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RESEARCH

H9N2 AI and NDV shedding pattern in SPF and commercial layer vaccinated chicken with trivalent vaccine containing H9N2 AI, LaSota, classical M41 and var 2 IB viruses challenged at 1, 3 and 7 months post vaccination

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ABSTRACT

Background: Prolonged immune protection for H9N2 and Newcastle in layer chicken rearing is an important issue.
Objective: To evaluate the protection duration for H9N2 and NDV post vaccination by inactivated trivalent (H9N2, LaSota, M41 and Var2 IB) vaccine based mainly on repetition of challenge.
Methods: We prepared formalin inactivated combined vaccine for Avian Influenza (H9N2N), Newcastle (La), and IB (M41 and Var 2) by using montanide 71 adjuvant. The prepared vaccine was injected intramuscularly in specific pathogen free and commercial layer chicken, the injection was repeated as a booster vaccination one month after the first vaccination. Humoral immune response was measured by HI for both H9N2 and LaSota viruses weekly for one and a half month after the booster vaccination, then monthly until the end of duration. Challenge trials were carried out after a month from the first vaccination, and after two and six months from the booster vaccination.
Results: Usage of formalin inactivated combined H9N2, LaSota and IB (M41 and Var 2) vaccine with montanide 71 adjuvant decreases the shedding pattern of H9N2 and NDV in SPF and commercial layer chicken for six months after the booster vaccination.
Conclusion: Formalin inactivated, montanide 71 adjuvanted combined H9N2, LaSota and IB (M41 and Var 2) vaccine induces protection for 7 months against challenge with Avian Influenza (H9N2) and Newcastle viruses when vaccination repeated after one month from the first vaccination.

Keywords: H9N2; Newcastle; SPF chicken; layer chicken.

BACKGROUND

H9N2 is a low pathogenic influenza virus (LPIV) which makes low mortality and mild symptoms except when associated with immune suppression which induce severe symptoms and high mortality. This may cause economic losses in poultry industry (Bano *et al.*, 2003; Kwon *et al.*, 2008; El Naggar *et al.*, 2017). H9N2 was isolated from European aquatic birds from 1977 (Suss *et al.*, 1994), and the wild duck in Canada from 1976 (Sharp *et al.*, 1997). There were outbreaks of H9N2 during and since 1990 in some European, South African, American, Asian and Middle East countries (Naeem *et al.*, 1999; Alexander, 2000; Bano *et al.*, 2003; Capua and Aalxander, 2004; Alexander, 2007).

In Egypt Hussien and Elazab (2001) detected H9N2 in 2001. In 2003 NAMRU3 discovered that there are high heamagglutinin gene sequence similarity between The Egyptian isolate and one of recent Israeli strains (Arafa *et al.*, 2012).

Newcastle disease (ND) is an acute highly contagious viral disease of various species of birds which causes high mortality worldwide (Iram *et al.*, 2013: Miller *et al.*, 2015). It affects mainly the neurological, gastrointestinal and respiratory systems (Ganar *et al.*, 2014; Brown and Bevins, 2017; Su *et al.*, 2018). The first outbreaks of ND may be in the Western Isles of Scotland in 1896 (Macpherson, 1956), but the first recorded detection of NDV was in Java, Indonesia and

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Newcastle-‘on-Tyne, England (Doyle, 1927), and since then various genotypes have been circulating worldwide (Miller *et al.,* 2010). The clinical signs of ND in chicken depend on the virus strain which is divided into five pathotypes: viscerotropic or neurotropic velogenic; mesogenic; lentogenic and asymptomatic (Alexander, 2000 b).The virus virulence depends on the amino acid sequence at the cleavage site of the F gene (Aldous *et al.,* 2003).

The vaccination mainly aims at decreasing the clinical signs and the virus shedding, and increasing the amount of virus required for infection (Lee and Suarez, 2005). The inactivated vaccine advantages are safety of using and giving strong prolonged humoral immune response, while the disadvantages are the need of high amount of antigen and adjuvant to induce protective immunity (Box *et al.,* 1982; Schijns and Tangeras, 2005) and giving low mucosal and cellular immunity (El Naggar *et al.,* 2017). The advantages of using emulsions are enhancement of antibody production and prolongation of the antigen releasement (Stone, 1997). Montanide ISA71 adjuvant is one of mineral oil/surfactant based adjuvants, it is prepared for use as an emulsion with aqueous Ag solution (Lone *et al.,* 2017).

In this study, we prepared inactivated combined H9N2, LaSota, M41 IB and variant IB vaccine based on montanide 71 then evaluated the immune response and its duration for H9N2 and NDV by repetition of challenge trials and HI test.

MATERIALS AND METHODS

SPF ECEs

Eggs were obtained from Nile SPF Farm, Kom Oshiem, Fayom, Egypt, and used for virus propagation and titration, and assurance of complete virus inactivation (OIE, 2018).

Antigen

H9N2: (A/chicken/Egypt/D4692A/2012), NDV: LaSota and virulent strain (NDV-B7-RLQP-CH-EG-12) with accession Number (112RKQKR*F117KM288609) (sequence of F gene) and IBV: Var2(IBV-S1/VSVRI_G9/Egy 013) with accession Number (KP729422) (sequence of S1 gene) and IB M41 were kindly provided by Department of Poultry Vaccines Production Unit, Veterinary Serum and Vaccine Research Institute, Abbasia, Egypt.

Characterization of the viruses

Characterization of the viruses was made by National Laboratory for Veterinary Control on Poultry Production, Animal Health Research Institute, Egypt. Real-time (RT) PCR was made to H9N2 sample to identify the virus and make sure it’s free from H5N1, ND or IB viruses. Identification of LaSota, IB M41 and variant IB viruses was made by HI test by reference antigen, RT-PCR and sequence analysis respectively.

Inactivation of viruses

Inactivation of viruses was carried out using formalin in a final concentration of 0.1% of the total volume. Samples from each inactivated virus were tested for complete inactivation in 10-day-old SPF ECE for two successive blind passages.

Adjuvant

Montanide™ ISA 71 VG mineral oil based adjuvant was used to make water-in-oil (W/O) emulsion.

