Journal of Current Veterinary Research

ISSN: 2636-4026

Journal homepage: http://www.jcvr.journals.ekb.eg

Newcastle Disease Virus Vaccines Based On Genotype VII Strains Provide Efficient Protection Against Challenge With Circulating Very Virulent Field Virus (Genotype VII) In Broiler Chickens

Ahmed. S. Kamal, Hesham. A. Sultan

Department of birds and rabbit medicine, Faculty of veterinary medicine, University of Sadat city, Menufiya, Egypt.

***Corresponding Author:** ahmed.said@vet.usc.edu.eg ***Submitted:** 16 Aug. 2019 **Accepted:** 22 Sept. 2019*

ABSTRACT

Newcastle disease virus (NDV) outbreaks still occur and cause disastrous economic losses in broiler chickens despite intensive vaccination policies using genetically different vaccines from the circulating strains. This study aimed to compare protection conferred by different vaccination strategies using genotype matched (genotype VII) and mismatched vaccine (genotype I and II) against challenge with currently circulating viruses in broiler chickens. 175 one- day-old broiler chicks were divided in to 5 groups. Groups 1 to 4 (G1, G2, G3 and G4) were vaccinated using genotype I (Ulster 2C strain), genotype II (VG/GA strain) and genotype VII (KBNP-4152R2L strain) vaccines according to manufacturer's instructions and group 5 (G5) was kept as control. Protection assessed based on clinical protection, seroconversion, shedding and histopathology. Chickens vaccinated with live genotype II vaccine at 7 and 21 days of age and killed genotype VII vaccine at 7 days of age (G2) were completely protected clinically with no mortality compared with control non vaccinated chickens (G5) that showed typical NDV clinical picture with (100%) mortality. while, other vaccinated groups showed lower level of clinical protection with (2.8%) mortality for each group. Viral shedding was greatly reduced in groups vaccinated with inactivated recombinant genotype VII vaccine (G2 and G3) which recorded (0% & 0%) and (16.6% & 0%) at 7 & 10 days post challenge, respectively compared with groups vaccinated with inactivated genotype II vaccine (G1 and G4) regardless the genotype of the combined live vaccine (either genotype II or VII). Moreover, histopathology of control non vaccinated challenged group (G5) revealed severe lymphocytic depletion and necrosis, epithelial degeneration beside neurological lesions. It was concluded that using genotype matched NDV vaccine with the currently circulating field strains can provide adequate clinical protection and minimize virus shedding that can help in decreasing virus load in the environment.

Keywords: *Newcastle, genotype VII, Live recombinant vaccine, KBNP-C4152R2L strain.*

INTRODUCTION

Newcastle disease (ND) is one of the most serious diseases threatening worldwide poultry production for what it causes a disastrous economic losses resulting from extensive weight losses and severe mortality rates in broiler chickens. NDV is a member of the genus Avulavirus in the family Paramyxoviridae. It was divided in to two main classes, class I & class II at which the class II viruses were divided in to 18 genotypes (Diel *et al.,* 2012; Miller and Koch, 2013). Recently, Dimitrov et al. (2019 a) updated a unified phylogenetic classification system and revised nomenclature for NDV at which s three new class II genotypes have been created associated with reduction of the number of sub-genotypes to obtain XXI NDV genotypes within class II.

Newcastle disease infections still occur in Egyptian poultry farms despite using multiple types of commercially available NDV vaccines and vaccination strategies. These available vaccines which are mostly of genotype I and II could partially or completely protect chickens from mortalities and clinical signs but not able to prevent viral dissemination from infected birds to the environment which is a potential serious problem (Ellakany *et al.,* 2019 and Sedeik *et al.,* 2019). Both F and HN genes of NDV are the major virulence determinants of NDV strains. They are present on the surface of virus envelop enabling the attachment of the virus to the target cell. The cleavage site sequence of the F protein determines the virulence and tropism of NDV viruses. Virulent strains of NDV contain multi-basic amino acids at the cleavage site of F gene thus enabling systemic spread of these viruses (Ogasawara *et al.,* 1992). While, the HN gene is responsible for virus release from infected cells and can potentiate the fusion activity of F gene. Antibodies against both F and HN are neutralizing antibodies (Seal, 2004 and Miller and Koch, 2013). Moreover, a single point mutation in HN gene linear epitope (E347K) can allow the virus to evade from its specific antibodies. This point mutation was reported in many genotype VII NDV strains in the world as reported by (Cho *et al.,* 2008). This mutation in addition to the high genetic difference between the conventional genotype I or II vaccine strains and the currently circulating genotype VII field isolates can explain the frequent exposure of poultry farms to virulent NDV outbreaks despite using extensive vaccination strategies (Cho *et al.,* 2008; Hu *et al.,* 2009 and 2011; Liu *et al.,* 2018; Ahmed, 2018 and Absalon *et al.,* 2019). In the current study, we aimed to compare between efficacy conferred by recombinant genotype VII ND vaccines (genotype matched) and other genotype mismatching vaccines (genotype I and II) in commercial broiler chickens on the basis of clinical protection, viral shedding and histopathological lesions.

MATERIALS AND METHODS

Challenge Virus

VV-NDV challenge virus (Chicken/USC/Egypt/2014) strain was kindly supplied by Prof. Dr. Hesham sultan, professor of birds and rabbit diseases, Faculty of Veterinary Medicine, University of Sadat City which was previously characterized molecularly as vv-NDV genotype VIId under accession no; (KM659400) on Gene bank. The virus challenge dose; $EID_{50} = 10^{5.5}$ by 0.5 ml via intra muscular route at 32 days of age.

Newcastle disease virus (NDV) vaccines

Live vaccines

Commercially available genotype II (heterologous to genotype VII challenge virus) NDV vaccine (Avinew Neo® vaccine- 2000 doses- Merial, France) in the form of freeze dried effervescent tablet containing ≥ 5.5 $\log_{10}$ EID_{50} live Newcastle Disease virus (VG/GA-AVINEW) strain per each dose, in addition to live recombinant genotype VII (Homologous to genotype VII challenge virus) NDV vaccine (Dalguban® N^+ live vaccine - KBNP-C4152R2L strain – Himmvac., KBNP,INC, Korea) were used via occulonasal route according to manufacturer's instructions.

Inactivated vaccines

Inactivated genotype I (heterologous to genotype VII challenge virus) NDV vaccine (Impost® inactivated Oil adjuvant Vaccine -

Ulster 2C strain - 1000 doses- Merial, France) and Inactivated recombinant genotype VII (Homologous to genotype VII challenge virus) NDV vaccine (Dalguban® N^+ oil vaccine - KBNP-C4152R2L strain – Himmvac., KBNP, INC, Korea) were used to vaccinate birds subcutaneously by 0.3 ml and 0.5 ml for each bird respectively according to manufacturer's instructions..

