

# Determination of the distribution of lentogenic vaccine and virulent Newcastle disease virus antigen in the oviduct of SPF and commercial hen using immunohistochemistry

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## a b s t r a c t

The control of Newcastle disease (ND) in South Africa has proved difficult since 2002 following the introduction of lineage 5d/VIID Newcastle disease virus (NDV) strain (“goose paramyxovirus” – GPMV) to which commercially available ND vaccines appeared less effective. Most of the ND infections, even in fully vaccinated hens were characterized consistently by a drop in egg production. In this study, commercial and SPF hens-in-lay were vaccinated with La Sota vaccine and challenged with a GPMV isolate. Immunohistochemical labeling was used to determine the distribution of viral antigen in the oviduct of the hens. Following reports that cloacal vaccination offered better protection against egg production losses than the oro-nasal route, the efficacy of cloacal and ocular routes of vaccination against challenge were compared. Results showed that La Sota vaccine offered birds 100% protection against the virulent ND (GPMV) virus challenge from clinical disease and death, but not against infection and replication of the GPMV, as birds showed varying degrees of macropathology. Histopathology of the oviduct of infected birds revealed multifocal lymphocytic inflammation in the interstitium as well as mild glandular ectasia and mild edema. Finely granular NDV-specific immunolabeling was demonstrated in the cytoplasm of epithelial cells and mononuclear (lymphohistiocytic) cells in the interstitium of the oviduct. Both vaccine and virulent GPMV showed greatest tropism for the uterus (versus the magnum and isthmus). There was no clear difference in the protection of the oviduct and in the distribution of oviductal GPMV antigens between the two routes of vaccination.

## Keywords:

GPMV  
Hens  
Immunohistochemistry  
La Sota vaccine  
Newcastle disease

## 1. Introduction

Newcastle disease (ND) is a highly contagious and widespread disease of avian species causing severe economic losses in domestic poultry, especially chickens (Alexander, 2000). This disease is still one of the most important in poultry production worldwide, and remains a serious problem in spite of control measures, including vaccination applied since the 1950s (Czeglédi et al., 2006). Vaccination as a control measure has been used to keep the disease under control in most countries where poultry is raised commercially and where the disease is endemic (Alexander, 2001). However, several reports had indicated that the sub-optimal performance of commercially available ND vaccines against virulent

Newcastle disease virus (NDV) exists (Burridge et al., 1975; Kapczynski and King, 2005; Miller et al., 2007).

Immunohistochemistry (IHC), as a diagnostic tool has been used to diagnose and study the pathogenesis of Newcastle disease in tissues from various avian species (Lockaby et al., 1993; Ojok and Brown, 1996; Al-Garib et al., 2003; Oldoni et al., 2005). Generally regarded as a fairly sensitive alternative to virus isolation (the gold standard in Newcastle disease diagnosis) and serology, IHC offers a rapid means of identifying various pathogens, including viruses (OIE, 2010). IHC is performed on formalin-fixed, paraffin-embedded (FFPE) tissues and in many cases viral antigen can be detected in autolyzed tissues. The advantage is that diagnosis can still be made in cases where fresh sera or tissues are unavailable (Lockaby et al., 1993; Shi et al., 2001). Its diagnostic sensitivity and specificity is also comparable to those of RT-PCR and in-situ hybridization (Wakamatsu et al., 2007). Similarly, IHC has been found to be more sensitive in early detection of Newcastle disease viral antigen in

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visceral tissues of birds within 2 days of infection. However, no statistical significant difference exists between the probability of diagnosing Newcastle disease by virus isolation and immunohistochemistry in tissues generally (Kuiken et al., 1999). Despite the fact that virulent strains of NDV are known to induce pathology in the reproductive tract of infected poultry, only two prior studies looked at the reproductive pathology associated with NDV (Biswal and Morrill, 1954; Rao et al., 2002).

As Kapczynski and King (2005) and Czeglédi et al. (2006) had previously proved that the currently available vaccines induced better protection against viruses that were isolated in past epizootics (1950s) than against most of the viruses currently circulating in the poultry industry, and field observations suggested suboptimal performance of commercially available vaccines in the field, the efficacy study of the commercially available ND vaccines against infection by currently circulating ND viruses was undertaken. This study also determined the pattern of tissue tropism by ND (vaccine and virulent ND virus) in the reproductive tract of laying hens following viral introduction into the host through vaccination or challenge. This work also disproved the assumption that superior immunity against ND might be achieved in the oviduct through cloacal application of ND vaccine.

## 2. Materials and methods

### 2.1. Virus

The challenge virus used was a local (South African) velogenic Newcastle disease virus (NDV) strain, but locally known as goose paramyxovirus (GPMV). The Mean Death Time (MDT) and intracerebral pathogenicity index (ICPI) were determined previously to be 48 h and 1.85, respectively. Based on polymerase chain reaction (PCR) and molecular sequencing, it was classified as highly pathogenic and of the genotype 5d/VIId (GenBank Ref. # FJ985978). The virus challenge dose used was  $10^{7.0}$  EID<sub>50</sub>/0.1 mL/bird.

### 2.2. Experimental design

Eighty two (82) week-old specific pathogen-free (SPF) White Leghorns ( $n = 40$ ) and 52 week-old commercial Hyline Brown hens

**Table 1a**  
Description of experimental chicken and allocation to groups.

Group	Number of specific pathogen free chicken in the group	Number of commercial chicken in the group	Description
1	10	Nil	White leghorn, SPF, cloacal-vaccinated, challenged
2	10	Nil	White leghorn, SPF, eye-drop-vaccinated, challenged
3	10	Nil	White leghorn, SPF, cloacal-vaccinated, unchallenged
4	10	Nil	White leghorn, SPF, eye-drop-vaccinated, unchallenged
5	Nil	10	Hyline brown, cloacal-vaccinated, challenged
6	Nil	10	Hyline brown, eyedrop-vaccinated, challenged
7	Nil	10	Hyline brown, cloacal-vaccinated, unchallenged
8	Nil	10	Hyline brown, eyedrop-vaccinated, unchallenged
Positive control	10	Nil	White leghorn, SPF, non-vaccinated, challenged
Negative control	2	Nil	White leghorn, SPF, non-vaccinated, unchallenged