Vaccine formulation

A ratio of 30/70 (v/v) aqueous/oil was prepared by mixing the aqueous medium into the Montanide™ ISA 71 VG with vigorous stirring (for 15-30 min) as recommended by SEPPIC Co; the manufacture Co.

Challenge trial

Specific pathogen-free (SPF) and commercial layer 21 days old chicken, both are divided into two groups, one group was vaccinated with 1 cm from the prepared combined vaccine via intramuscular route and the other group was kept non-vaccinated as a control negative group. After 4 weeks post vaccination 13 chicken from each group were challenged with 10^6 EID_{50} NDV via intramuscular rout and 13 chicken from each group were challenged with 10^6 EID_{50} H9N2 virus via intranasal and intraocular route.

Intramuscular 1 cm booster vaccination was given to the vaccinated group after one month from the first vaccination, then repetition of the challenge was carried out typically as the first time twice, after two and six months from the booster vaccination.

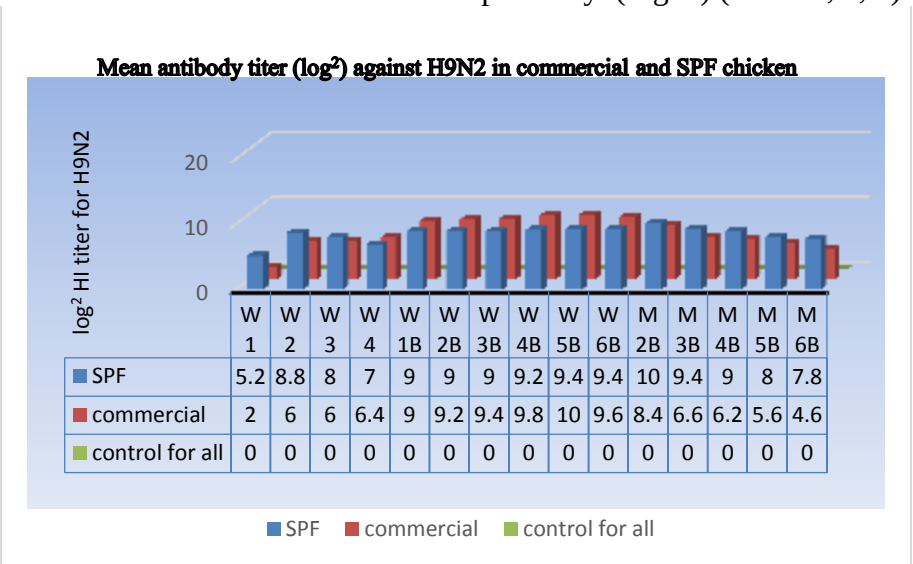
Serum samples were collected weekly till one and a half month post booster vaccination and then monthly, for detection of serum Abs by HI test for NDV and H9N2. Oropharyngeal swabs from groups that were challenged, were collected at 3, 5, 7 and 10 days post challenge and tested for virus shedding by real-time PCR. Tracheal and renal tissue were collected from H9N2 challenged birds and tracheal and spleen tissue were collected from NDV challenged birds for histopathological examination.

RESULTS

Serological response

HI test for H9N2

H9N2 Abs were detected by HI test until six months from the booster vaccination. The titer starts at 1st week from 2 and 5 $\log^2$ in commercial and SPF respectively then increase to reach the peak about 10 $\log^2$ at 5 weeks and 2 months after booster vaccination in commercial and SPF chicken respectively. The titer decreases to 4.6 and 7.8 $\log^2$ in commercial and SPF chicken at the 6^{th} month after booster vaccination respectively. (Fig. 1) (Table 1, 2, 3)



	W 1	W 2	W 3	W 4	W 1B	W 2B	W 3B	W 4B	W 5B	W 6B	M 2B	M 3B	M 4B	M 5B	M 6B
SPF	5.2	8.8	8	7	9	9	9	9.2	9.4	9.4	10	9.4	9	8	7.8
commercial	2	6	6	6.4	9	9.2	9.4	9.8	10	9.6	8.4	6.6	6.2	5.6	4.6
control for all	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Fig. 1: Antibody titers $\log^2$ to H9N2 in SPF and commercial chicken using HI Test.

B: after booster vaccination, control group not vaccinated by any vaccine.

Table 1: The mean Log^2 ± SD of H9N2 HI antibody titer for SPF and commercial chicken after the first vaccination.

Chicken type	Weeks post vaccination			
	1	2	3	4
SPF	5.2±0.84[a]	8.8± 0.84[a]	8± 0.71[a]	7±0.71[a]
Commercial	2±0.71[b]	6±1[b]	6±0.71[b]	6.4±0.55[a]

Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 between chicken groups

Table 2: The mean Log^2 ± SD of H9N2 HI antibody titer for SPF and commercial chicken up to 6 weeks after the booster vaccination.

Chicken type	Weeks after booster vaccination					
	1	2	3	4	5	6
SPF	9±01.22[a]	9±0.71[a]	9±0.71[a]	9.2±1.10[a]	9.4±1.14[a]	9.4±0.55[a]
Commercial	9±0.71[a]	9.2±0.45[a]	9.4±0.55[a]	9.8±0.84[a]	10±0.71[a]	9.6±0.55[a]

Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 between chicken groups.

Table (3): The mean Log^2 ± SD of H9N2 HI antibody titer for SPF and commercial chicken after the booster vaccination from 2 to 6 months.

Chicken type	Months after booster vaccination				
	2	3	4	5	6
SPF	10.2± 0.84[a]	9.4± 1.14[a]	9± 0.71[a]	8± 0.71[a]	7.8±0.84[a]
Commercial	8.4± 0.55[b]	6.6± 0.89[b]	6.2±0.84[b]	5.6±0.89[b]	4.6±0.89[b]

Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 between chicken groups

HI test for NDV

NDV Abs were detected by HI test until six months from the booster vaccination. The titer starts from 6 and 8 log^2 at the 2nd weeks after vaccination in commercial and SPF chicken respectively, then increase to reach the about 10 log 2 at 2 weeks after booster vaccination in SPF chicken, while in commercial between the first and fifth week after the booster vaccination. The test end by titer about 6.5 log^2 at the 6th months in both chicken type. (Fig. 2) (Table 4, 5, 6).

