protein raises questions about the implications in NDV serological diagnostic.

Some studies have demonstrated that the F protein is stable; its conformational structure is conserved, and that amino acid changes related to antigenic variation of conformational epitopes are poorly known (Cho et al., 2008; Yusoff et al., 1989). However, other data have contradicted such a conclusion and evidenced significant antigenic variability on the F protein epitopes (Panshin et al., 1998).

In earlier studies, it has been shown that the F protein is activated after an initial interaction mediated by the HN protein has occurred by recognizing the host cells sialic acid (Kumar et al., 2011). The co-expression of HN protein and its interaction with the F protein is known to be crucial for the host infection (Gravel & Morrison, 2003; Zaitsev et al., 2004). Considering this step in the infective process, the HN protein appears to be exposed to a significant selection pressure that may explain its higher rate in genetic variability compared to the F protein as previously supported by other authors (Miller et al., 2013). Our findings are fully aligned with the aforementioned since antibody reactivity against the HN protein was observed in fewer NDV isolates compared to that of the F protein **(Figure 3**).

The conservancy of neutralizing epitopes has been shown to vary between reference strains, lentogenic and velogenic isolates which could be largely explained by existing antigenic variability (Jin et al., 2021). For example, the mutation E347K has been reported in the HN345-353a.a epitope in Korean strains, being a potentially useful marker of antigenic variability in circulating NDV (Cho et al., 2008). Nevertheless, it is also important to mention that highly conserved epitopes have also been described among several strains, such as the linear HN 242-256aa epitope, which is recommended to be a target for vaccine design (Jin et al., 2021).

Low cross-reactivity has already been demonstrated between reference strains and NDV isolates of the VII genotype already been identified in Colombia (Berhane et al., 2017; Huang et al., 2015). For example, the use of LaSota strain in live vaccines has failed to protect poultry flocks from genotype VII strains (Huang et al., 2015; Yi et al., 2011). Genotype VII is an antigenic variant that can escape immune response originated by vaccines (Cho et al., 2008). As NDV phylogenetic classification is built on the diversity of the F gene, it would be of high interest to determine to which genotypes correspond the studied NDV isolates and correlate those findings with our results concerning antibody responses, especially for the NC001 and NC039 NDV isolates that did not show antibody reactivity for some of the samples evaluated in this study.

All the results considered substantiate the need to extend future studies on the antigenic variability of the circulating NDV, which will allow the development of novel diagnostic and preventive approaches for NDV by the characterization of conserved and exposed linear epitopes which have a high potential for the design of vaccines (Chandra & Singh, 2012).

Conclusions

In this study, several vaccination schedules implemented in the Colombian territory could be documented and related to extensive immunization programs existing in other Latin American countries where NDV is also endemic. Our results concerning the antibody responses to lentogenic and velogenic isolates uggest antigenic variability in the territory that had been reported in previous studies. Considering the importance of linear epitopes in vaccine design and diagnostic tools, further research regarding the F and HN sequence diversity is needed to improve control and prevention strategies in the future.

Acknowledgements

This study was funded by the Colombian Ministry of Science (MinCiencias) with the 828 call “Segunda Convocatoria de innovación entre universidades y empresas para la promoción y validación de productos derivados del aprovechamiento sostenible de la biodiversidad en el Departamento de Cundinamarca – 2018”.

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