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Article:

Comparative histopathological evaluation of different vaccines in broiler chickens experimentally infected with low pathogenic avian influenza (H9N2) and Newcastle disease virus (Genotype VII d)

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Abstract

Newcastle disease is one of the most critical poultry diseases threatening the poultry industry and production. It is a highly contagious infection that affects a wide range of poultry species, including chickens. This experiment was designed to examine the role of live vaccine (IB and ND live freeze-dried vaccine) and five different killed vaccines (H9N2 + ND) for their effect on immune organs (bursa of fabricius) and proventriculus after experimental H9N2 infection at 14 days old and challenged with virulent NDV at 21 days old in broiler chickens. Birds were arranged into six separate groups. Group 1 was immunized with (live vaccine and Nobilis H9N2 + ND P.), group 2 was vaccinated with (live vaccine and MEFLUVAC TM H9 + ND7), group 3 was vaccinated with (live vaccine and CEVAC® NEW FLU H9 K), group 4 was immunized with (live vaccine and Gallimune Flu H9 M.E.), group 5 was vaccinated with (live vaccine and ValleyVac H9 – NDG7) and group 6 was considered as control positive (challenged non-vaccinated). Killed vaccines were applied by the s/c injection route at 4 days old, while the live vaccine was given by eye drop route at 7 days old. Group 6 shows the severest clinical signs and lesions in the bursa and proventriculus, while the remaining groups show milder signs. Groups 1 and 5 show the milder signs and histopathological lesions. This indicates the importance of a combination of both live and killed vaccines in vaccination programs for poultry farms, especially for long lasting birds (layers and breeders).

Keywords: Newcastle disease, LPAI, Histopathology, vaccine, challenge

Introduction

Newcastle disease (ND) is one of serious diseases threatening the poultry industry all over the world. It is caused by avian paramyxovirus (APMV), family *Paramyxoviridae* and genus *Orthoavulavirus*. Newcastle disease viruses (NDVs) have five pathotypes: Asymptomatic enteric strain, Lentogenic strain, Mesogenic stain, Viscerotropic velogenic strain, and neurotropic velogenic strain. NDV belongs to serotype 1 of APMV **[1]**. At present, a total of 22 serotypes of APMV (APMV-1 to APMV-22) have been identified in different species of wild and domesticated birds **[2].** A wide range of host species (wild, domestic and cage birds) can be infected naturally or experimentally, while the chickens remain the highly susceptible species to infection with NDV **[3]**. In case of natural infection, the incubation period of the disease varies between 2 and 15 days, with an average of 5-

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6 days **[4]**. The clinical signs of the digestive form of ND are greenish diarrhea, reduced feed and water intake **[5]**. Respiratory signs are sneezing, gasping, nasal discharge, coughing and birds may also exhibit tracheal rales, conjunctivitis, facial swelling and sinusitis **[6, 7]**. Neurological signs are incoordination, torticollis, circling, paralysis, tremors and ataxia. They are more common in neurotropic Newcastle disease **[6]**. Newcastle disease clinical manifestations and avian influenza are closely similar to each other, complicating the diagnosis and intervention of the disease **[8]**. Some Poultry diseases such as Newcastle disease, can be diagnosed easily by its distinctive histopathological lesions **[9, 10]**. Many vaccines are used worldwide to control Newcastle disease such as live and inactivated NDV vaccines **[11, 12]**. Live vaccines that are used for controlling ND contain lentogenic NDV strains such as (LaSota, F, B1, V4, I2, VG/GA). The most widely used strain is LaSota due to its maximum immunogenicity **[13]**. Inactivated vaccines are often used in combination with live ones to vaccinate breeders and layers for producing high levels of antibodies **[14]**. Avian influenza (AI) is a highly contagious viral infection caused by family Orthomyxoviridae and belongs to type A avian influenza **[15]**. Recently, several studies have highlighted the role of LPAI H9N2 in inducing immunosuppression in poultry farms by depletion and apoptosis of some immune cells or changing the differentiation of inflammatory cytokines or lymphocytes **[16-20]**. It has been demonstrated that H9N2 virus can either act as immunosuppressive **[21]** or cause damage to the epithelial tissue of respiratory tract, encouraging other viral or secondary bacterial infections **[22, 23]**. Many research studies have been made on the interaction between ND and AI (LPAI and HPAI) viruses through both in vitro and in vivo techniques in poultry species **[24-27]**. The objective of this study was to compare the gross and pathological changes in different immunized groups with live and killed vaccines after experimental infection with H9N2 at 14 days and vNDV (Genotype VII d) at 21 days of age.