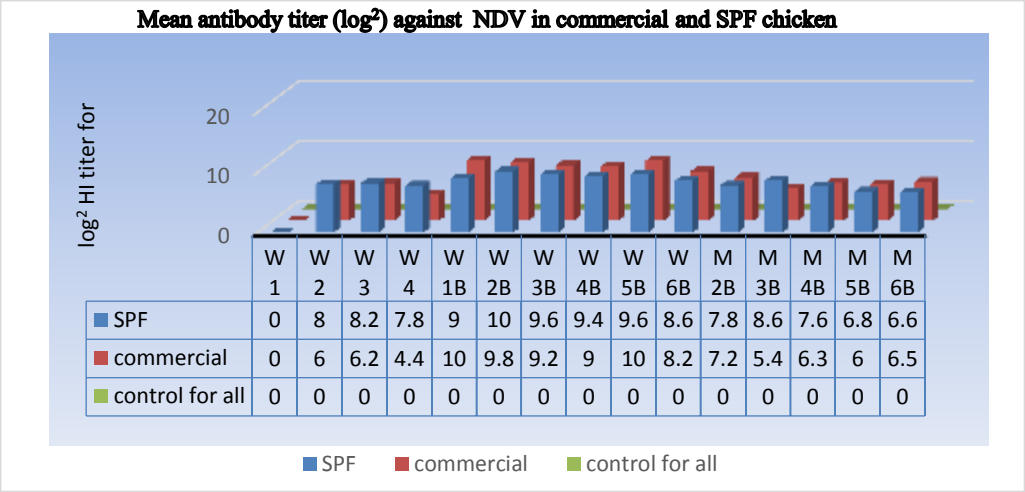


Fig. 2: Antibody titers log^2 to LaSota in SPF and commercial chicken using HI Test. B: after booster vaccination, control group not vaccinated by any vaccine.

Table 4: The mean Log^2 ± SD of LaSota HI antibody titer for SPF and commercial chicken after the first vaccination.

Chicken type	Weeks post vaccination		
	2	3	4
SPF	8± 0.71[a]	8.2± 0.84[a]	7.8±0.84[a]
Commercial	6±1[b]	6.2±0.84[b]	5.5±0.58[b]

Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 between chicken groups.

Table 5: The mean Log^2 ± SD of LaSota HI antibody titer for SPF and commercial chicken up to 6 weeks after the booster vaccination.

Chicken type	Weeks after booster vaccination					
	1	2	3	4	5	6
SPF	9±0.71[a]	10.2± 0.45[a]	9.6± 0.55[a]	9.4± 0.89	9.6± 0.89[a]	8.6±0.89[a]
Commercial	10±0.71[b]	9.8±0.84[a]	9.2±0.45[a]	9± 0.71[a]	10± 1[a]	8.2±0.45[a]

A Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 between chicken groups.

Table 6: The mean Log^2 ± SD of LaSota HI antibody titer for SPF and commercial chicken after the booster vaccination from 2 to 6 months.

Chicken type	Months after booster vaccination				
	2	3	4	5	6
SPF	7.8± 0.84a	8.6±0.89a	7.6± 0.55a	6.8± 0.45a	6.6±0.55a
Commercial	7.2± 1.30a	5.4± 1.52b	6.3±0.50b	6±0.82a	6.5±0.58a

Means with different letters (a, b) within the same column are significantly different at P value ≤ 0.05 between chicken groups

Shedding ratio after challenge with H9N2 virus in SPF and commercial chicken

The shedding in oro-pharynx after challenge with H9N2 for vaccinated and positive control groups in SPF and commercial chicken at interval days was measured by RT-PCR. It was observed that the control groups had the highest virus shedding values. No shedding was detected in samples in the 3rd and 10th days post challenge (DPC) in all vaccinated groups. In SPF chicken the third challenge show shedding in the 5th DPC only but another vaccinated challenges show shedding in the 5th and 7th day. In commercial chicken the shedding was detected in all challenge in the 5th and 7th DPC. All groups showed no mortalities, Table (7, 8).

Table 7: Shedding ratio after challenge with H9N2 virus in SPF chicken.

groups	3 DPC		5 DPC		7 DPC		10 DPC	
	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})
1st challenge	-ve	-	+ve	$2.551X10^5$	+ve	$2.265X10^5$	-ve	-
2nd challenge	-ve	-	+ve	$1.665X10^5$	+ve	$1.268X10^5$	-ve	-
3rd challenge	-ve	-	+ve	$1.098X10^4$	-ve	-	-ve	-
Control +ve	+ve	$6.419X10^5$	+ve	$1.978X10^6$	+ve	$4.209X10^6$	+ve	$1.600X10^7$

EID_{50}=Embryo infective dose 50% control +ve: challenged not vaccinated chicken DPC: day post challenge.

Table 8: Shedding ratio after challenge with H9N2 virus in commercial chicken.

groups	3 DPC		5 DPC		7 DPC		10 DPC	
	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})
1st challenge	-ve	-	+ve	$1.468X10^5$	+ve	$1.065X\ 10^5$	-ve	-
2nd challenge	-ve	-	+ve	$3.336X10^4$	+ve	$2.003X10^4$	-ve	-
3rd challenge	-ve	-	+ve	$9.816X10^3$	+ve	$4.394X10^3$	-ve	-
Control +ve	+ve	$6.419X10^5$	+ve	$1.978X10^6$	+ve	$4.209X10^6$	+ve	$1.600X10^7$

EID_{50}=Embryo infective dose 50% control +ve: challenged not vaccinated chicken DPC: day post challenge

Shedding ratio after challenge with velogenic NDV genotype VIId virus in SPF and commercial chicken

The shedding in oro-pharyngeal after challenge with virulent NDV for vaccinated and positive control groups at interval days measured by real-time RT-PCR. It was observed that the control groups had the highest virus shedding values. No shedding was detected in the second and third SPF challenge in all interval days, while the first SPF challenge showed no shedding in the 10^{th} DPC only. No shedding was detected in commercial first challenge in 3^{rd} and 5^{th} days, the second commercial challenge showed no shedding in the 5^{th} day only, while the third challenge showed shedding in all interval DPC. All control chicken died between 5^{th} and 6^{th} day post challenge and no mortalities among vaccinated chicken, table (9, 10).