( $n = 40$ ) were procured from two reputable poultry establishments and assigned randomly into eight groups (groups 1–4 for SPF and groups 5–8 for commercial hens; Table 1a) of 10 hens per isolator/group. 12 extra SPF birds were also procured for the control experiment (Table 1a). The birds were allowed to acclimatize for 2 days after which they were vaccinated with NEW VAC-LS® Newcastle disease vaccine (La Sota strain, live virus – Forte Dodge®, Brazil; FD6033A; Batch No: 002/07) at the manufacturer’s recommended dose. Birds in groups 1, 3, 5 and 7 were vaccinated via the cloacal route while birds in groups 2, 4, 6 and 8 were vaccinated via eyedrop. A chicken each was removed from each group and euthanized for pathological examination on days 2, 4, 6, 8, and 10 post-vaccination (PV). On day 12 PV, the remaining birds were challenged via eyedrop with the NDV challenge virus. Ten White Leghorn SPF birds were used as the positive control. The positive control birds were not vaccinated but challenged with the same NDV at the same dose and by the same route. Two SPF birds were kept as negative control birds. They were neither vaccinated nor challenged. Serological responses were monitored in the groups through the use of NDV ELISA and manufacturers instruction (Newcastle Disease Virus Antibody Test Kit – FlockChek\*; IDEXX Laboratories Inc., Maine, USA).

### 2.3. Clinical observations, euthanasia and tissue sampling

All birds were observed twice daily throughout the trial, at intervals of not less than 8 h. One bird from each of the eight groups was humanely euthanized on days 2, 4, 6, 8 and 10 post-vaccination (PV) and post-challenge (PC) and the various parts of the oviduct sectioned. Birds were monitored according to the Poultry Reference Centre’s Standard Operating Procedure (PAS/PRC/035). Euthanasia was done by asphyxiation with carbon dioxide (CO<sub>2</sub>) according to the Poultry Reference Centre’s Standard Operating Procedure (SOP 0104) which was previously reviewed and approved by the Animal Use and Care Committee (AUCC); University of Pretoria.

All the birds were necropsied immediately after death (due to challenge or by euthanasia). Organs were examined for signs of ND such as tracheitis, necrohemorrhagic foci in cecal tonsils, proventriculi, etc. One to two centimeters (1–2 cm) of each part of the oviduct (magnum, isthmus and uterus) were collected in 10% neutral buffered formalin (NBF) in pre-labeled containers and allowed to fix for 24 h. Experimental birds were identified by routes of vaccination, date of euthanasia and the tissue sampled. At sectioning and histological processing of tissues, sample (S) numbers and the year of processing were added to the earlier identifications. Date/days of euthanasia were identified as: 2, 4, 6, 8 and 10 PV (post-vaccination) and 2, 4, 6, 8, and 10 PC (post-challenge), while organs were identified as either uterus, magnum or isthmus.

### 2.4. Histopathology

Tissues fixed in NBF were routinely processed into paraffin blocks, and 4 µm-thick sections were cut for hematoxylin-and-eosin staining (HE) according to the Standard Operating Procedure (SOP) of the Histopathology Laboratory, Pathology Section, Department of Paraclinical Sciences, University of Pretoria (Bancroft and Gamble, 2002).

### 2.5. Immunohistochemistry

Three-micrometer thick paraffin-embedded tissue sections were cut, mounted on positively charged Superfrost® Plus glass slides, dried overnight in a 58 °C oven, and labeled using a standard IHC protocol (California Animal Health and Food Safety, Paramyxo-

virus Type-1 IPX Stain #DHIS-02-716) (Manzer, 2008), modified to suit the SOP of the IHC Laboratory, Pathology Section, Department of Paraclinical Sciences, University of Pretoria. Following routine deparaffinization, the endogenous peroxidase activity in tissues was quenched in 3% Hydrogen Peroxide (in methanol) for 15 min (Clift et al., 2009). Tissue sections were subsequently subjected to heat-induced antigen retrieval, whereby slides were incubated in Dako Target Retrieval Solution (1× concentration) with 3.5 mg Protease, Type XIV (Sigma product #P5147-5G) and microwaved at 96 °C for 40 min. This was followed by blocking of non-specific protein binding sites with NovoLink™ Protein Block (#RE7102) (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) for 5 min. Slides were further immersed in 2% milk powder solution (Elite Fat-Free Instant Milk Powder, Clover S.A Pty, South Africa, H2038-3-2-500G) for 30 min at room temperature (approximately 25 °C) to block non-specific tissue antigen. The primary anti-NDV monoclonal antibody (mAb), pure mouse ascites, 10-5E6, 1985, specific for the phosphoprotein (P) genome of NDV, (courtesy Mark Peeples, Rush-Presbyterian-St. Luke's Medical Centre, Chicago, USA), diluted in 2% milk powder solution was used at 1:500 dilutions to stain the slides (Lockaby et al., 1993) for 1 h. Antigen detection was further accomplished using the NovoLink™ Polymer Detection System (RE7150-K), and a Vector NovaRed substrate (Vector Laboratories, Marion, Iowa, USA). Sections were lightly

counterstained (for 1–2 min) with Mayer's hematoxylin and coverslipped with Balsam oil for permanent record purposes.

For negative reagent control purposes, a mAb against Wesselsbron disease replaced the NDV mAb, keeping all other variables constant including the concentration and incubation time of the NDV mAb. This was done to assess the specificity of the NDV mAb.

## 2.6. The nature of positive labeling and target cells for NDV

Pattern of positive labeling in NDV IHC is usually stippled to granular, red to brownish depending upon the intensity of counterstaining, while labeling are mostly intracytoplasmic or not clearly cell-associated. Target cells are mostly epithelial cells (surface and glandular), mononuclear cells in the interstitium and in lymphoplasmacytic foci – possibly histocytes and lymphoid cells. Proper identify of the mononuclear cells will need double-immunolabeling procedures.