Serology

Hemagglutination inhibition (HI) test was performed to determine bird's immune response to NDV according to **(OIE, 2012)**. Five serum samples were collected from each group at 14, 21, 28 and 39 days of age. Two-fold dilutions of the serum samples were tested against two different types of NDV antigens that were prepared through inocuolation of either live genotype II (VG/GA strain) - Avinew® vaccine or live recombinant genotype VII (KBNP-C4152R2L strain) - Dalguban® N+ live vaccine through ECE followed by collection of the allantoic fluid at the level of the 1st passage **(Roohani *et al.*, 2015).**

Conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

Sampling

Six pools of tracheal and cloacal swabs (Three each) were collected from each group for detection of vv-NDV viral shedding through respiratory and or gastrointestinal routes directly before challenge and either 3, 7 and 10 days post challenge. The swabs were immersed in 400 µl of 1ml DMEM media with antibiotic solution and stored at -80 C until further analysis.

RNA extraction

The viral RNAs were extracted directly using Patho Gene-spin™ DNA/RNA Extraction Kit (50 preps, Intron, Korea) according to the manufacturer's instructions.

RT-PCR kits

MyTaq™ One-Step RT-PCR Kit (Bioline-USA) was used to detect vv-NDV RNA in the tested swabs using specific primers.

Oligonucleotide primers

A 254bp PCR product of the F gene of virulent NDV was amplified with specific primers; **A**; 5'-TTGATGGCAGGCCTCTTGC-3' and **C**; 5'-AGCGT(C/T)TCTGTCTCCT-3'. Reverese transcribtion was carried out by incubation at 45 ºC for 20 minutes followed by polymerase activation at 95 ºC for 1 minute. After denaturation for 4 min at 94°C, the PCR consisted of 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C.The PCR products were analyzed by electrophoresis on a2% agarose gel stained with ethidium bromide **(Kant *et al.*, 1997).**

Histopathological examination

Tissue samples include trachea, cecal tonsils, spleen, bursa, proventriculus, brain and kidneys were fixed in 10% buffered formalin solution, processed and stained with haematoxylin and eosin (HE) **(Desingu *et al.*, 2017)**.

Chickens and experimental design

One hundred and Seventy five- day old (Cobb) commercial broiler chicks were housed in separated pens with food and water providedad libitum. They were divided into five groups (N = 35 each) for evaluation of laboratory vaccination experiment **(Table, 1)**.

RESULTS

Clinical signs, Mortality and P.M gross lesions

Clinical signs differed from mild or no signs in some vaccinated groups to severe respiratory, neurological and gastrointestinal disorders in the control group. G2 revealed complete absence of clinical signs after challenge and didn't recorded any mortalities (0%) in comparison with the other groups that showed variable degrees of clinical symptoms, mortalities and post mortem gross lesions. G5 (control non vaccinated challenged) showed an exaggerated degree of clinical signs after challenge including severe respiratory distress associated with mucoid nasal discharge, severe depression, off food, severe neurological signs including wing drooping, lameness, tremors and torticollis in addition to severe watery whitish and greenish diarrhea. The birds in G5 showed the typical P.M gross lesion of NDV including severe hemorrhages on the proventricular mucosa, sever tracheal and lung congestion, button like ulcers on the intestinal mucosa, severe hemorrhages on the cecal tonsils and kidney enlargement and congestion **(Fig.1)**. The mortality pattern in the control group began

from the 3rd day post challenge (DPC) and continued to increase in the 4th day post challenge (DPC) to reach 100% at the 5th day post challenge (DPC) mimicking the field infection **(Table, 2).** On the other hand, the clinical signs were greatly reduced in G1 (Vaccinated with live genotype II and inactivated genotype I vaccines), G3 (Vaccinated with live and inactivated recombinant genotype VII vaccines) and G4 (Vaccinated with live recombinant genotype VII and inactivated genotype I vaccines) especially the neurological signs in comparison with the control group. Upon that, G1 showed only mild greenish diarrhea with complete absence of other clinical signs. While, one bird in both G3 and G4 only showing lameness at the 4th day post challenge (DPC). The mortality percentages were the same (2.8%) in the three groups **(Table, 2)** and exhibited a slight degree of P.M. lesions compared to the control group.

Viral shedding

No viral shedding neither from tracheal nor cloacal swabs was detected from all groups at the day of challenge (0%). Whereas after challenge, viral shedding positive swabs was significantly reduced in G2 and G3 after challenge at 3, 7 and 10 days post challenge in comparison to the control group that showed (100%) of viral shedding at the 3rd day post challenge (DPC). On the other hand, G1 and G4 showed higher degrees of viral shedding at 3 and 7 DPC. (50% & 33.3%) vs. (100% & 50%), respectively and the viral shedding were not completely prevented at the 10th D.P.Ch. (16.6%) in comparison with G2 & G3 as shown in **(Table, 3).**

Serological response

Serum samples (N=5) were collected at 14, 21, 28 and 39 days of age from all groups. All vaccinated groups elicited a gradual increase in immune response expressed as Geometric mean titers (G.M.T.) log_2 to NDV after vaccination at 14, 21 & 28 days of age while the control non vaccinated group (G5) showed gradual decrease in the MDA titers till reach (0.5 & 0.4 G.M.T. log_2) at 28 days of age for G-II and G-VII antigens, respectively.

By using G-II antigen, G1 recorded the highest immune response among all vaccinated groups at 28 days of age which was (5 Log2-G.M.T.) followed by G2, G4 and G3 which recorded G.M.T. of (4, 3.9 and 2.7 Log2), respectively. Moreover, when the G-VII antigen was used, results showed that the immune response to NDV was higher in G2, G3 and G4 which elicited higher G.M. titers of (4.8, 4.5 & 4.9) log2, respectively. After challenge, all groups showed a high rapid increase of antibody titer which recorded higher values when the G-VII antigen was used than when using the G-II antigen (Table, 4, Figs. 2 and 3).

Histopathology:

The control non vaccinated challenged group (G5) showed severe multifocal coagulative necrosis in splenic cells associated with moderate lymphocytic depletion and moderate to severe haemorrhages in addition to mild to moderate congestion on splenic BL.Vs**.** In addition bursa of fabricious showed severe hyperplasia of the lining epithelium associated with severe extensive lymphocytic depletion in addition to moderate degeneration and necrosis of the bursal tissue which led to high level of cryst formation beside moderate to severe oedema**.** Whereas, cecal tonsils showed severely distended crypts containing high amount of necrotic debris associated with severe hemorrhage and lymphocytic infiltration including macrophages beside lymphocytic degeneration and necrosis**.** While, brain showed severe demyelination of brain tissue associated with severe gliosis and perivascular cuffing in addition to moderate to severe congestion of the BL.Vs **(Fig.8)**. On the other hand, the other vaccinated groups showed a greater reduction in the histopathological lesions in all organs especially the lymphoid organs.