Table (9): shedding ratio after challenge with velogenic NDV genotype VIId virus in SPF chicken.

groups	3 DPC		5 DPC		7 DPC		10 DPC	
	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})
1st challenge	+ve	$3.800X10^5$	+ve	$8.282X10^4$	+ve	$1.382X\ 10^4$	-ve	-
2nd challenge	-ve	-	-ve	-	-ve	-	-ve	-
3rd challenge	-ve	-	-ve	-	-ve	-	-ve	-
Control +ve	+ve	$1.468X10^6$	+ve	$6.392X10^5$				

EID_{50}=Embryo infective dose 50% control +ve: challenged not vaccinated chicken DPC: day post challenge.

Table 10: Shedding ratio after challenge with velogenic NDV genotype VIId virus in commercial chicken.

groups	3 DPC		5 DPC		7 DPC		10 DPC	
	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})	result	Shedding amount (EID_{50})
1st challenge	-ve	-	-ve	-	+ve	$3.119X\ 10^5$	+ve	$2.975X\ 10^5$
2nd challenge	+ve	$9.303X10^2$	-ve	-	+ve	$1.973X\ 10^5$	+ve	$1.983X\ 10^5$
3rd challenge	+ve	$5.596X\ 10^4$	+ve	$4.633X\ 10^4$	+ve	$7.224X\ 10^4$	+ve	$3.313X\ 10^4$
Control +ve	+ve	$1.468X10^6$	+ve	$6.392X10^5$				

EID_{50}=Embryo infective dose 50% control +ve: challenged not vaccinated chicken DPC: day post challenge.

Table 11: Mean HI titer for H9N2 and LaSota post the first challenge in commercial chicken and the second challenge in SPF chicken.

	Mean AB titter in HI test (log^2) in the second SPF chicken challenge	Mean AB titter in HI test (log^2) in the first commercial chicken challenge

	H9N2 challenged group	NDV challenged group	H9N2 challenged group		NDV challenged group	
	2WPC	2WPC	1WPC	2WPC	1WPC	2WPC
vaccinated	10	11	4	9	5	11
Control positive	0	0	0	0	0	0

WPC=Week post challenge control+ve: challenged not vaccinated chicken

Histopathological examination post challenge

Results of SPF chicken histopathological examination of organs collected from birds after 7 days post challenge with H9N2 virus

The non-vaccinated challenged birds showed thickening of the tracheal mucosa due to edema and mononuclear cells infiltration, other showed degeneration and necrosis of the lining epithelium, while Kidney exhibited degeneration of renal tubules with interstitial mononuclear cells infiltration and congested blood vessels. Contracted and hollowed glomeruli were observed.

Chicken challenged after month from the first vaccination Showed thickening of the lining epithelium due to edema, and mononuclear cells infiltration, in addition to bloody exudate in the lumen, while Kidney showed congested blood vessels intertubular hemorrhage. Chicken challenged after two months from booster vaccination showed focal hyperplasia of lining tracheal epithelium, while Kidney showed congested blood vessels. Chicken challenged after six months from booster vaccination Showed hyperplasia of lining tracheal epithelium with mononuclear cells infiltration in lamina propria, while Kidney showed denudation and degeneration of the renal tubules and congested blood vessels in addition to focal histeocytes aggregation. The severity of lesions appear in table (11).

Table 11: Results of SPF chicken histopathological examination of organs collected from birds after 7 days post challenge with H9N2 virus

Group/organs	Trachea	Kidney
1st challenge	++	+
2nd challenge	-	+
3rd challenge	++	++
Control positive	++	++

- : normal +: mild ++: moderate +++: severe

Results of commercial chicken histopathological examination of organs collected from birds after 7 days post challenge with H9N2 virus

The non-vaccinated challenged birds showed thickening of the tracheal mucosa due to edema and mononuclear cells infiltration, other showed degeneration and necrosis of the lining epithelium, while Kidney exhibited degeneration of renal tubules with interstitial mononuclear cells infiltration and congested blood vessels. Contracted and hollowed glomeruli were observed.

Chicken challenged after month from the first vaccination Showed apparently normal tracheal architecture, while Kidney showed degenerated tubules and intertubular focal infiltration with mononuclear cells. Chicken challenged after two months from booster vaccination showed l hyperplasia of lining tracheal epithelium, while Kidney showed congested blood vessels. Chicken challenged after six months from booster vaccination Showed focal hyperplasia of lining tracheal epithelium, while Kidney showed degeneration of the renal tubules and focal intertubular infiltration of histeocytes and congested blood vessels. The severity of lesions appear in table (12).

Table (12): Results of commercial chicken histopathological examination of organs collected from birds after 7 days post challenge with H9N2 virus

Group/organs	Trachea	Kidney
1st challenge	-	++
2nd challenge	+	+
3rd challenge	+	++
Control positive	++	++

- : normal +: mild ++: moderate +++: severe

Results of SPF chicken histopathological examination of organs collected from birds after 7 days post challenge with velogenic NDV genotype VIId virus

The non-vaccinated challenged birds showed hyperplasia of lining tracheal epithelium and activation of mucous glands and congested blood vessels, other case showed necrosis of the tracheal epithelium and submucosal edema, while spleen showed proliferation of capillary sheaths, depletion of lymphocytes and mild focal hemorrhage.

Chicken challenged after month from the first vaccination Showed mild hyperplasia of lining tracheal epithelium, while Spleen showed diffuse depletion of lymphocytes. Chicken challenged after two months from booster vaccination Showed thickening of the tracheal mucosa due to edema and mononuclear cells infiltration, while Spleen showed diffuse depletion of lymphocytes and congested blood vessels. Chicken challenged after six months from booster vaccination Showed hyperplasia of lining tracheal epithelium and nodules of monocular cells in lamina propria, while Spleen showed proliferation of capillary sheaths. The severity of lesions appear in table (13).