## 2.7. Slide examination and statistics

Labeled slides were examined at 400× magnifications, using an Olympus BH-2 light microscope. Positive labeling was noted either intracellularly or extracellularly (not clearly cell-associated) in the oviduct. Per section, one “hot spot” was identified (defined as the field with the greatest amount of immunolabeling) and within the “hot spot”, cells were counted and grading was performed according to Brown et al. (1999) as follows (Table 1b):

The numerical scores of 0, 1, 2, and 3 were entered into an Excel Spreadsheet (Microsoft Corporation, Redmond, Washington, USA) and added to get a cumulative score for each group. Mean scores for groups and vaccination routes including standard deviations (see Tables 2b and 3b) were calculated at 95% confidence level by selecting parametric methods and using Graphpad Quickcalcs® (Free Online Statistical Calculator, 2010). The different routes of

**Table 1b**  
Scoring of positively labeled immunohistochemically stained cells.

Scores	No. of labeling per field (400× magnification)	Numerical values
– Negative	No immunolabeling seen	0
+ Positive	1 positively-labeled cell seen	1
++ Positive	2 ≤ 5 positively-labeled cells seen	2
+++ Positive	6 or more positively-labeled cells seen	3

**Table 2a**  
Distribution of positive NDV-specific labeling (La Sota vaccine) post-vaccination in tissues of the oviduct of SPF and commercial hens as detected by immunohistochemical (IHC) technique.

Commercial hens					SPF hens				
Day Euth.	Sample No.	Uterus	Magnum	Isthmus	Day Euth.	Sample No.	Uterus	Magnum	Isthmus
2PV(1)	CV5 S1947-08	+	+	+	2PV(1)	CV1 S1946-08	–	–	–
2PV(1)	CV7 S1948-08	++	–	–	2PV(1)	EV2 S1949-08	–	–	–
2PV(1)	EV6 S1951-08	–	–	–	2PV(1)	EV4 S1950-08	–	–	–
4PV(2)	CV5 S1953-08	–	–	++	4PV(2)	CV1 S1952-08	+	+	–
4PV(2)	CV7 S1955-08	–	–	–	4PV(2)	CV3 S1954-08	–	–	–
4PV(2)	EV6 S1958-08	+++	++	–	4PV(2)	EV2 S1956-08	–	–	–
4PV(2)	EV8 S1959-08	–	–	+	4PV(2)	EV4 S1957-08	–	–	–
6PV(3)	CV5 S1963-08	+	++	+	6PV(3)	CV1 S1961-08	+++	–	–
6PV(3)	CV7 S1964-08	–	–	–	6PV(3)	CV3 S1962-08	++	+	++
6PV(3)	EV6 S1967-08	–	–	–	6PV(3)	EV2 S1965-08	–	–	–
6PV(3)	EV8 S1968-08	–	–	–	6PV(3)	EV4 S1966-08	–	–	–
8PV(4)	CV5 S1989-08	–	–	–	8PV(4)	CV1 S1987-08	–	–	+
8PV(4)	CV7 S1990-08	+	+	–	8PV(4)	CV3 S1988-08	–	–	–
8PV(4)	EV6 S1993-08	–	–	–	8PV(4)	EV2 S1991-08	+++	+++	+++
8PV(4)	EV8 S1994-08	–	–	–	8PV(4)	EV4 S1992-08	++	+	–
10PV(5)	CV5 S1997-08	+	–	–	10PV(5)	CV1 S1995-08	+	–	–
10PV(5)	CV7 S1998-08	+	+	+	10PV(5)	CV3 S1996-08	+++	+++	+++
10PV(5)	EV6 S2002-08	++	+	–	10PV(5)	EV2 S2000-08	+	+	–
10PV(5)	EV8 S2003-08	++	–	–	10PV(5)	EV4 S2001-08	–	–	–

– = Negative for NDV-specific labeling; + = 1 positive cell or focal extracellular cluster of positive NDV-specific labeling per 400× magnification; ++ = 2–5 positive cells or focal extracellular clusters of positive NDV-specific labeling per 400× magnification; +++ = >5 positive cells or focal extracellular clusters of positive NDV-specific labeling per 400× magnification.

Day Euth. = Days euthanized; PV = post-vaccination; CV = cloacal vaccination; EV = eyedrop vaccination.

CV (1, 3, 5, and 7) = cloacal vaccination (isolators 1, 3, 5, and 7).

EV (2, 4, 6, and 8) = eye (ocular) vaccination (isolators 2, 4, 6, and 8).

CV3 S1996-08; EV2 S2000-08 will mean cloacal vaccination in isolator 3, sample number 1996 processed in 2008; and eye vaccination in isolator 2, sample number 2000 processed in 2008, respectively.

**Table 2b**

Statistical analysis of mean scores for experimental chickens post-vaccination.

	Oviduct (all parts)		Uterus		Magnum		Isthmus	
	CV	EV	CV	EV	CV	EV	CV	EV
<i>Commercial chickens</i>								
Mean score	0.57	0.41	0.70	0.78	0.50	0.33	0.50	0.11
Standard deviation	0.68	0.84	0.67	1.20	0.71	0.71	0.71	0.33
Numbers	30	27	10	9	10	9	10	9
p-value	0.4338		0.8621		0.6146		0.1511	
<i>SPF chickens</i>								
Mean score	0.70	0.52	1.00	0.67	0.50	0.56	0.60	0.33
Standard deviation	1.09	1.01	1.25	1.12	0.97	1.01	1.07	1.00
Numbers	30	27	10	9	10	9	10	9
p-value	0.5188		0.5496		0.9044		0.5842	

CV is cloacal-vaccinated birds and EV is eyedrop-vaccinated birds.

**Table 3a**

Extent of infection and distribution of virulent NDV post-challenge as detected by IHC in the oviduct of vaccinated SPF and commercial hens.