DISCUSSION

Although the occulo-nasal route is the natural route of NDV transmission (Miller and Koch, 2013) the chickens was infected with the challenge virus using intramuscular route to ensure delivery of the correct virus dose to each bird as it may be lost in the digestive tract and destroyed by acidic PH of the digestive enzymes (Moses *et al.*, 1947).

The infected commercial broilers with genotype VII virus exhibited the clinical disease typical for VV-NDV symptoms beginning from the 3rd day after challenge especially at the control non vaccinated challenged group (G5). The mortality started at the 3rd D.P.Ch and continued to increase to reach up to 100% in the control group in the 5th D.P.Ch and continued to the 8th D.P.Ch in other vaccinated groups which represent the mortality curve obtained by field exposure. These findings agree with previously reported findings by (Susta *et al.,* 2011 and Moussa *et al.,* 2015). Typical P.M. lesions were observed at the control non vaccinated challenged group as hemorrhage on proventricular glands, cecal tonsils and payr's patches with congestion of bursa, liver and spleen which are similar to previous observations by (Susta *et al.,* 2011 and 2014 and Miller and Koch, 2013).

Based on these results, it was concluded that clinical protection against NDV can be obtained through using either genotype I & II based commercial vaccines or recombinant genotype VII based vaccines against challenge with VV-NDV genotype VII as previously reported by (Kapczynski *et al.,* 2013 and Yang *et al.,* 2017).

Both F and HN surface genes of NDV are the major virulence determinants of NDV strains (Ogasawara *et al.,* 1992). Antibodies against both F and HN are neutralizing antibodies (Seal, 2004 and Miller and Koch, 2013). Moreover, a single point mutation in HN gene linear epitope (E347K) can allow the virus to evade from its specific antibodies. This point mutation was reported in many genotype VII NDV strains worldwide as reported by (Cho *et al.,* 2008). This mutation in addition to the high genetic difference between the conventional genotype I or II vaccine strains and the currently circulating genotype VII field isolates can explain the frequent exposure of poultry farms to virulent NDV outbreaks despite using extensive vaccination strategies.

From this point of view, using of genotype matched vaccines that are homologous to the currently circulating field isolates by exchanging the F and HN genes of La Sota with the F & HN genes of KBNP-C4152 genotype VII strain is essential to induce higher protection against clinical disease and shedding by production of specific neutralizing antibodies (Cho *et al.,* 2008; Hu *et al.,* 2009 and 2011; Liu *et al.,* 2018; Ahmed, 2018 and Absalon *et al.,* 2019). These reports support our results where chickens vaccinated with the homologous inactivated recombinant genotype VII oil NDV vaccine in G2 showed complete protection against clinical disease.

On the other hand, chickens vaccinated with live recombinant genotype VII vaccine (G4) didn't gave a complete protection against clinical signs or mortality in comparison with G2. Moreover, chickens vaccinated with inactivated recombinant genotype VII oil NDV vaccine (G3) showed similar results. This could be occurred due to low ICPI of live recombinant genotype VII NDV vaccine as reported by (Cho *et al.,* 2008) which it may lead to lower ability of the immune system to build up an effective primary immune response to the vaccine especially when used as a primer vaccine at early life or may be attributed to some defect in the cellular immune response produced after vaccination with the live recombinant genotype VII NDV vaccine.

Viral shedding is a potential reliable indicator for assessment of NDV vaccination efficacy. An effective vaccine not only that protects against clinical disease but also which is capable to reduce virulent virus dissemination from infected birds to the environment. However, NDV strains are belonging to one serotype, there is a genetic difference between strains each other's that led to division of these strains in to different genotypes. Most of commercially available NDV vaccines are belonging to genotype I & II. These vaccines can protect against clinical disease but not able to completely prevent virulent virus shedding from infected birds due to major genetic distance between these genotypes and the currently circulating field virus which is mostly of genotype VII (Miller *et al.,* 2007; Cho *et al.,* 2008; Roohani *et al.,* 2015; Wajid *et al.,* 2018 and Absalon *et al.,* 2019).

Our results showed that a major reduction in viral shedding was achieved through combining either inactivated or live recombinant genotype VII NDV vaccines beside vaccines produced from genotype I or II or combination between live and inactivated recombinant genotype VII NDV vaccines in different vaccination program than when using vaccines of other genotypes alone. This was clearly detected in G2 and G3 that showed major reduction of the virulent virus shed either from respiratory or digestive tracts (50 %, 0% & 0%) vs. (83.5%, 16.6% & 0%) at 3, 7 & 10 D.P.Ch, respectively. So, using genotype matched vaccine can achieve better protection and ensure a strict disease control.

Haemagglutination inhibition test considered one of the major assays to assess the protection level conferred by NDV vaccines (Kapczynski and King, 2005). In this study, it was concluded that sera collected from groups that were vaccinated with recombinant genotype VII live & or inactivated vaccines showed higher immune response to NDV when tested using antigen that is homologous to the vaccine strain and the challenge virus than when tested using heterologous antigen (genotype II antigen) (Table. 4, Fig. 3 & Fig. 4).Therefore, it is recommended to estimate the serological immune response against NDV vaccines using genotype matched antigen to give more accurate results which correlates to protection level as previously reported by (Miller *et al.,* 2007; Roohani *et al.,* 2015 and Wajid *et al.,* 2018).

Since, the histopatological lesions are considered a reliable marker to assess the protective efficacy produced by a give vaccination program. Histopathological examination revealed that vv-NDV genotype VII infection severely affects bird immune system and causes severe tissue destruction in bursa, spleen and cecel tonsils. Thus, VV-NDV genotype VII can lead to immunosuppression as shown on (Fig. 8). The control non vaccinated challenged group (G5) showed severe multifocal coagulative necrosis in splenic cells associated with moderate lymphocytic depletion and moderate to severe hemorrhages in addition to mild to moderate congestion on splenic BL.Vs (Fig.8). In addition bursa of fabricious showed severe hyperplasia of the lining epithelium associated with severe extensive lymphocytic depletion in addition to moderate degennearation and necrosis of the bursal tissue which led to high level of cryst formation beside moderate to severe oedema (Fig.8). Whereas, cecal tonsils showed severely distended crypts containing high amount of necrotic debris associated with severe hemorrhage and lymphocytic infiltration including macrophages beside lymphocytic degeneration and necrosis (Fig.8). These results were supported by many previous reports that studied the effect of genotype VII NDV on tissues of immune system (Susta *et al.,* 2014; Miller and Koch, 2013; Desingu *et al.,* 2017 and Dimitrov *et al.,* 2019 b).