Material and methods

Ethical approval

The protocol of this study was approved by the ZU-IACUC committee with approval number (ZU-IACUC/2/F/92/2024).

Experimental design

Ninety, one-day old commercial broiler chicks were obtained from a local hatchery in Egypt. Chicks were raised in separate pens and divided into six groups (n=15) in clean and disinfected chambers in the Department of Poultry Diseases, Faculty of Veterinary Medicine, Zagazig University. Clean feed and water were provided ad libitum. All the environmental conditions (temperature, ventilation, humidity and light) were adjusted according to each stage of age of birds.

Vaccines

Killed vaccines

On day 4th of age, the birds were divided into six groups in separate pens. Five groups were vaccinated with killed vaccines, while the last group (6th group) is a control positive (challenged non-vaccinated). Group (1) was vaccinated with (Nobilis H9N2 + ND P.) containing strain (A/CK/UAE/415/99) and strain clone 30. It is manufactured by Merck Sharp & Dohme Animal Health, S.L. SA Salamanca- Spain. It is applied by the subcutaneous injection route (0.25 ml/bird). Group (2) was vaccinated with (MEFLUVAC TM H9 + ND7) composed of (A/ck/Egypt/ME/543V/2016) strain and NDV recombinant GVII strain. It is manufactured by MEVAC (made in Egypt). The vaccine was applied by s/c injection route (0.5 ml/bird). Group (3) was vaccinated with (CEVAC® NEW FLU H9 K) composed of H9N2 (sub-type G1-like sub linage) strain and LaSota strain. It is manufactured by Ceva Inc., Budapest, Hungary. Chicks were vaccinated by s/c injection route (0.2 ml/bird). Group (4) was vaccinated with (Gallimune Flu H9 M.E.) containing (A/chicken/Iran/Av1221/1998) strain and Ulster 2C strain. It is produced by Merial Inc., Lyon, France. The vaccine was injected subcutaneously (0.2 ml/bird). Group (5) was vaccinated with (ValleyVac H9 – NDG7). Composing of (A/chicken/Egypt/S10490/2015) strain and NDV GVII strain. It is produced by the Egyptian Company for Biological & Pharmaceutical Industries 101 extension of the sixth industrial zone-6th of October City, Egypt. Chicks were injected by s/c route (0.5 ml/bird). HPAI (H5) vaccine: The vaccine is prepared from H5N1 subtype (Re-5) strain. It is manufactured by Pulike (Nj) Biological Technology Company Nanjing, China, and applied by s/c injection route (0.3 ml/bird) to all ninety birds at 7 days old.

Live vaccines

Infectious bronchitis (IB) and Newcastle disease (ND) live freeze-dried vaccine. The Vaccine is composed of IB (Massachusetts H120) strain and (PHY. LMV.42) strain. It is produced by Ceva Inc., Budapest, Hungary, and applied by the intraocular route to 7 days old chicks (all groups except group 6). Infectious bursal disease (IBD) vaccine is composed of the intermediate (LC-75) strain. It is manufactured by Lohmann Company, Germany, manufactured for Elanco and applied via the intraocular route to all ninety birds at 10 days old.