Table 13: Results of SPF chicken histopathological examination of organs collected from birds after 7 days post challenge with velogenic NDV genotype VIId virus

Group/organs	Trachea	Spleen
1st challenge	-	++
2nd challenge	+	++
3rd challenge	+	++
Control positive	++	+

-: normal +: mild ++: moderate +++: severe

Results of commercial chicken histopathological examination of organs collected from birds after 7 days post challenge with velogenic NDV genotype VIId virus

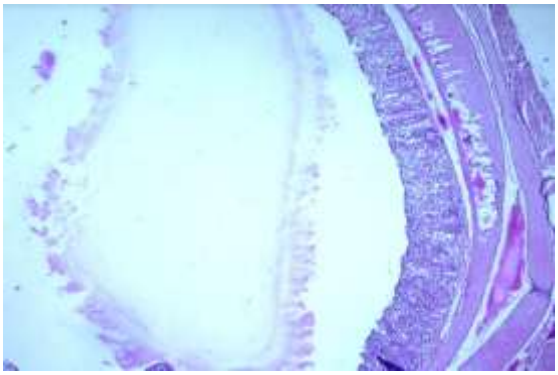
The non-vaccinated challenged birds showed hyperplasia of lining tracheal epithelium and activation of mucous glands and congested blood vessels, other case showed necrosis of the tracheal epithelium and submucosal edema, while spleen showed proliferation of capillary sheaths, depletion of lymphocytes and mild focal hemorrhage.

Chicken challenged after month from the first vaccination Showed hyperplasia of lining tracheal epithelium and activation of mucous glands, while Spleen showed depletion of lymphocytes, cellular necrosis and congested blood vessels. Chicken challenged after two months from booster vaccination Showed thickening of the tracheal mucosa due to edema and mononuclear cells infiltration, while Spleen exhibited multifocal depletion of lymphocytes. Chicken challenged after six months from booster vaccination Showed thickening of lining tracheal epithelium with nodules of monocular cells in lamina propria in addition to degeneration of tracheal cartilage with heterophils infiltration, while Spleen showed focal depletion of lymphocytes. The severity of lesions appear in table (14).

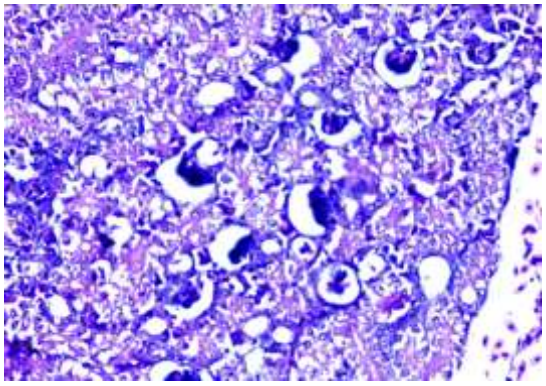
Table (14): Results of commercial chicken histopathological examination of organs collected from birds after 7 days post challenge with velogenic NDV genotype VIId virus

Group/organs	Trachea	Spleen
1st challenge	++	++
2nd challenge	++	++
3rd challenge	++	++
Control positive	++	+

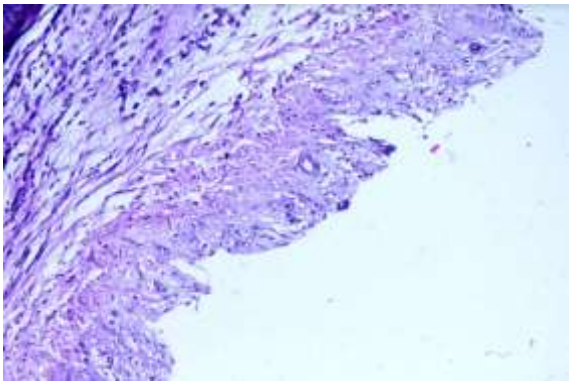
-: normal +: mild ++: moderate +++: severe



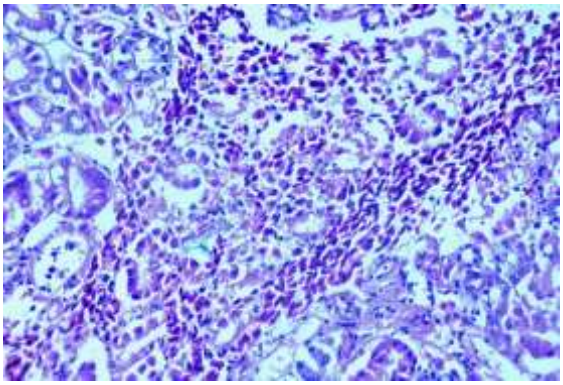
Snap 1: thickening of the tracheal mucosa in Control +ve H9N2, second ND SPF and Commercial challenged chicken due to edema and mononuclear cells infiltration (H&E 50).



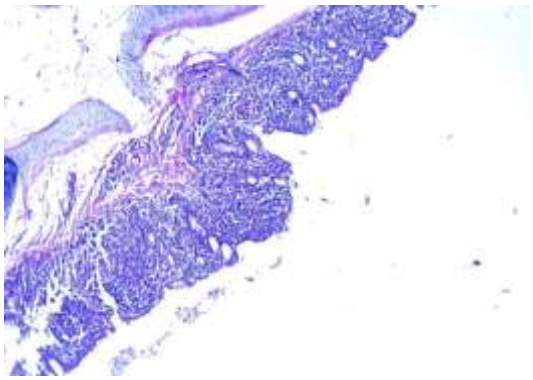
Snap 2: kidney of +ve control, second SPF and commercial chicken H9N2challenge showed degenerated tubules congested blood vessels (H&E 200).



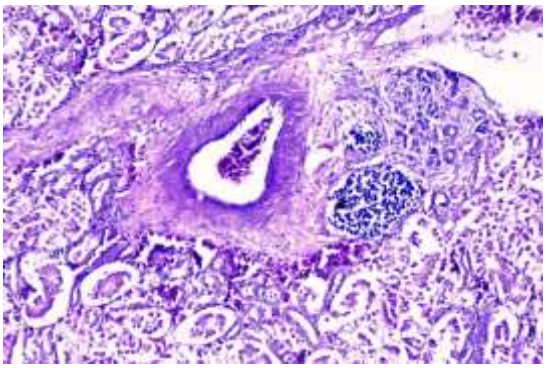
Snap 3: Trachea of control +ve H9N2 chicken showed necrosis of the lining epithelium, (H&E 200).