To detect the possible cause of nervous signs occurred after challenge with VV-NDV genotype VII, histopathological lesions were observed from brain tissue which recorded severe demyelination of brain tissue associated with severe gliosis and perivascular cuffing in addition to moderate to severe congestion of the BL.Vs (Fig. 8), as previously recorded by (Ecco *et al.,* 2011; Miller and Koch, 2013 and Desingu *et al.,* 2017).

Histopathological lesions observed in proventriculus, trachea and kidneys of VV-NDV genotype VII infected chickens were similar to lesions previously observed (Miller and Koch, 2013). Interestingly, the histopathological lesions scores in all organs tested were minimized in all NDV vaccinated groups compared to the control non vaccinated group.

CONCLUSION

Based on results obtained in this study it concluded that commercially available NDV vaccines based on strains from genotype I, II, and VII can provide a sufficient clinical protection from clinical disease occurrence but not all of them can reduce or prevent viral dissemination to the environment. Moreover, introducing inactivated recombinant vaccines based on VV-NDV genotype VII in NDV vaccination programs is recommended.

Therefore, it can be advisable to vaccinate broiler chickens with genotype matched NDV vaccine which is similar to the currently circulating field strains to allow better protection based on virus shedding which in turn can minimize the opportunities for occurrence of new outbreaks. In addition, from this study, it's not preferred to use live recombinant genotype VII NDV vaccine as a primer vaccine in early life of the bird as it could not provide the expected protection level.

Table (1): Experimental grouping, used vaccines and vaccination regimes as well as assessment of immune response in commercial broiler chickens.

Group No.	Birds No.	NDV vaccination regime		Challenge [e] Age / day	Assessment of protection
		Vaccine genotype	age/days		
1	35	Live G-II [a] Inactivated G-I [b]	7, 21 7	32	**1**. Clinical signs **2**. Pm. gross lesions **3**. Mortality % **4**. Seroconversion **5**.Detection of viral shedding **6**. Histopathology
2	35	Live G-II Inactivated G-VII	7, 21 7		
3	35	Live G-VII [c] Inactivated G-VII [d]	7, 21 7		
4	35	Live G-VII Inactivated G-I	7, 21 7		
5	35	-------	--------		

a. Live NDV vaccine Avinew Neo (VG/GA- AVINEW strain) belonging to genotype II NDV. Each dose contains ≥ 5.5 $\log_{10}$ EID_{50} via occulonasal route.
b. Inactivated oil emulsion NDV vaccine (Impost) (Ulster 2C strain) belonging to genotype I NDV by 0.3 ml via subcutaneous route.
c. Live genotype VII (Dalguban N+) NDV vaccine (KBNP- C4152R2L strain) administered via occuolonasal route.
d. Inactivated oil emulsion genotype VII NDV vaccine (Dalguban N+- oil vaccine) administered by a dose of 0.5 ml through S/C injection.
e. Challenge with velogenic viscerotropic Newcastle disease virus (genotype VII) **(Chicken/USC/Egypt/2014**), EID_{50} = $10^{5.5}$ by 0.5 ml via intra muscular route.

Table (2): Mortality pattern and percentage recorded in different broiler chickens challenged groups:

Group NO.	Birds No.	NDV vaccination regime		Challenge	Mortality pattern (D.P.Ch) [a]										Mortality %
		Vaccine genotype	age/days	Age / day	1	2	3	4	5	6	7	8	9	10	
1	35	Live G-II Inactivated G-I	7, 21 7	32	-	-	-	-	1	-	-	-	-	-	2.8
2	35	Live G-II Inactivated G-VII	7, 21 7		-	-	-	-	-	-	-	-	-	-	0
3	35	Live G-VII Inactivated G-VII	7, 21 7		-	-	-	-	1	-	-	-	-	-	2.8

4	35	Live G-VII Inactivated G-I	7, 21 7	-	-	-	-	1	-	-	-	-	-	2.8
5	35	-------	--------	-	-	7	18	10	-	-	-	-	-	100

a- D.P.Ch= Days post challenge.

Table (3): VV-NDV viral shedding in different broiler chickens challenged groups:

Group No.	Swabs No / group (N= 6)		Viral shedding at days post challenge examined by RT-PCR											
			0 [c]			3			7			10		
	Tracheal swabs	Cloacal swabs	TR[a]	CL[b]	%	TR	CL	%	TR	CL	%	TR	CL	%
1	3	3	0/3	0/3	0	0/3	3/3	50	0/3	2/3	33.3	0/3	1/3	16.6
2	3	3	0/3	0/3	0	1/3	2/3	50	0/3	0/3	0	0/3	0/3	0
3	3	3	0/3	0/3	0	2/3	3/3	83.3	0/3	1/3	16.6	0/3	0/3	0
4	3	3	0/3	0/3	0	3/3	3/3	100	1/3	2/3	50	0/3	1/3	16.6
5	3	3	0/3	0/3	0	3/3	3/3	100	-	-	-	-	-	-

a. TR= Tracheal swabs**.** **b.** CL= Cloacal swabs. **c**. Just before challenge.

Table (4): ND HI antibody titers in broiler chickens vaccinated with different NDV vaccination regimes and challenge with vv-NDV genotype VII using cross HI:

Group No.	Challenge Age / day	G.M.T [a] Age / days							
		G-II antigen				G-VII antigen			
		14	**21**	**28**	**39**	**14**	**21**	**28**	**39**
1	32	1.8	3.2	5	7.4	1.9	3.1	4	7.2
2		1.5	3.1	4	8.2	2.8	3.5	4.8	8.4
3		1.5	2.3	2.7	6.4	2.7	3.2	4.5	8.4
4		0.9	3.1	3.9	6.6	2.8	3.2	4.9	8
5		1.6	0.7	0.5	6	1.4	0.6	0.4	8.6

f. a. GM. = geometric mean of HI titer $\log_2 \leq 2$ Log_2 considered negative **(OIE, 2012)**.