Virus

The first challenge virus used in the experiment was avian influenza virus (H9N2) at 14 days of age to all listed 6 groups. It was a reference LPAI (H9N2) strain with accession number of ok148893. The infective dose was adjusted to 10^6 embryo infective dose 50 (EID_{50}) / ml and the birds were challenged via the ocular route. The second challenge was done at 21 days of age with Newcastle disease virus was applied via the ocular route to all birds. It was a reference genotype VIId vNDV strain (NDV/ chicken/Egypt/1/2015), with an accession number KX231852 **[28]**. The infective dose was adjusted to $10^{7.7}$ embryo infective dose 50 (EID_{50})/ml.

Clinical signs and postmortem lesions

After experimental infection with LPAI (H9N2) and vND virus (Genotype VIId), all birds in the groups were examined daily for any signs of disease and dead birds were necropsied to view the postmortem changes in organs.

Sampling

Bursa of fabricius and proventriculus were collected from dead birds post challenge and from the remaining birds after 7 days post challenge for histopathological study.

Histopathology

Broiler chickens were euthanized in a human manner and exposed to necropsy after 7 days post challenge. Bursa and proventriculus were collected and fixed in 10% neutral buffered formalin. Then they were dehydrated in a graded alcohol series, cleared with xylene, embedded in paraffin wax, sectioned at 4-5 µm thickness, and stained with hematoxylin and eosin for histopathological examination by light microscopy **[29]**. Stained tissue sections were examined by light microscopy (Olympus, Japan) and photographed using a digital camera (Olympus, Japan).

Results

After challenge with H9N2 virus at 14 days old, birds show signs as general signs (depression, ruffled feathers, decrease in feed and water intake and decrease in body weight), respiratory signs (sneezing, conjunctivitis, nasal and ocular discharge). The signs were milder in groups 1, 2, 3, 4 and 5 in comparison to group 6 (non-vaccinated). After challenge with vND virus at 21 days old, birds displayed clinical manifestations such as depression, conjunctivitis, anorexia, greenish diarrhea, and respiratory rales. It is more severe in group 6 (control positive) than other groups (immunized with killed H9+ND as listed above and IB and ND live freeze-dried vaccine). In case of challenging with H9N2 virus, necropsied birds revealed splenomegaly, swelling of kidneys, tracheitis and congestion of lungs after 7 days post infection. The severity of lesions is higher in group 6 than other vaccinated groups (1, 2, 3, 4 and 5). On the other hand, after challenging with vND virus by 7 days, necropsied birds show hemorrhage and necrosis of cecal tonsils, hemorrhage on glands of proventriculus, necrosis and ulceration of intestine, enlarged spleen, tracheitis and hemorrhage on rectum. The lesions were more severe in group 6 (non-vaccinated) than other vaccinated groups. illustrated in **Figure 1.**

Histopathology

In this study we collect proventriculus and bursa of fabricius at 28 days of the experiment (14 days from challenging with H9N2 and 7 days from challenging with vND virus). In case of bursa, it shows necrosis of bursal follicles, increases thickness of interfollicular tissue, depletion of lymphocytes and interfollicular inflammatory cells infiltration. Group 1 shows mild lymphocytic depletion and necrotic changes of epithelial tissue covering. Group 2 shows sloughing of epithelial tissue and mild lymphocytic depletion. Group 3 shows severe depletion of lymphoid cells in the bursal follicles and marked inter-follicular inflammatory cell infiltration. Group 4 shows severe multiple areas of necrosis of lymphoid cells and sloughing in the bursal follicles. Group 5 shows moderate lymphocytic depletion and sloughing of epithelial tissue covering. Group 6 shows central necrosis of bursal follicles with an increase in thickness of interfollicular C.T. The degree of lesion of bursa of fabricius ranges between mild and highly severe, as illustrated in **Table 1** and **Figure 2.** By examination of the proventriculus under a microscope, it shows different lesions as inflammation and necrosis of mucosa, thickening of mucosal layer as a result of inflammatory cells infiltration, and lymphocytes aggregates in the proventricular gland lobule. Group 1 shows focal lymphocytic aggregation in the proventricular gland lobule. Group 2 shows mucosal inflammation, profuse infiltration with inflammatory cells, and submucosal hemorrhages. Group 3 shows severe proventricular gland lobule necrosis and infiltration with inflammatory cells. Group 4 shows severe proventricular gland lobule necrosis and inflammatory cell infiltration. Group 5 shows mucosal layer necrosis and inflammatory cells infiltration in both mucosa and proventricular gland lobule. Group 6 shows mucosal inflammation caused thickening of the mucosal layer due to profuse infiltration with inflammatory cells and focal lymphocytic aggregation in the proventricular gland lobule. The degree of lesion was viewed in **Table 1** and **Figure 3.**