Commercial hens					SPF hens				
Day Euth	Sample No.	Uterus	Magnum	Isthmus	Day Euth	Sample No.	Uterus	Magnum	Isthmus
2PC(6)	CV5 S2060-08	+	–	–	2PC(6)	CV1 S2058-08	+++	+++	–
2PC(6)	CV7 S2061-08	+	–	–	2PC(6)	CV3 S2059-08	+++	+++	+++
2PC(6)	EV6 S2064-08	+	++	+	2PC(6)	EV2 S2062-08	+++	+	–
2PC(6)	EV8 S2065-08	++	+	+	2PC(6)	EV4 S2063-08	+	–	–
4PC(7)	CV5 S2112-08	+	+	–	4PC(7)	CV1 S2110-08	++	++	+
4PC(7)	CV7 S2113-08	+	–	+	4PC(7)	CV3 S2111-08	Removed from the study		
4PC(7)	EV6 S2116-08	++	++	++	4PC(7)	EV2 S2114-08	+++	+	–
4PC(7)	EV8 S2117-08	++	++	+	4PC(7)	EV4 S2115-08	+++	+++	++
6PC(8)	CV5 S2167-08	++	++	–	6PC(8)	CV1 S2165-08	++	++	+
6PC(8)	CV7 S2168-08	++	+	–	6PC(8)	CV3 S2166-08	++	++	++
6PC(8)	EV6 S2171-08	+	–	–	6PC(8)	EV2 S2169-08	+++	+++	++
6PC(8)	EV8 S2172-08	+	+	+	6PC(8)	EV4 S2170-08	++	–	–
8PC(9)	CV5 S2175-08	+++	++	–	8PC(9)	CV1 S2173-08	+	+	–
8PC(9)	CV7 S2176-08	++	–	+	8PC(9)	CV3 S2174-08	++	+	+
8PC(9)	EV6 S2179-08	++	+	+	8PC(9)	EV2 S2177-08	+++	+++	++
8PC(9)	EV8 S2180-08	–	–	+	8PC(9)	EV4 S2178-08	Removed from the study		
10PC(10)	CV5 S2199-08	+++	+	+	10PC(10)	CV1 S2197-08	+++	+++	+++
10PC(10)	CV7 S2200-08	–	–	–	10PC(10)	CV3 S2198-08	++	+	–
10PC(10)	EV6 S2203-08	+	+	+	10PC(10)	EV2 S2201-08	++	++	+
10PC(10)	EV8 S2204-08	+	+	+	10PC(10)	EV4 S2202-08	–	–	–

– = Negative for NDV-specific labeling; + = 1 positive cell or focal extracellular cluster of positive NDV-specific labeling per 400× magnification; ++ = 2–5 positive cells or focal extracellular clusters of positive NDV-specific labeling per 400× magnification; +++ = >5 positive cells or focal extracellular clusters of positive NDV-specific labeling per 400× magnification.

Day Euth. = day euthanized; PC = post-challenge; CV = cloacal vaccination; EV = eyedrop or ocular vaccination; S = histopathology sample registration number.

CV (1, 3, 5, and 7) = cloacal vaccination (isolators 1, 3, 5, and 7).

EV (2, 4, 6, and 8) = eye (ocular) vaccination (isolators 2, 4, 6, and 8).

CV3 S1996-08 and EV2 S2000-08 will mean cloacal vaccination in isolator 3, sample number 1996 processed in 2008; and eye vaccination in isolator 2, sample number 2000 processed in 2008, respectively.

Birds CV3 S2111-08 and EV4 S2178-08 oviduct had adenocarcinoma and cannot be sampled, and were therefore removed from the study.

vaccination were compared for each section of the oviduct and the whole oviduct using unpaired Student *t*-test and significant result was fixed at *p*-value of 0.05. Shapiro–Wilk test was used to test for normality of data with a correlation coefficient (*r*) of 0.2620 and a critical value of 0.9715 (Harwell et al., 1992; Lix et al., 1996).

### 3. Results

#### 3.1. Clinical signs and mortality

All vaccinated birds appeared healthy post-vaccination and post-challenge. However, there appeared to be a transitory drop in egg production immediately following vaccination. Two birds (one SPF and one commercial) died on day 2 PV from causes not associated with the trial and were therefore not included in the

analysis. Subsequent to challenge, egg production dropped sharply to almost no production and was associated with the production of abnormally formed eggs. Given the small number of birds in the trial and the removal of birds on a daily basis for sampling – these results were not statistically significant, but were consistent with the expected effect of the vaccination and the challenge on egg production. There was no death from ND-related causes.

The positive control birds (unvaccinated but challenged) showed ND-related clinical signs on day 1 PC. Four of the birds had ruffled feathers and passed greenish feces on day 1 PC. Two hens were euthanized according to the research design while the remaining eight (100%) positive control birds appeared depressed and anorexic at the second observation on day 2 PC. By the second observation on day 3 PC, one positive control bird was found dead. Five more of the positive control birds died on day 4 PC, one on day 5 PC, and by the morning of day 6 PC, all the positive control birds

**Table 3b**  
Statistical analysis of mean scores for experimental chickens post-challenge.

	Oviduct (all parts)		Uterus		Magnum		Isthmus	
	CV	EV	CV	EV	CV	EV	CV	EV
<i>Commercial chickens</i>								
Mean score	0.87	1.10	1.60	1.30	0.70	1.10	0.30	1.00
Standard deviation	0.94	0.55	0.97	0.67	0.82	0.74	0.48	0.47
Numbers positive	30	30	10	10	10	10	10	10
<i>p</i> -value	0.2438		0.4313		0.2675		0.0042	
<i>SPF chickens</i>								
Mean score	1.81	1.48	2.22	2.22	2.00	1.44	1.22	0.78
Standard deviation	1.00	1.25	0.67	1.09	0.87	1.33	1.20	0.97
Numbers positive	27	27	9	9	9	9	9	9
<i>p</i> -value	0.2849		1.00		0.3101		0.4011	

CV is cloacal-vaccinated birds and EV is eyedrop-vaccinated birds.

were dead from viral challenge-associated causes. Egg production dropped to 0% by day 5 PC.

The two negative control birds (neither vaccinated nor challenged) did not manifest any signs of disease throughout the vaccination trial period, nor was egg production affected.