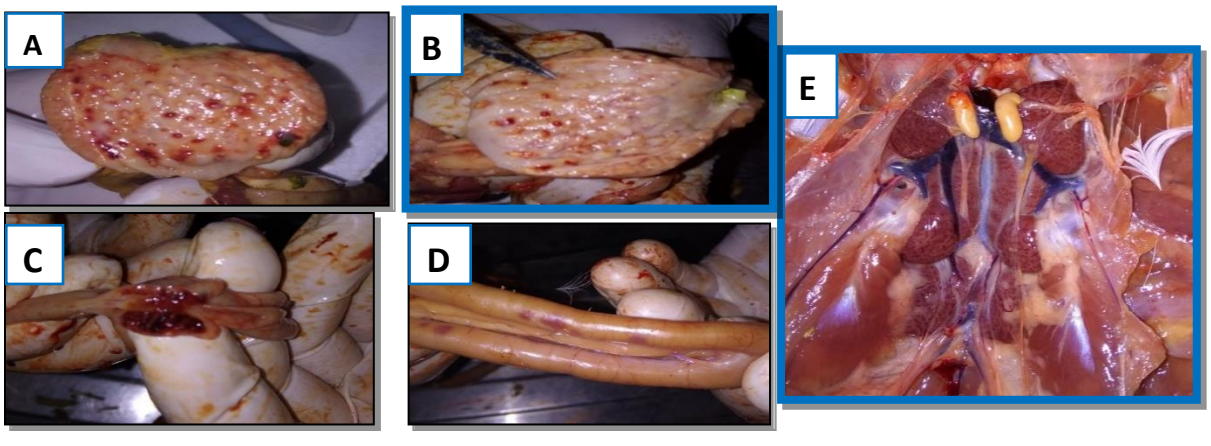

Fig. (1): Showing the detected P.M gross lesions of G5 (Control non vaccinated challenged) (A, B, C, D& E) at the 3rd day post challenge (DPC) with vv-NDV genotype VII strain "chicken/USC/Egypt/2014". (A & B) Showing Petechial haemorrhagic spots on the proventricular glands, (C) showing severe haemorrhages on the cecal tonsils, (D) shows Button like ulcer on the intestinal wall. While, (E) showing congestion and enlargement of kidneys.

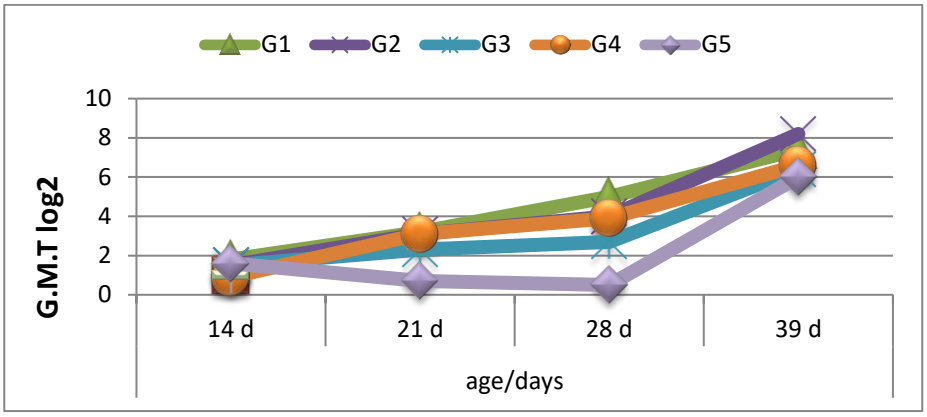

Fig. (2): HI antibody levels of broiler chickens in different vaccination- challenge groups using G-II antigen (VG/GA strain).

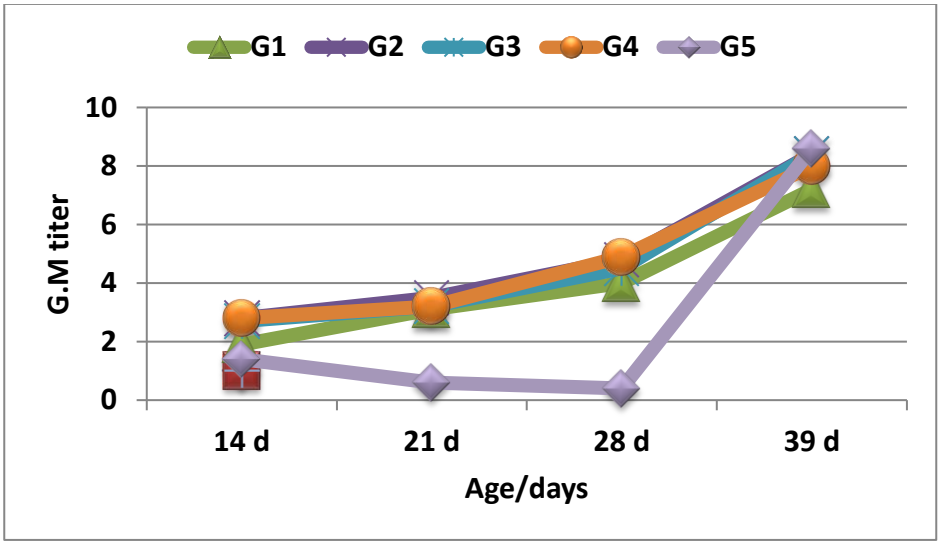

Fig. (3): HI antibody levels of broiler chickens in different vaccination- challenge groups using G-VII antigen (KBNP- C4152R2L strain).