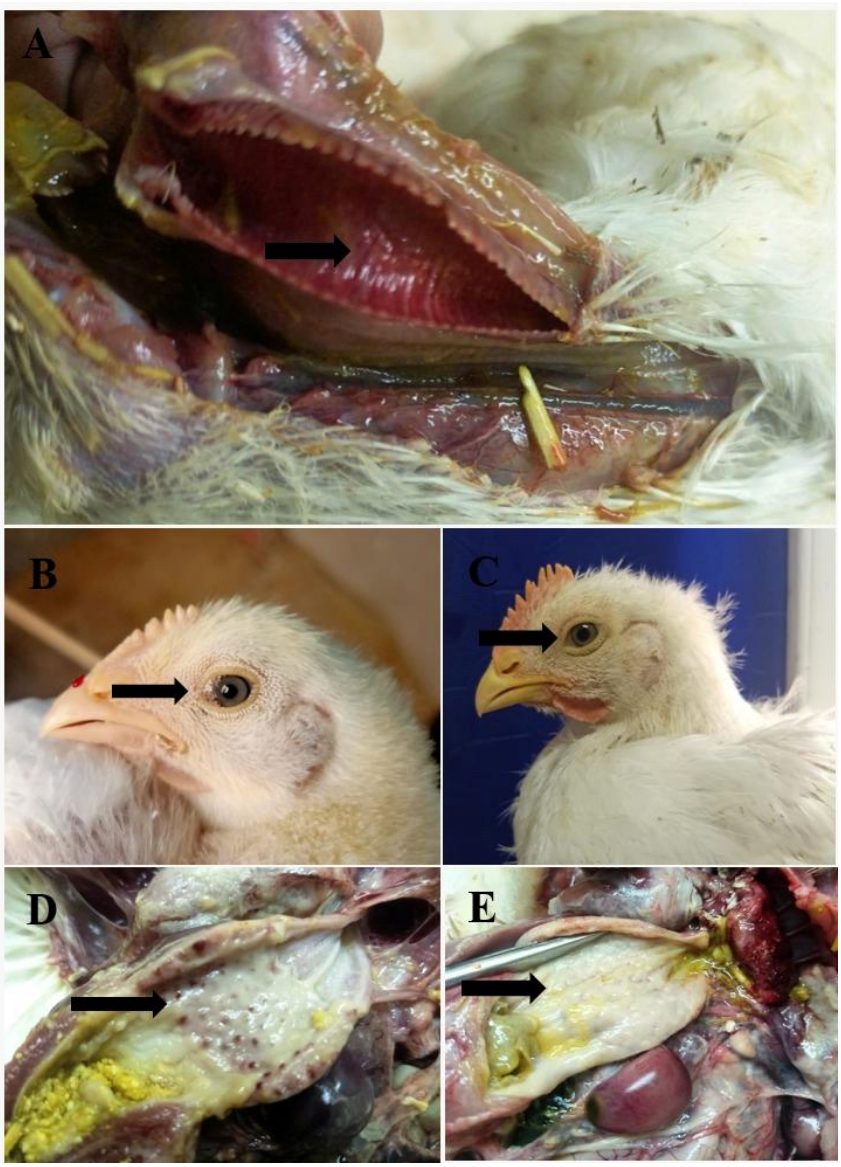

Figure (1): A) Trachea from group (6) broilers showing congested trachea. **B)** Bird from group (6) showing ocular discharge and swelling of head. **C)** Bird from group (1) showing normal eye appearance. **D)** Proventriculus from group (6) birds showing hemorrhage in proventricular glands. **E)** Proventriculus from group (1) illustrating normal appearance.