### 3.2. Serology (ELISA)

Prior to vaccination, all the SPF experimental birds had no detectable antibody to NDV while the commercial birds had titers of between  $2^5$  and  $2^{17}$ . Post-vaccination titers of the SPF birds that were bled remained at undetectable levels for the first six days PV, and gradually rose to between  $2^5$  and  $2^9$  and sometimes  $2^{12}$ , an indication of seroconversion in the SPF groups (data not shown). For the commercial birds (PV COMM), the PV titers fluctuated between  $2^{11}$  and  $2^{16}$  (data not shown).

Post-challenge, the antibody titers in SPF birds were between  $2^3$  and  $2^{16}$  while they fluctuated between  $2^8$  and  $2^{18}$  in commercial birds.

### 3.3. Gross pathology

None of the birds euthanized after vaccination showed any gross pathology.

Eight days after challenge one of the vaccinated hens had necrohemorrhagic foci in the cecal tonsils, while five hens showed similar lesions on day 10 post challenge. Four of the affected hens had been vaccinated by the ocular route and two by the cloacal route. Four of the five hens that had necrohemorrhagic foci in cecal tonsils on day 10 PC were commercial hens with a history of multiple ND vaccinations, while only one was an SPF hen that had received only a single ND vaccination.

At necropsy, two of the SPF hens were found to have uterine adenocarcinoma which made it impossible to sample the oviduct; these birds had to be removed from the trial. Seven others SPF hens had small, flaccid and inactive oviducts. The ovarian follicles of these seven hens were also inactive and consist of small immature ova.

The macropathology observed in the positive-control birds was variable, depending on the duration of the infection. Birds euthanized on day 2 PC had no gross lesions. The bird that died on day 3 PC, had pin-point hemorrhages on the serosal surface of the spleen, slight hemorrhage in the lumen of the proventriculus, congested ovarian follicles, and a segmentally congested duodenum that was filled with greenish watery content. Five birds died on day 4 PC, four of them had matted vents stained with green or white-tinged fecal material. Macroscopic lesions included congested tracheas, kidneys and lungs, pericardial hemorrhages and necrohemorrhagic foci in the cecal tonsils. Only one bird had hemorrhage in the lumen of the proventriculus. Also, all the positive

control birds (5) that died on day 4 PC showed marked degeneration of the ovarian follicles, which was characterized by resorption of the yolk, ill-defined external follicle outlines, and congested follicles, with some having yolk material lying freely in the abdominal cavity. The bird that died on day 5 PC had a diffusely congested trachea, several pin-point white spots on the serosal surface of the spleen, a congested heart and hemorrhagic foci in the proventriculus and cecal tonsils. The duodenum of this bird was also diffusely hemorrhagic and the ovarian follicles were hemorrhagic and appeared degenerated. Similar lesions were observed in the last bird that died on day 6 PC. In this bird there was also severe splenomegaly due to widespread hemorrhage.

### 3.4. Histopathology

Histopathological findings in all the three sections of oviducts (PV and PC) were variable but the lesions were generally mild in nature and mostly observed in the oviduct of post-challenge birds. They included: mild interstitial edema; focal to dispersed interstitial accumulations of lymphocytes and plasma cells (Fig. 1a); sporadic glandular ectasia with occasional pink granular intraluminal content (Fig. 1a); mild interstitial fibrosis with noticeable, widespread glandular drop-out/atrophy. The listed changes were observed throughout the oviduct but were most pronounced in the uterus with only few of these lesions seen in the magnum and the isthmus. Sections of the oviduct from the positive control birds revealed similar but more severe lesions compared to those observed in the oviducts of the post-challenge birds.

### 3.5. Immunohistochemistry (IHC)

Sections were obtained from a total of 83 birds – (38 birds post-vaccination, 38 birds post-challenge, two negative control birds and five positive control birds, Tables 2a and 3a). The use of the mAb to investigate viral distribution/tropism in the reproductive tract resulted in the observation of both intracellular and extracellular (or not clearly cell-associated) NDV-specific positive labeling, visualized as red-orange to tan-colored, finely granular/stippled labeling that tended to be focally clustered (Figs. 1b–d). Of the 76 trial hens (excluding the controls), 56 (73.68%) were positive and 20 (26.32%) were negative for NDV-specific labeling in their oviducts. A total of 20/38 (52.63%) hens euthanized post-vaccination, and 36/38 (94.74%) euthanized post-challenge were positive for NDV-specific labeling in their oviducts. Intracellular labeling was clearly intracytoplasmic (Fig. 1c and d) with a definite tropism for epithelial cells (surface and glandular) throughout the oviduct (Fig. 1c and d). Positive labeling was also frequently associated with mononuclear cells (macrophages/histocytes and/or lymphocytes) within focal or dispersed mononuclear infiltrates throughout the oviductal interstitium (Fig. 1a–d). Occasional fibroblast-



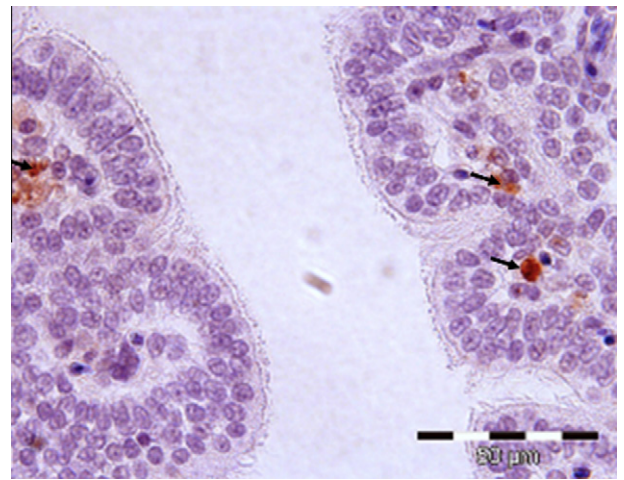
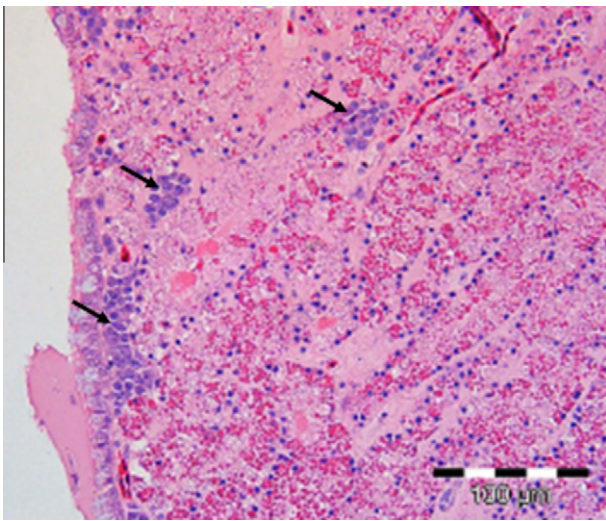