Snap 4: Kidney of first H9N2 SPF chicken challenge showed intertubular hemorrhage (H&E 400).

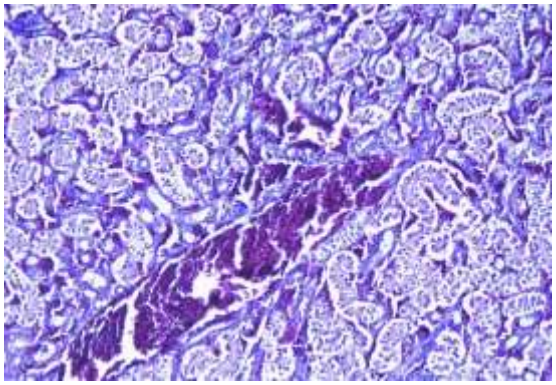


Snap 5: Trachea of first H9N2 SPF chicken challenge showed



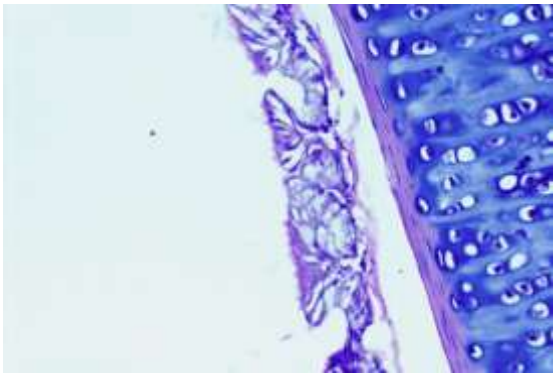
Snap 6: Kidney of first H9N2 Commercial chicken challenge

thickening of the lining epithelium due to edema, and mononuclear cells infiltration, in addition to bloody exudate in the lumen (H&E 100).

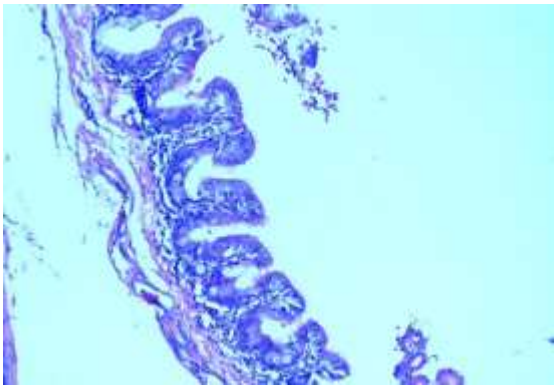


Snap 7: Kidney of first H9N2 commercial chicken challenge showed apparently normal architecture. (H&E 400).

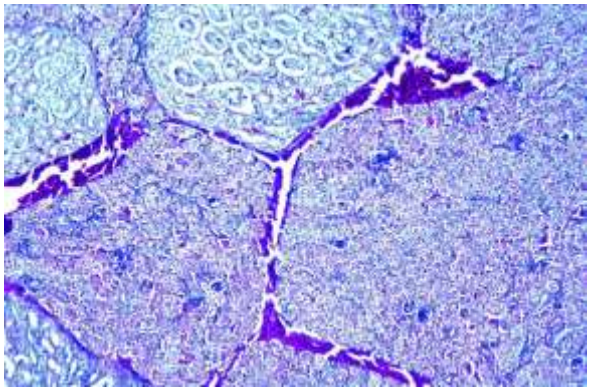
showed intertubular focal infiltration with mononuclear cells. (H&E 400).



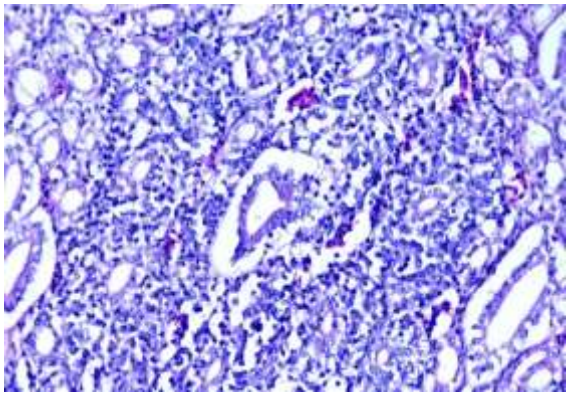
Snap8: Trachea of second SPF and third commercial chicken H9N2 challenge showed focal hyperplasia of lining epithelium (H&E 200).



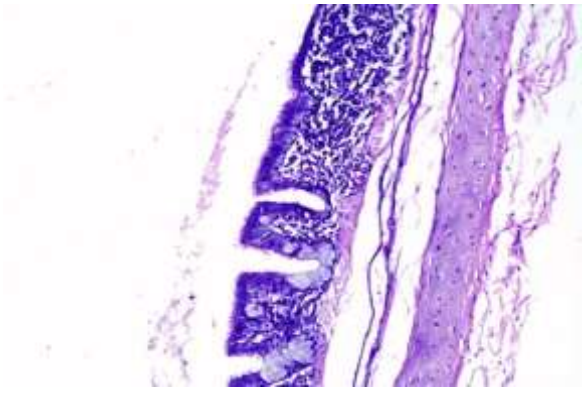
Snap 9: Trachea of second H9N2 Commercial chicken challenge showed hyperplasia of lining epithelium (H&E 200).



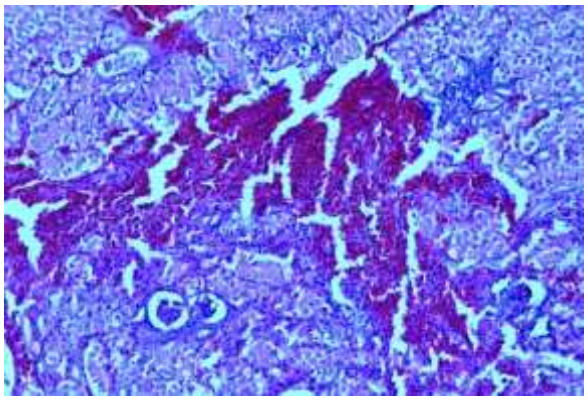
Snap 10: Kidney of third H9N2 SPF chicken challenge showed congested blood vessels with denudation and degeneration of the renal tubules. (H&E 100).