Table (1): Score of pathological lesions in bursa of fabricius and proventriculus 7 days post challenge with H9N2 virus (at 14 days) and ND virus (at 21 days)

	Bursa of fabricius				Proventriculus			
Groups	**Mild**	**Moderate**	**Severe**	**Highly severe**	**Mild**	**Moderate**	**Severe**	**Highly severe**
Group 1	+	-	-	-	+	-	-	-
Group 2	+	-	-	-	-	-	+	-
Group 3	-	-	+	-	-	-	+	-
Group 4	-	-	+	-	-	-	+	-
Group 5	-	+	-	-	-	+	-	-
Group 6	-	-	-	+	-	-	-	+

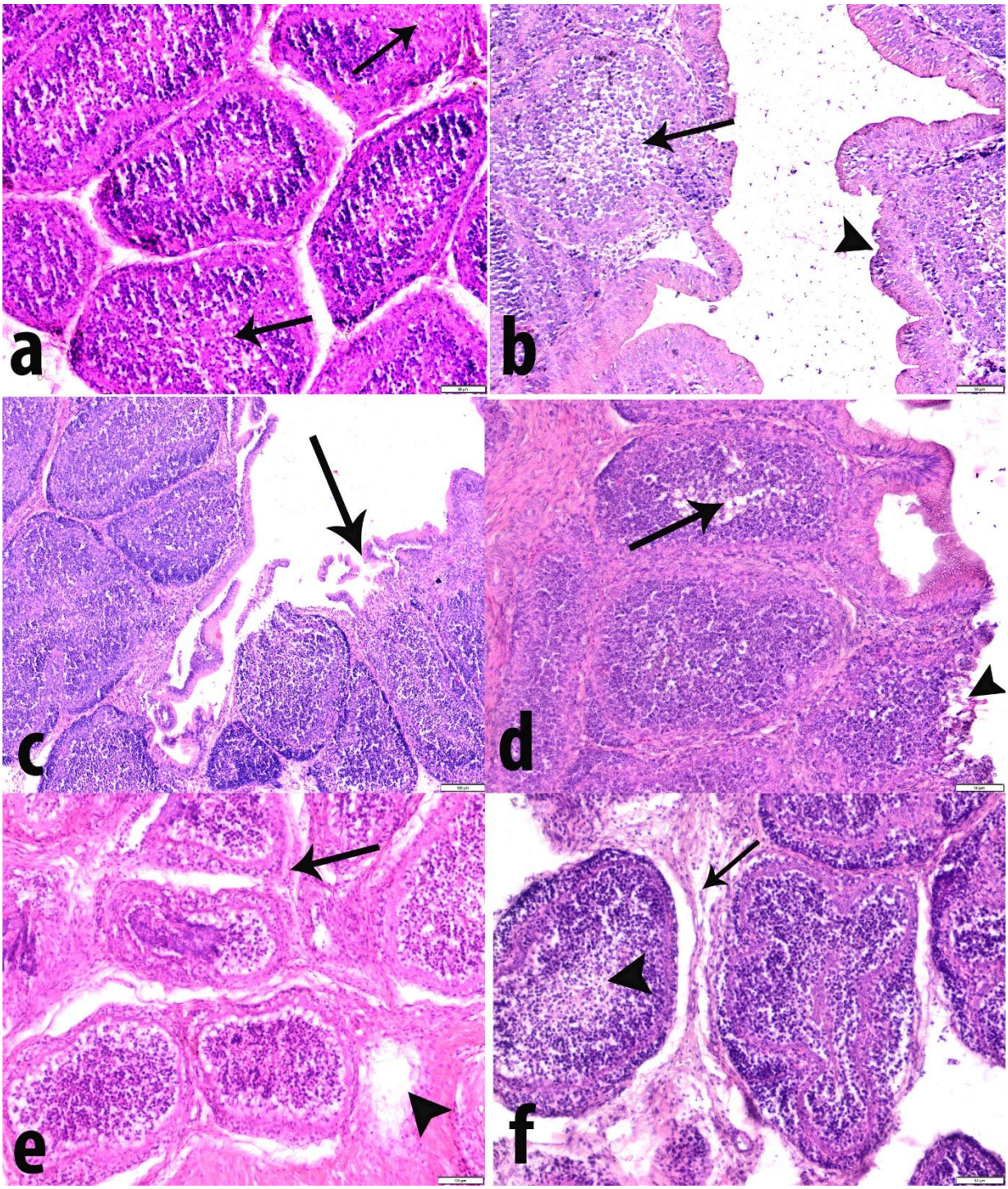

Figure (2): a) bursa of group (6) broilers showing central necrosis of bursal follicles **(arrow),** with an increase in thickness of interfollicular C.T **(HE, Bar = 50 µm). b)** bursa from group (1) showing mild lymphocytic depletion **(arrow)** and necrotic changes of epithelial tissue covering **(HE, Bar = 50 µm). C)** bursa from group (2) showing sloughing of epithelial tissue layer **(arrow)** and mild lymphocytic depletion in bursal follicles **(HE, Bar = 50 µm). d)** bursa from group (5) showing moderate lymphocytic depletion(arrow) and sloughing of epithelial tissue covering**(arrowhead) (HE, Bar = 50 µm)**. **e)** bursa from group (4) showing severe multiple areas of necrosis of lymphoid cells and sloughing in the bursal follicles **(arrowhead)** and increase in thickness of interfollicular C.T **(arrow) (HE, Bar = 100 µm). f)** bursa from group (3) severe depletion of lymphoid cells in the bursal follicles and marked inter-follicular inflammatory cell infiltration **(arrow) (HE, Bar = 50 µm)**.