Fig. 1 (continued)

**Fig. 1.** (a) Magnum; Hen No. CV3 S2166-08; SPF hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 6PC. Mononuclear infiltrations (arrows) in the interstitium. Mayer's hematoxylin and eosin (H & E). (b) Magnum; Hen No. CV7 S1990-08; Commercial hen vaccinated with La Sota via cloacal route; Day 8PV. Presence of NDV-specific positive labeling within a lymphoid aggregate (arrow). IHC, Polymer system, Mayer's hematoxylin counterstain. (c) Isthmus; Hen No. CV1 S2197-08; SPF hen vaccinated with La Sota via cloacal route and challenged with a GPMV isolate; Day 10PC. Presence of ND viral antigen-specific positive labeling in subepithelial mononuclear cells (arrows). IHC, Polymer system, Mayer's hematoxylin counterstain. (d) Uterus; Hen No. EV4 S2115-08; SPF hen vaccinated with La Sota by eyedrop route and challenged with a GPMV isolate; Day 4PC. Presence of NDV-positive labeling in glandular epithelium (arrows). IHC, Polymer system, Mayer's hematoxylin counterstain.

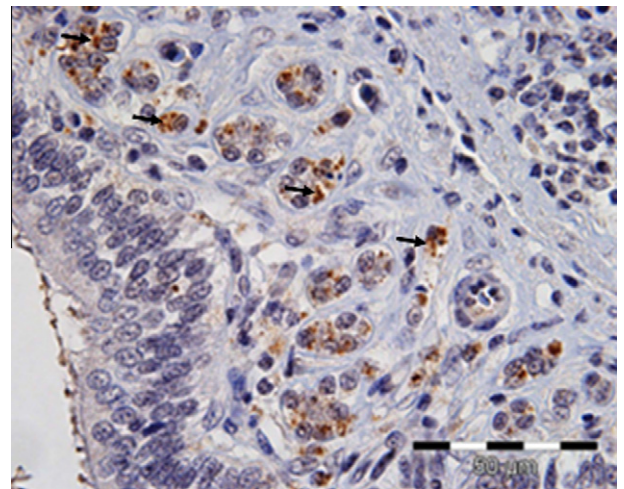


Fig. 1 (continued)

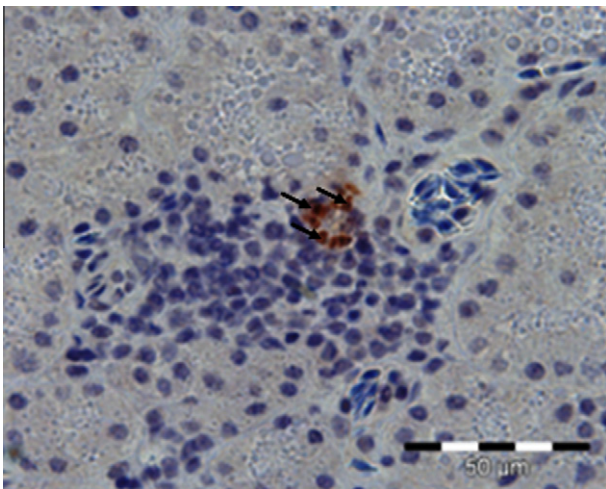


Fig. 1 (continued)

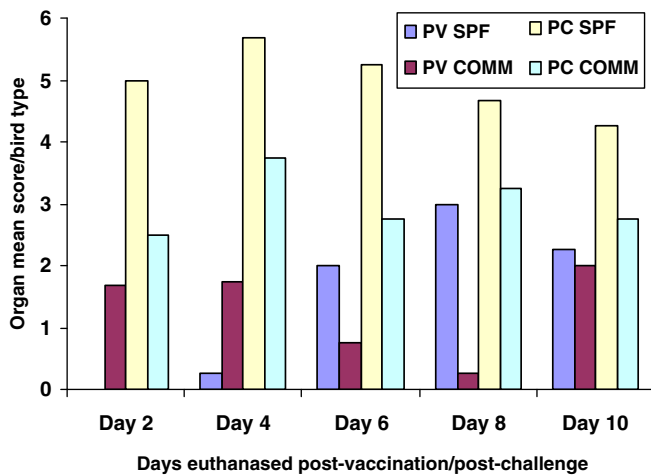
like connective tissues cells also appeared to contain NDV-specific labeling.

The distribution of NDV-specific positive labeling within the oviduct was similar for both the vaccinated and challenged birds. However, specific labeling was more prevalent in the oviduct of the challenged hens compared to the hens that were only vaccinated (Tables 2a and 3a). Positive labeling was not observed in any part of the oviduct from the negative control hens. In addition, no specific labeling was observed in sections of the oviducts from positive hens where the NDV mAb was replaced with the mAb against Wesselsbron disease virus. These results affirm the specificity of the NDV mAb utilized in the present study.