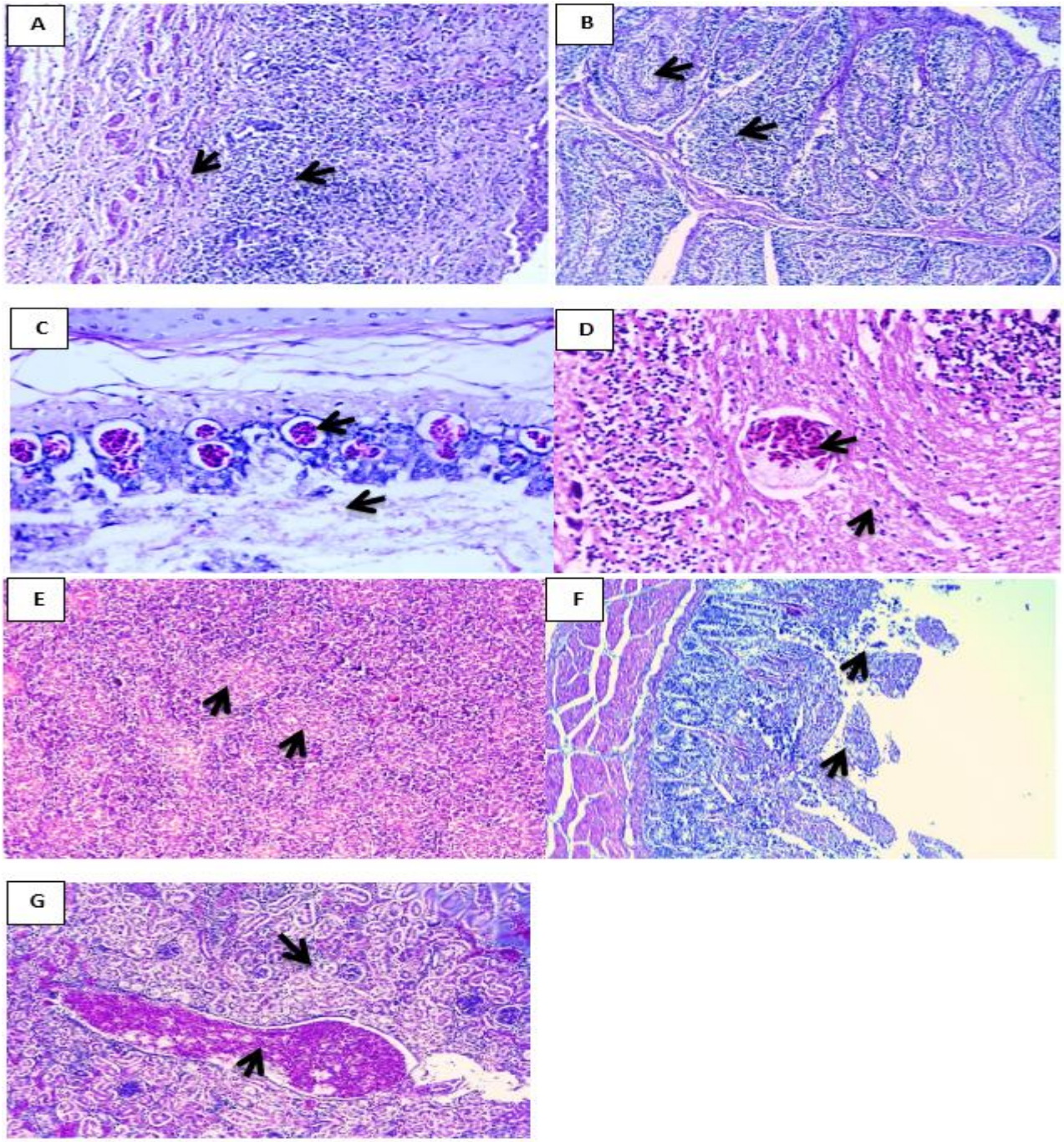

Fig. (4): Histopathological lesion of G1 **(A, B, C, D, E, F& G), (A)** Proventriculus of showing necrosis and sloughed epithelium H&E X200. **(B)** Bursa showing depletion of lymphocytes H&E X100. **(C)** Trachea of showing mild focal hyperplasia of lining epithelium with mucosal congestion H&E X400. **(D)** Cerebrum showing thrombus formation and demyelination H&E X400. **(E)** Spleen showing mild depletion H&E X100**. (F)** Cecal tonsils showing sloughed lining epithelium H&E X100. **(G)** Kidney showing interstitial congestion and focal mononuclear cells infiltration H&E X100.

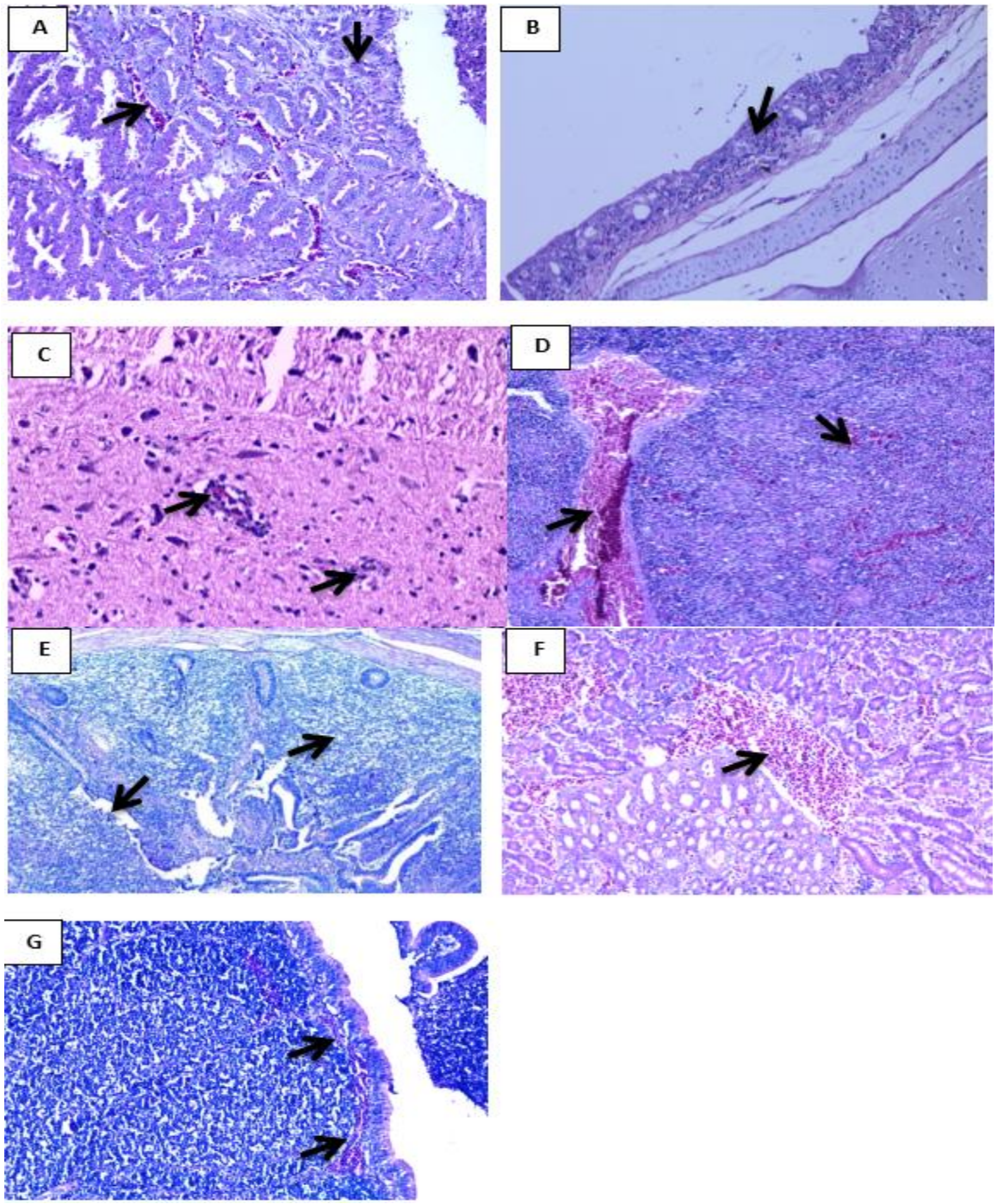

Fig (6): Histopathological lesion of G3 **(A, B, C, D, E, F & G), (A)** Proventriculus showing multifocal mucosal and submucosal mononuclear cells infiltration and congestion of blood vessels H&E X200. **(B)** Trachea showing mild thickening of the mucosa due to mononuclear cells infiltration and congestion H&E X400. **(C)** Cerebrum showing demylination H&E X200. **(D)** Spleen showing mild depletion of lymphocytes and congestion of blood vessels H&E X100. **(E)** Cecal tonsil showing severe depletion and necrosis of lymphocytes in the lamina propria H&E X100. **(F)** Kidney showing congested blood vessels H&E X200. **(G)** Bursa showing mild sub mucosal congestion H&E X200.