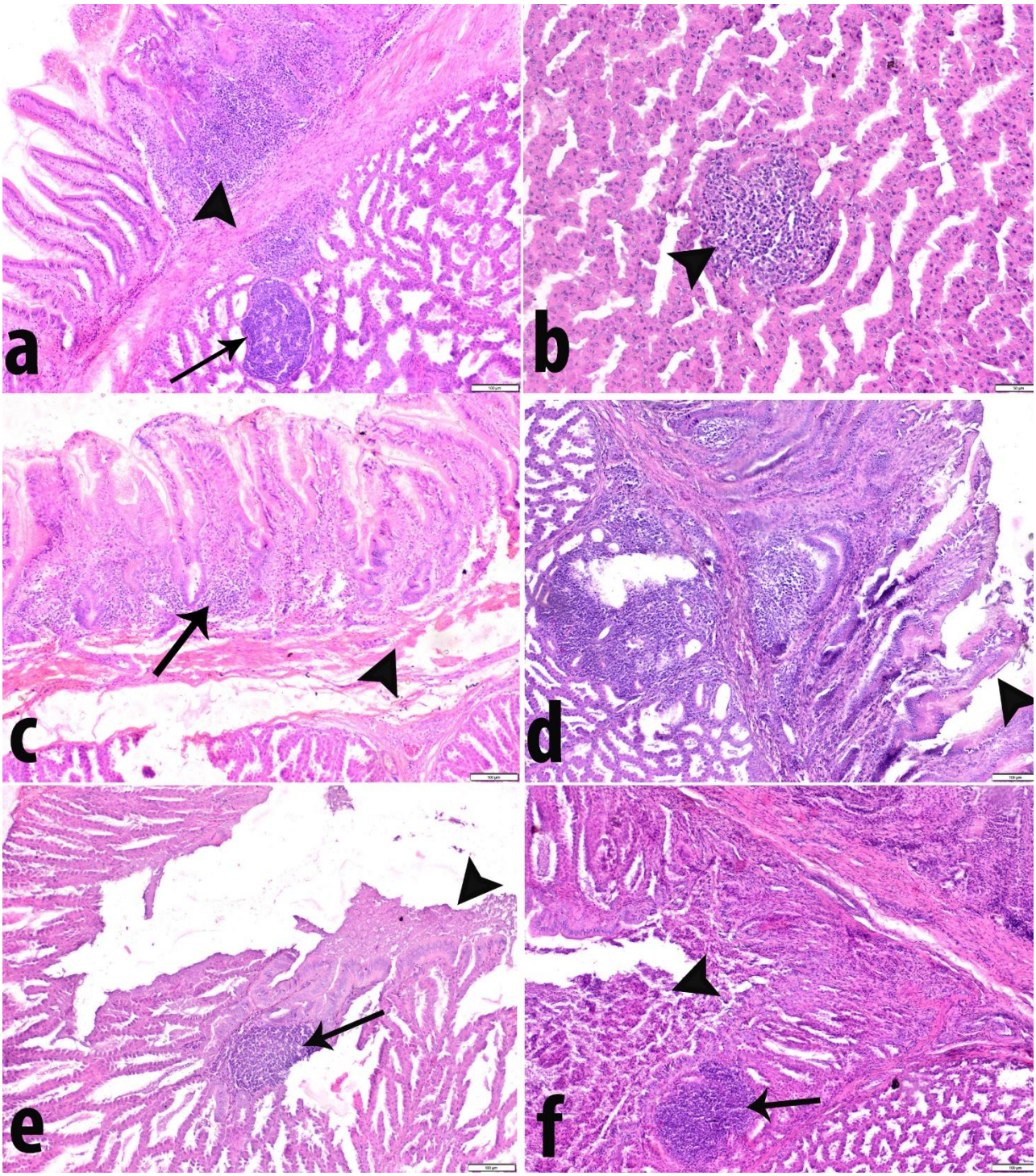

Figure (3): a) proventriculus of group (6) broilers showed mucosal inflammation caused thickening of the mucosal layer due to profuse infiltration with inflammatory cells **(arrowhead) and** focal lymphocytic aggregation in the proventricular gland lobule **(arrow). (HE, Bar = 100 µm). b)** proventriculus from group (1) showing focal lymphocytic aggregation in the proventricular gland lobule **(arrowhead). (HE, Bar = 50 µm). C)** proventriculus from group (2) showed mucosal inflammation, profuse infiltration with inflammatory cells **(arrow),** and sub-mucosal hemorrhages **(arrowhead) (HE, Bar = 100 µm). d)** proventriculus from group (5) showing mucosal layer necrosis **(arrowhead),** inflammatory cells infiltration in both mucosa and proventricular gland lobule **(HE, Bar = 100 µm)**. **e)** proventriculus from group (4) showing severe proventricular gland lobule necrosis **(arrowhead)** and infiltration with inflammatory cells **(arrow) (HE, Bar = 100 µm). f)** proventriculus from group (3) showing severe proventricular gland lobule necrosis **(arrowhead)** and infiltration with inflammatory cells **(arrow) (HE, Bar = 100 µm)**.