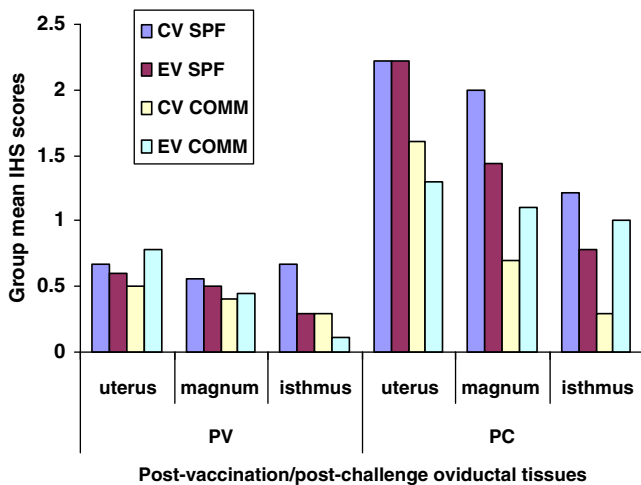
### 3.6. IHC labeling of the oviduct of hens post-vaccination (unchallenged hens)

The distribution of NDV-specific positive labeling in the oviduct of commercial and SPF hens vaccinated with La Sota vaccine is presented in Table 2a. Within the 10-day post-vaccination period, positive labeling was demonstrated in the oviduct of 20 (52.63%) hens, while the oviducts of 18 (47.37%) hens were negative for NDV-specific labeling, majority of which are SPF hens (10 SPF hens). Of the 20 positive hens, 11 (57.89%) were commercial and 9 (47.37%) were SPF. The mean range IHC labeling scores for the uterus, magnum and isthmus, increased from 0.6 to 1; 0.2 to 0.63 and 0.2 to 0.5, respectively from day 2PV to day 10PV (Fig. 4). The greatest amount of labeling was consistently observed in the uterus, 44.74% (17/38), followed by the magnum 31.58% (12/38) and the isthmus 23.68% (9/38) (Table 2a). Specific labeling was observed in the oviduct of commercial hens as early as day 2 PV. The SPF birds first showed NDV-specific positive labeling in oviductal tissues on day 4 PV averaging 0.25, and the mean score increased gradually to 3.0 on day 8 PV before it declined to an average of 2.25 by 10 PV (Figs. 2 and 3).

Cloacally-vaccinated SPF birds showed slightly more positive IHC labeling in the oviduct compared to SPF birds vaccinated via



**Fig. 2.** La Sota NDV vaccine and virulent GPMV distribution in the oviductal tissues of SPF and commercial hens post-vaccination and post-challenge. PV SPF = Post-vaccination SPF hens; PV COMM = post-vaccination commercial hen; PC SPF = post-challenge SPF hens; PC COMM = post-challenge commercial hens.

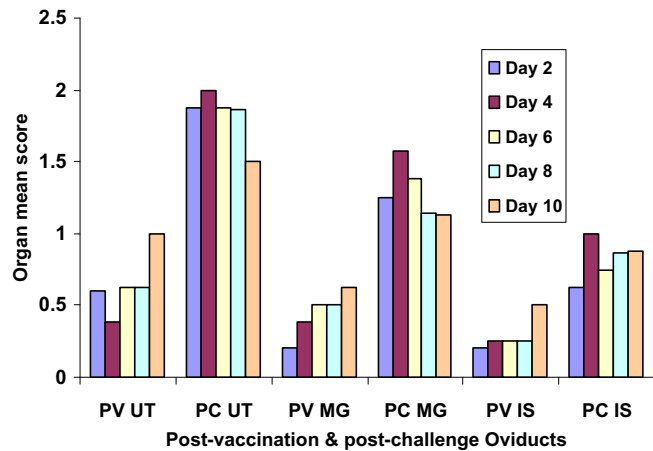


**Fig. 3.** Distribution of La Sota NDV vaccine virus post-vaccination and virulent GPMV post-challenge in the oviduct of SPF and commercial hens vaccinated via cloacal and eyedrop routes. CV SPF = cloacally-vaccinated SPF; EV SPF = eyedrop vaccinated SPF = CV COMM = cloacally-vaccinated commercial hens; eyedrop vaccinated commercial hens; PV = post-vaccination; PC = post-challenge.

eye-drop (cumulative score of 21 versus 14 respectively,  $p$ -value = 0.5188). A similar bias was observed in the commercial hens (cumulative score of 17 versus 11 respectively,  $p$ -value = 0.4338). These trends were not statistically significant in the uterus ( $p$  = 0.8621); magnum ( $p$  = 0.6146) and the isthmus ( $p$  = 0.1511) of commercial hens.

### 3.7. IHC labeling of the oviduct of hens post-challenge (challenged hens)

Within the 10-day post-challenge, positive labeling was demonstrated in the oviduct of 36 (94.74%) hens, while the oviducts of 2 (5.26%) hens were negative for NDV-specific labeling. Not only was this higher than the proportion positive after vaccination, but positive labeling scores were also higher than after vaccination (cumulative score of 163 versus 62 respectively). Scores in SPF hens were not significantly higher in the uterus ( $p$  = 0.0931) and



**Fig. 4.** Distribution of La Sota NDV vaccine post-vaccination and virulent GPMV post-challenge in the tissues of the oviduct of both SPF and commercial hens. PV UT = post-vaccination uterus; PC UT = post-challenge uterus; PV MG = post-vaccination magnum; PC MG = post-challenge magnum; PV IS = post-vaccination isthmus; PC IS = post-challenge isthmus; day = days of euthanasia.

the magnum ( $p$  = 0.7696) than in the commercial hens (Table 3a). The PC positive labeling scores fluctuated, but generally they remained high with mean scores of above 0.5 throughout the 10 days post-challenge period (Fig. 4). Positive labeling in the uterus was more extensive than in the magnum and isthmus (Table 3a). This pattern was maintained throughout the post-challenge period.

## 4. Discussion

In this study, vaccination with La Sota protected all the trial birds (both SPF and commercial laying hens) from clinical disease and death related to NDV challenge using GPMV. The fact that most of the birds (immunologically naïve SPF and routinely vaccinated commercial laying hens) had high NDV antibody titer after vaccination and prior to challenge indicated that seroconversion had occurred. The unvaccinated control birds were not protected and all died from NDV within six days post-challenge. They displayed clinical signs and lesions that are consistent with velogenic NDV infection in non-immunized birds (Biswal and Morrill, 1954; McFerran and McCracken, 1988; Parede and Young, 1990; Hamid et al., 1991; Ojok and Brown, 1996).