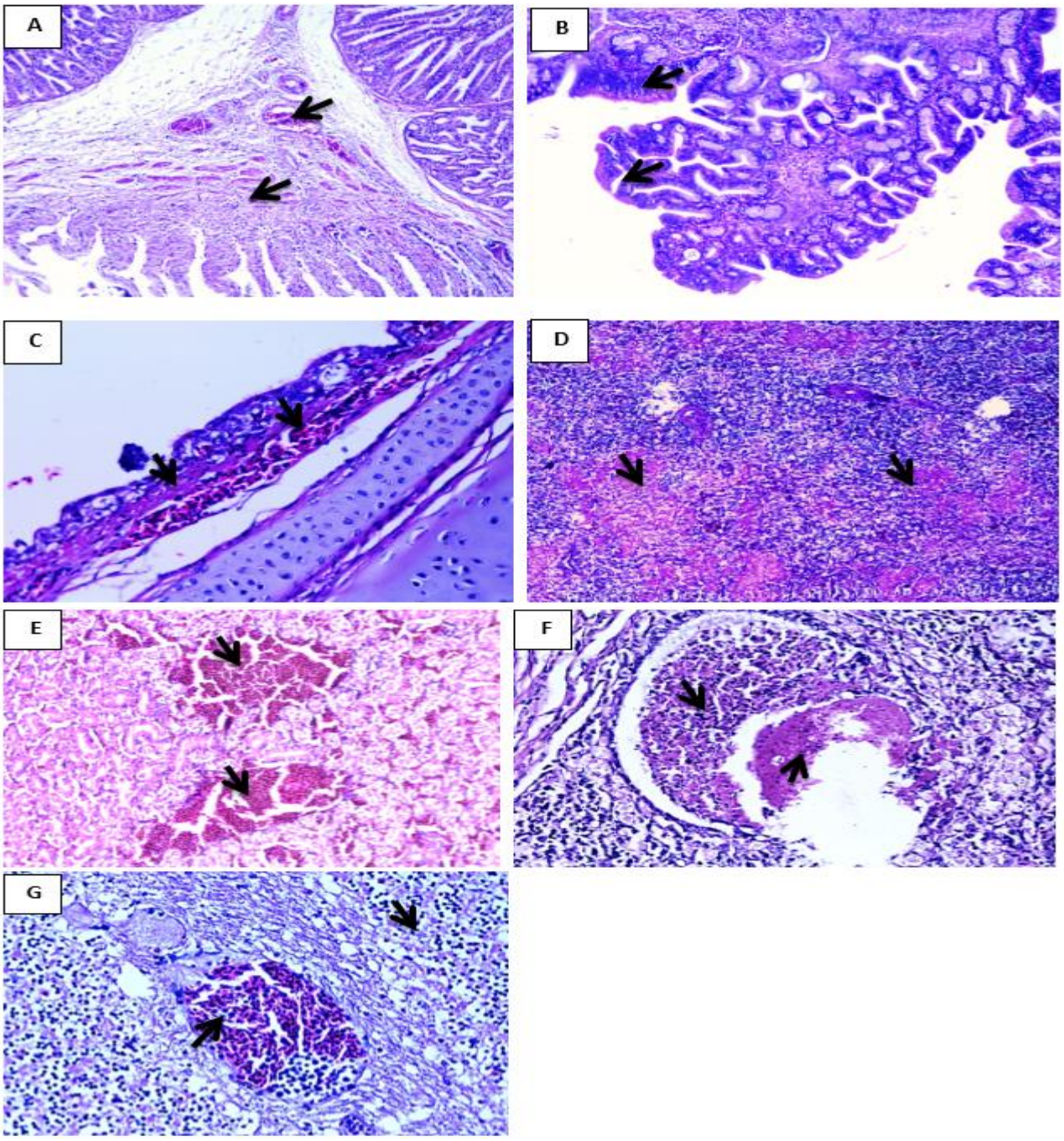

Fig. (8): Histopathological lesion of G5 **(A, B, C, D, E, F & G), (A)** Proventriculus showing interglandular edema and mononuclear cell infiltration and congested blood vessels H&E X100. **(B)** Bursa showing severe hyperplasia of the lining epithelium with epithelization H&E X200. **(C)** Trachea of showing mild sub mucosal edema and congestion H&E X 400. **(D)** Spleen showing multifocal coagulative necrosis H&E X100. **(E)** Kidney showing congested blood vessels H&E X200**. (F**) Cecal tonsil showing severely distended crypts containing necrotic debris and macrophages and severe hemorrhages H&E X400. **(G)** Cerebrum showing demyelination, congested blood vessels and perivascular cuffs H&E X400.