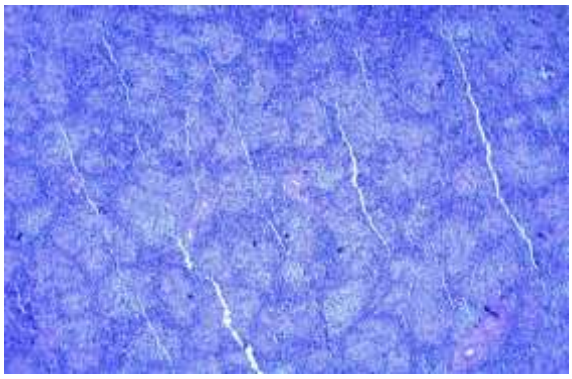
Snap 11: Kidney of third H9N2 SPF chicken challenge showed degeneration of the lining epithelium of the renal tubules with focal histeocytes aggregation. (H&E 400).



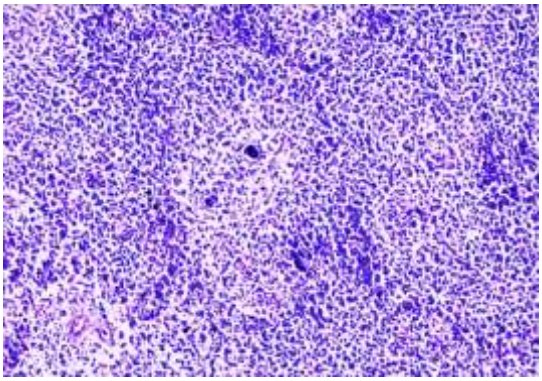
Snap 12: Trachea of third H9N2 SPF chicken challenge showed hyperplasia of lining epithelium with mononuclear cells infiltration in lamina propria (H&E 200).



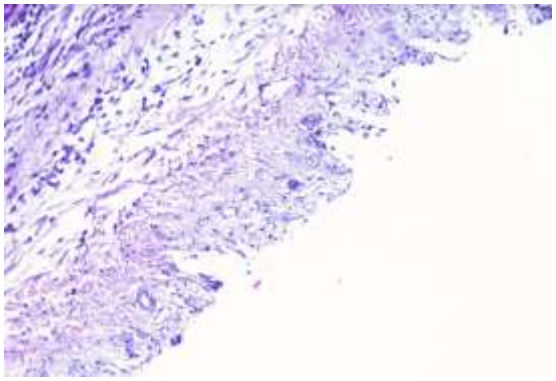
Snap 13: Kidney of third H9N2 Commercial chicken challenge showed focal aggregation of histeocytes (H&E 400).



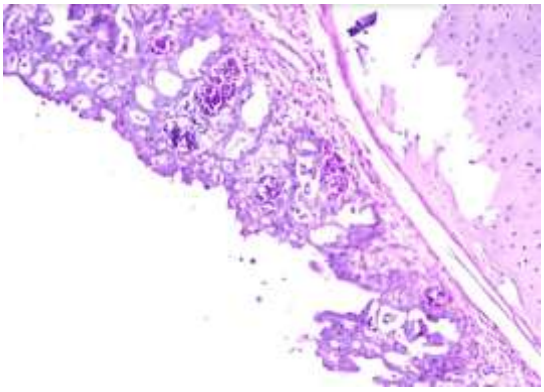
Snap 14: Spleen of ND control +ve and third SPF challenged group showed proliferation of capillary sheaths (H&E 50).



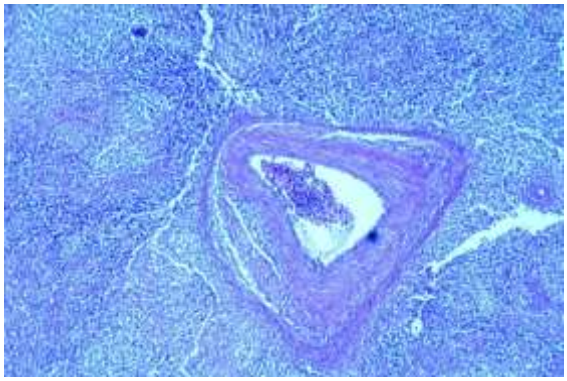
Snap 15: Spleen of ND control group showed depletion of lymphocytes and mild focal hemorrhage (H&E 50).



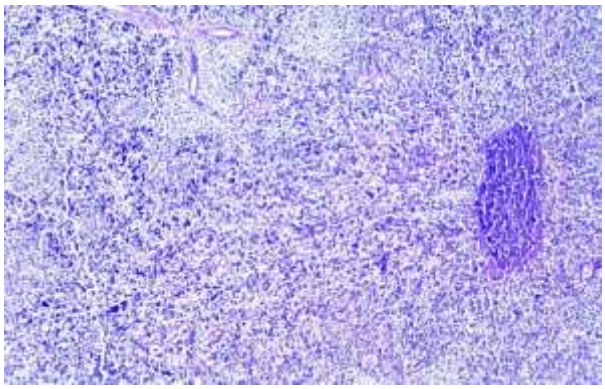
Snap 16: Trachea of ND +ve control group showed necrosis of the tracheal epithelium and submucosal edema (H&E 100).



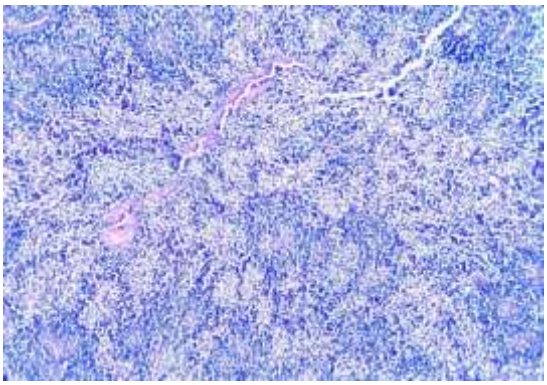
Snap 17: Trachea of ND +ve control, first commercial and third SPF challenged group showed hyperplasia of lining epithelium and activation of mucous glands and congested blood vessels (H&E 200).