Biswal and Morrill (1954) and Rao et al. (2002) had showed that the reproductive tract is targeted by both vaccine strain and field isolates of NDV. Therefore, the mild temporary drop in egg production witnessed post-vaccination in this study indicated that vaccination with La Sota vaccine did have a transient negative effect on egg production. Vaccine and vaccination have been reported as “stressor” and can affect Follicular Stimulating Hormone/Lutenizing Hormone (FSH/LH) secretion (hormonal control) possibly through the stress-corticosterone pathway (Mészáros, 1983; Allan and Borland, 1979). The findings in this study agreed with this postulation.

The high post-challenge NDV-specific positive labeling in the tissues of the oviduct indicated that the infected cells could be impeded from performing their specialized functions (Sharma and Adlakha, 1995), and in this case, production of poor quality eggs could be the end results. Since both the lining and glandular epithelium of both the uterus and magnum of birds exposed to the virulent NDV challenge virus were affected, as evidenced by the presence of ND viral antigen in these tissues, the oviductal dynamics (cilia movements) and mineral balances were severely affected in the ND-infected birds (Rao et al., 2002; Holm et al., 2003).

Previous reports by Sjaastad et al. (2003) that follicular degeneration affects the quality of egg shell and albumen of eggs laid



by ND-infected birds through inadequate production of steroid hormones was confirmed in this study as challenged control birds had follicular degeneration and yolk resorption which lead to the production of soft-shelled and shell-less eggs with watery albumen; a similar reports had been presented previously by Biswal and Morrill (1954) and McFerran and McCracken (1988). Drop in egg production and degeneration of follicles which lead to arrested ovulation and delayed or no-oviposition, as recorded in the positive control hens have been reported as a consistent feature of infection with all pathotypes of ND (Biswal and Morrill, 1954; Al-Garib et al., 2003).

Other studies have suggested that if properly applied, La Sota vaccine confer greater protection against NDV challenge than other lentogenic ND vaccines such as Ulster 2C, B1 and F (Thornton et al., 1980; Rehmani, 1996). The level of protection offered by La Sota vaccine in SPF and commercial hens in the present study concurs with findings from previous studies although no effort was made to compare La Sota with other lentogenic vaccines in this study (Parede and Young, 1990; Beard et al., 1993; Kapczynski and King, 2005; Miller et al., 2007; Perozo et al., 2008). A single application of La Sota ND vaccine conferred protection against clinical ND in the present study since none of the immunologically naïve SPF birds manifested clinical signs or died after challenge (Rehmani, 1996). However, whether a single La Sota vaccination will protect against other more virulent NDV apart from the one used in this study cannot be confirmed in this report.

Previous studies had indicated that vaccination of poultry against ND can only protect birds from the more serious consequence of virulent NDV infection (severe clinical signs and mortality) but not infection and replication of the virulent strains of the virus (Parede and Young, 1990; Hamid et al., 1991; Alexander, 2001; Kapczynski and King, 2005; Miller et al., 2007). Indeed, in the present study, despite the level of protection afforded by vaccination, challenge virus did infect and replicate in host tissues and organs as was indicated by the gross pathology observed in clinically healthy challenged birds necropsied at the termination of the trial.

In this study, intracloacal and intraocular routes of vaccination were compared in order to assess their effectiveness in conferring protection against infection and decreased egg production. Though previous reports have shown that the route of vaccination can influence the level of protection conferred by the same vaccine against challenge (Kojnok et al., 1977; Rehmani, 1996), the present study showed no significant difference in protection of the oviduct against NDV infection, between the two application routes. However, slightly more NDV-specific positive labeling was observed in the oviducts of cloacally-vaccinated (CV) birds, an indication that the vaccine virus can infect and replicate in tissues without a corresponding higher antibody titer. This observation could not be linked to superior protection from challenge, in this study.

Furthermore, earlier works indicated that NDV antigen cannot be detected earlier than 3 days PV (Rao et al., 2002; Perozo et al., 2008), viral antigen was evidently demonstrated on day 2 PV in commercial birds (probably this was associated with prior NDV vaccination) and on day 4 PV in SPF birds (Table 2a).

In this study, more vaccine and the virulent challenge viral antigen was detected in sections of uterus than in sections of magnum and isthmus. Biswal and Morrill (1954) had confirmed that functional damage was more severe in the uterus than in other parts of the oviduct in past studies. The magnum appeared to be more susceptible than the isthmus region, a possible reason for the poor albumen quality of eggs laid by ND-infected birds.

The distribution of NDV antigen in the oviduct of trial hens in the present study confirmed that the reproductive tract of vaccinated birds is susceptible to viral infection and replication. Target cells included lining and glandular epithelial cells, fibroblast-like

cells in the interstitium and mononuclear cells (macrophages/histocytes and lymphocytes) in the oviductal tissues (Fig. 1d). The fibroblast-like and mononuclear cells could not be identified with certainty in the present study, because double-staining immunodetection techniques were not applied to facilitate the conclusive identification of these cells. However, epithelial cells, fibroblast, macrophages and lymphoid cells have been identified as target cells for infection and replication of NDV by other workers (Lockaby et al., 1993; Lam et al., 1996; Ojok and Brown, 1996; Al-Garib et al., 2003; Oldoni et al., 2005). Other workers have reported variable pathology in the oviduct of ND-infected birds, ranging from infiltration of inflammatory cells and the formation of lymphoid aggregates, to degenerative changes in ciliated epithelium, atrophy and necrosis of glandular epithelium and edema of submesothelium in most of the tissues (Biswal and Morrill, 1954; Rao et al., 2002).

In conclusion, the fact that ND viruses can infect well-immunized flocks remains a continuing threat to the poultry industry. The susceptibility and colonization of the reproductive tract by both vaccine and virulent field ND virus isolates and the resulting pathologies from the activity of the infecting virus explains the effect of NDV on egg production. The development of improved vaccines against ND that will more effectively reduce the infectivity and shedding of virulent viruses during infection in vaccinated flocks will be essential for the long term control of this disease.

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