Discussion

Newcastle disease remains one of the most dangerous diseases affecting poultry farms and industry worldwide, leading to great economic losses every year due to high mortalities, retarded growth, and drop in egg production **[3, 30].** Many vaccines are used worldwide to decrease losses from the disease **[13].** H9N2 appears to be circulating in an undetectable manner in poultry farms in Egypt since it has been demonstrated on 2009-2010 by serology **[31].** Co-circulation of vNDV and H9N2 is regarded as one of the main hazards affecting poultry farms and industry in the Middle East and worldwide, in particular in Egypt, where both diseases are present until this moment. Many commercial products of vaccines have been applied against both diseases, but new cases still appears. In this study we focused on organs such as bursa of fabricius (immune organ) and proventriculus to evaluate the role of different vaccines against LPAI (H9N2) virus challenge (at 14 days-old) and vND virus (Genotype VIId) challenge (at 21 days-old). Vaccination was applied via ocular route for its efficiency to stimulate production of high level of antibodies in comparison with spray and drinking water methods **[32, 33].** Clinical manifestations that were observed are general signs (decrease feed and water intake, retarded growth, huddling together and ruffled feathers), respiratory (nasal and ocular discharge, rales, sneezing and swollen sinuses) and greenish diarrhea. Clinical signs as edema of eyelids, cyanosis, nasal discharge, white pasty or greenish diarrhea and nervous manifestations were also documented in previous study **[34, 35].** Gross findings that were demonstrated are hemorrhage and necrosis of cecal tonsils, necrosis and ulceration of intestine, hemorrhage on glands of proventriculus, tracheitis, enlarged spleen and hemorrhage on rectum. The same findings were demonstrated in turkeys in other research **[36, 37].** Histological examination of birds in this study suggests that both viruses (ND and H9N2) can replicate in lymphoid tissues as bursa of fabricius and cause severe damage to it. The same findings were also recorded in other study **[38].** In the present study we found lesions such as (necrosis of bursal follicles, depletion of lymphoid cells, inflammatory cells infiltration and increase thickness of interfollicular tissue). **Elmore, (2006)** stated that the lymphoid depletion that occurred in immune organs was a sequela to apoptosis or necrosis **[39].** On the other hand, another study revealed that it may be due to lymphoid cells migration from these organs **[40].** It was observed in other studies lesions as bursal tissue damage and it was explained and attributed to T cells which limits the replication of virus in the bursal tissue and stimulates damage of the bursal tissue and delays the recovery of it due to cytokines released and cytotoxic effect **[41]** leading to lymphoid cells apoptosis **[42].** Necrosis of bursal follicles was also demonstrated by other study **[43].** Lesions such as lymphoid depletion in follicles, necrosis, and infiltration of mononuclear cells were also detected in bursa of turkeys on days 6 and 8 PI then partial recovery and lymphoid hyperplasia occurred at day 14 PI **[36].** The size of bursa has decreased, and it is attributed to Lymphocytic depletion leading to suppressed immunity of birds **[36, 44].** For proventriculus examination in this protocol, it revealed inflammation and necrosis of mucosa, and thickening of mucosa. Other study documented necrotic and denuded surface epithelium with severe hemorrhages in sections from proventriculus **[37].** Other studies also showed proventriculitis, desquamation of proventriculus epithelial tissue and inflammatory cells infiltration to submucosal layer of proventriculus gland **[45].** The findings and observations in this research showed that vaccination programs, which are currently used against H9N2 and ND viruses can't prevent disease infection in the field. On the other hand, birds can achieve protection by decreasing the severity of the clinical manifestation and postmortem lesions of the disease.

Conclusion

In conclusion, combination of both live and killed vaccines in vaccination programs is necessary in poultry farms and must be combined with biosecurity measures in order to prevent the opportunity of spreading of the disease and decrease losses from it that occur every year.

Conflict of interest

No conflict of interest was reported by the authors.

Authors' contribution

Hesham Asaad: Investigation, Methodology, Visualization, Data curation, Writing- review & editing. Mostafa Saif-Edin: Conceptualization, Project administration and Supervision. Ragab S. Ibrahim: Visualization and Supervision. Moemen A. Mohamed: Visualization and Supervision. Ola Hassanin: Conceptualization, Data curation, Validation and Visualization. Mustafa Hamad: Methodology and resources. Mohamed Gamal: Histopathological examination of slides. Tamer Mahmoud Abdullatif: Writing – review & editing.

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