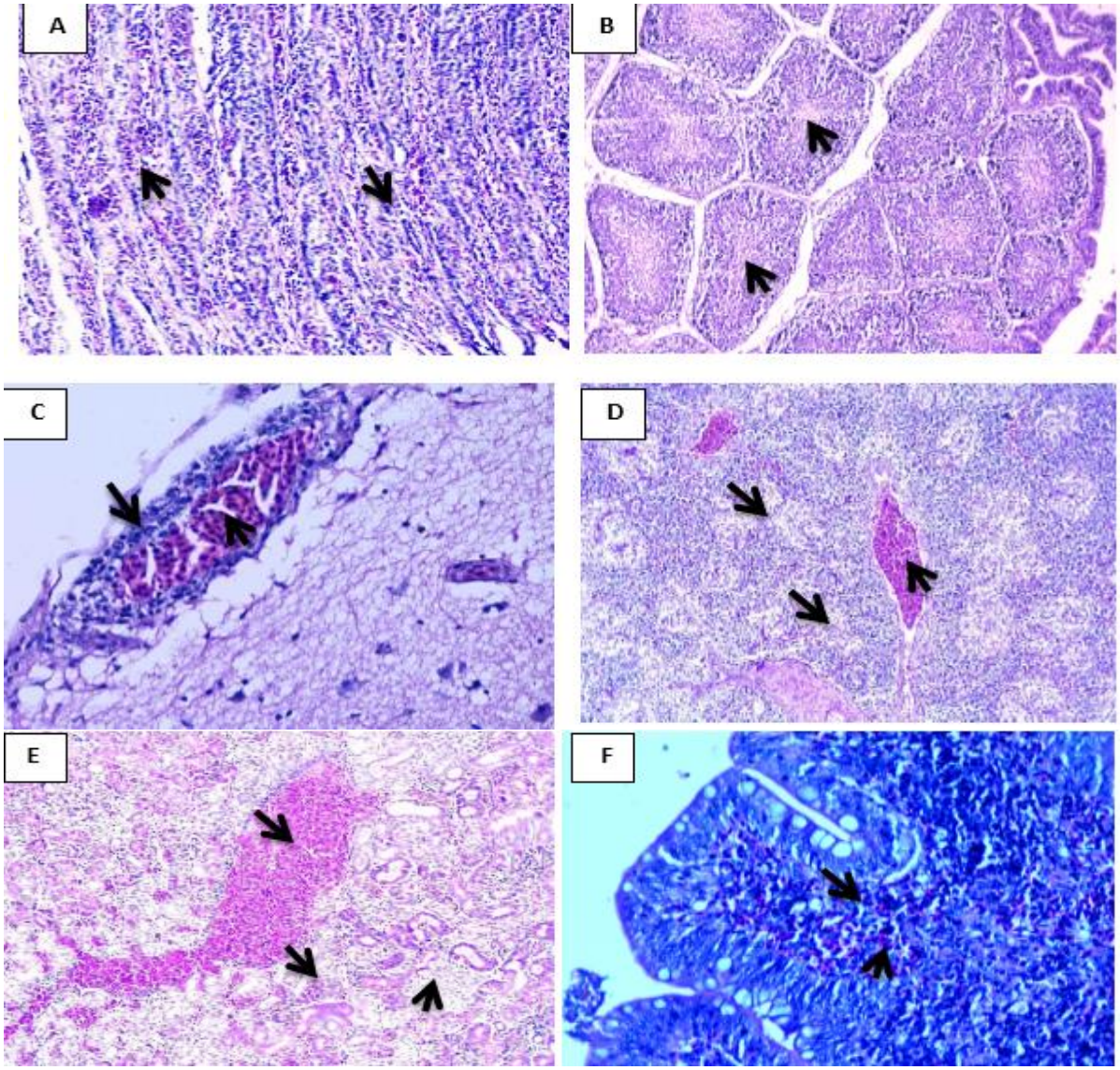

Fig (7): Histopathological lesion of G4 **(A, B, C, D, E, F), (A)** Proventriculus showing degenerated lining epithelium and mononuclear cells infiltration in lamina propria H&E X200. **(B)** Bursa showing depletion of lymphocytes H&E X100. **(C)** Cerebrum showing perivascular cuff, congested blood vessels surrounded by mononuclear cells H&E X400. **(D)** Spleen showing depletion of lymphocytes and congestion of blood vessels H&E X100. **(E)** Kidney showing severe congested blood vessels with interstitial mononuclear cells infiltration in addition to tubular degeneration with hyaline and heterophilic casts H&E X100. **(F)** Cecal tonsils showing few extravasated RBCS in the tips of villi H&E X400.

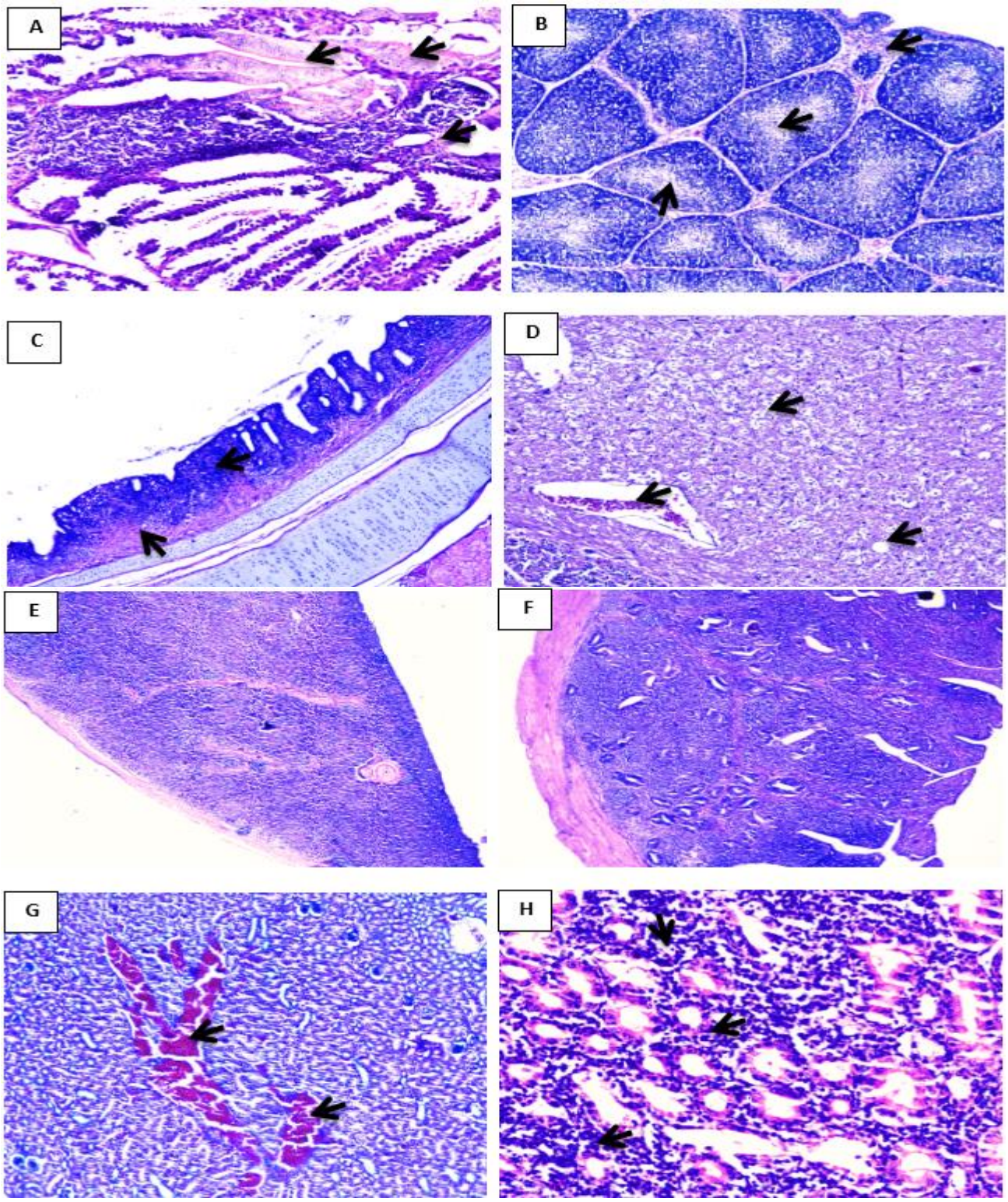

Fig. (5): Histopathological lesion of G2 **(A, B, C, D, E, F, G, H), (A)** Proventriculus showing multifocal mucosal and submucosal haemorrhage. **(B)** Bursa showing depletion of lymphocytes with few interfollicular extravasated blood H&E X100. **(C)** Trachea showing thickening of the mucosa due to mononuclear cells infiltration H&E X100. **(D)** Cerebellum showing congested blood vessels and focal spongiosis H&E X200. **(E)** Spleen showing apparently normal architecture H&E X50. **(F)** Cecal tonsil showing apparently normal architecture H&E X50. **(G)** Kidney showing intertubular congestion H&E X100. **(H)** Kidney showing focal mononuclear cells infiltration H&E X400.

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