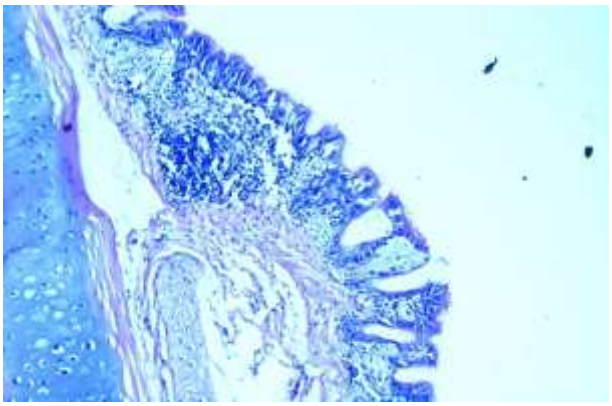
Snap 18: Spleen of first ND Commercial chicken challenge showed depletion of lymphocytes, cellular necrosis and congested blood vessels (H&E 100).



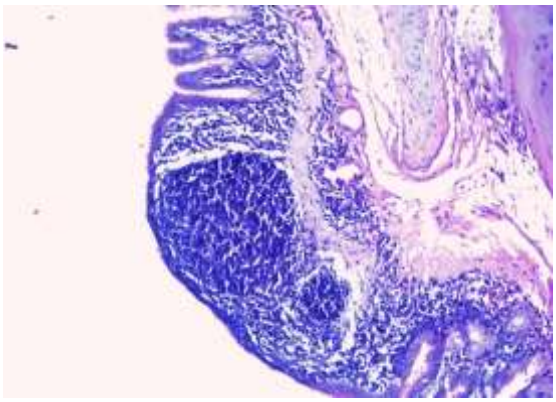
Snap 19: Spleen of second ND SPF chicken challenge showed diffuse depletion of lymphocytes and congested blood vessels (H&E 100).



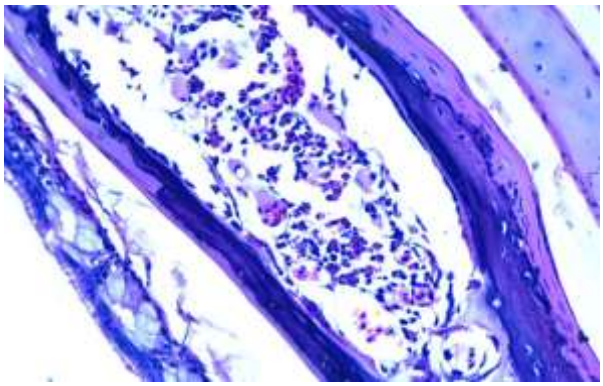
Snap 20: Spleen of second and third ND Commercial chicken challenge showed multifocal depletion of lymphocytes (H&E 100).



Snap 21: Trachea of third ND SPF chicken challenge showed nodule of monocular cells in lamina propria (H&E 200).



Snap 22: Trachea of third ND Commercial chicken challenge showed thickening of lining epithelium with nodules of monocular cells in lamina propria (H&E 200).



Snap 23: Trachea of third ND Commercial chicken challenge showed degeneration of the cartilage with heterophils infiltration (H&E 400).

DISCUSSION

Inactivated polyvalent oil emulsion vaccine could be a very useful solution for efficient and protective vaccination of poultry flocks and decrease efforts and money through increase the breadth of vaccine coverage by combining multiple micro-organisms protection into a single vaccination (Ali *et al.*, 2017).

In this study, we documented the serological and field immune response on long duration after vaccination to match with long rearing period. We prepared combined inactivated oil emulsion vaccine for AIV H9N2, NDV and IB (Var 2 and M41) with montanide 71 adjuvant, then we evaluated the level and duration of humoral immune response and the response to challenge test up to six months after booster vaccination for both H9N2 and NDV.

The humoral immune response for H9N2 was investigated by monitoring antibodies through HI test as shown in Figure (1) and table (1, 2, 3) in which the mean log^2 HI titer increased gradually to reach the highest titer about 10 Log^2 at the second month and the fifth week after booster vaccination for SPF and commercial chicken respectively, and then declined gradually to reach (7.8 and 4.6) log^2 for SPF and commercial chicken respectively at the six month after booster vaccination.

The humoral immune response for NDV was investigated by monitoring antibodies through HI test as shown in Figure (2) and table (4, 5, 6) in which the mean log^2 HI titer increased gradually to reach the highest titer 10.2 Log^2 at the second week after booster vaccination in SPF chicken, and then declined gradually to reach 6.6 log^2 at the six month after booster vaccination. The mean titer in commercial chicken increased gradually to reach between 10 and 9 Log^2 from the first and fifth week after BV and then decreased gradually to reach 6.5 log^2 at the six month after BV.

Challenge tests for H9N2 and NDV were repeated at month after vaccination and two and six months after booster vaccination. The results showed mild clinical signs in the control positive group of H9N2, while severe clinical signs with 100% mortality in the control positive group of NDV. At the same time, 100% of the vaccinated chicken were protected from mortality and showed no clinical signs of both viruses. Shedding test was carried out at the 3, 5, 7 and 10 DPC from oropharyngeal swabs by real time PCR revealed that this vaccine decreased H9N2 and NDV shedding with the comparison of positive control group, This agree with (OIE, 2012). Sera from the control positive group were zero by HI test at the first commercial and second SPF challenge (Table 11). The severity of lesion in histopathological examined organs (trachea in all challenged viruses, spleen in NDV challenged groups and kidney in H9N2 challenged groups) at 7 DPC in many vaccinated groups is lower or the same severity of the control positive group, but sometimes it's higher than the control positive.

CONCLUSION

For a six months after booster vaccination, this combined H9N2, NDV and IBV (M41 and var 2) vaccine induced humeral immunity for H9N2 and NDV and reduced viral shedding after challenge, so it has the advantages of a multivalent vaccine and also effective in a long duration rearing.

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