African Journal of Biotechnology Vol. 10(62), pp. 13664-13671, 12 October, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2506
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

In vivo pathogenicity of hydropericardium hepatitis syndrome (Angara disease)

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Accepted 1 September, 2011

This study was conducted on 175 one day old broiler chicks in order to observe *in vivo* pathogenicity of hydropericardium hepatitis syndrome (Angara disease). Chicks were grouped into A (25 chicks; reared at the Poultry Research institute (PRI), Rawalpindi) and B groups (150 chicks; reared at National Veterinary Laboratories (NVL) Islamabad in poultry rearing units). Feed and water were provided *ad-libitum* to all the birds in both groups. The LD_{50} (lethal dose $_{50}$) of the virus was determined at the age of 26 days of broiler chickens divided into sub-groups a, b, c, d and e (each having 5 birds). The LD_{50} titre of the viral suspension (10%) was prepared from liver extract and determined as $10^{-2.5}$ per ml. During the study, potency of the vaccines was determined by vaccinating 150 chicks of sub-group k, l, m, n and o (each having 30 birds) with a dose of 0.2 ml. The vaccinated and non-vaccinated chicks of sub-groups were challenged with viral dose of 2 ml at day 17 post-vaccination to know the protection potency of the vaccines. No chick showed clinical manifestation of disease up to five days post challenge. On the 6th day post challenge, all the chicks were slaughtered and subjected to postmortem. Some of the chicks showed the lesions of hydropericarium. Histopathological findings of liver of all sub-groups revealed different stages of necrosis, cloudy swelling, liquefactive necrosis, cytoplasmic blabing, fatty degeneration and intranuclear inclusion bodies of virus.

Key words: *In* vivo pathogenicity, hydropericardium hepatitis syndrome, Angara disease.

INTRODUCTION

Hydropericardium hepatitis syndrome (HHS) was first recognized in broiler flocks in Angara village near Karachi Metropolitan City of Pakistan, in the late 1987 (Jaffery, 1988). Since the disease emerged in this specific geographic area, HHS was initially referred to as “Angara Disease”. The disease is caused by an avian adenovirus serotype-iv in Pakistan. This virus is responsible for development of intranuclear inclusion bodies in the cells of liver, pancreas and kidneys. The syndrome was spread in the densely populated broiler growing areas all over the country within six months. The outbreaks of HHS were also recorded in Mexico in 1989 in the high density poultry producing states. The preliminary work on the pathogenicity and vaccine development was conducted by Ahmed et al. (1989), Anjum et al. (1989), Cheema et al. (1989) and Khawaja et al. (1988). The formalinized vaccines including aqua base liver organ (ABLO) and oil base tissue culture (OBTC) were developed for the control of the syndrome (Chishti et al., 1989; Afzal and Ahmed, 1990; Ahmed et al., 1990, 1991). Both types of vaccines were reported to provide 100% protection in vaccinated flocks (Ahmed et al., 1990). The objective of this study was to evaluate the pathogenicity of the recent field isolate of avian adenovirus.

MATERIALS AND METHODS

Rearing of chicks

A total of 175, day old quality broiler chicks were purchased from

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Table 1. Angara disease vaccines prepared from chicken liver organ and used for the study.

Subgroups of chick	Vaccines used	Age at		Dose of		Route of administration		Number of chicks in each subgroup
		Vaccination day	Challenge day	Vaccination (ml)	Challenge (ml)	Vaccination	Challenge	
k	ADVac-I	20	37	0.20	2	S/C	S/C	30
l	ADVac-II	20	37	0.20	2	S/C	S/C	30
m	ADVac-III	20	37	0.20	2	S/C	S/C	30
n	ADVac-IV	20	37	0.20	2	S/C	S/C	30
o	Control (unvaccinated)	-	37	-	2	S/C	S/C	30
Total	-	-	-	-	-	-	-	150

the market and divided into two groups (A and B), each having 25 and 150 chicks, respectively. Chicks in group A (25) were reared at Poultry Research Institute (PRI), Rawalpindi, and used to determine the biological titre, that is, LD_{50} of the virus suspension while chicks in group B (150) were reared at National Veterinary Laboratories (NVL) Islamabad in poultry rearing units and used for vaccine potency testing. Feed and water were provided *ad libitum* to all the birds in both groups. Blood samples for determination of antibody titres were collected from live birds at 0, 7, 14 and 28 days of age and tissue samples were collected from chicks after postmortem of the died birds.

Procurement of Angara disease vaccines

Four commercially available Angara disease vaccines were selected and purchased from the market and subjected to sterility and potency testing. Their brand names were kept confidential and granted codes as AD Vac-I, AD Vac-II, AD Vac-III and AD Vac-IV for study purpose.

Procurement of Angara disease virus

Ten percent infectious Angara disease liver suspension was obtained from the Angara Disease Vaccine Production Laboratory, Disease Section, Poultry Research Institute, Murree Road, Rawalpindi. This virus suspension was used to determine the biological titre, that is, lethal dose for 50% and challenge protection test.

Determination of biological titre (LD_{50}) of Angara disease (AD) virus

At the age of 26 days, the chicks in group A were divided into sub groups, that is, a, b, c, d and e each having five chicks and the biological titre, that is, lethal dose for 50% (LD_{50}) of the virus suspension (10% infectious liver suspension) per 1 ml was determined. Ten fold dilution of virus suspension was prepared in phosphate buffer saline (PBS) starting from 10^{-1} to 10^{-5} dilution. 1 ml of each dilution suspension, that is, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} was injected subcutaneously into five chicks of each sub group of a, b, c, d and e, respectively. Mortality was observed up to seven days post injection. Dead and survived birds in each group were counted. LD_{50} was calculated by the method described by Reed and Muench (1938).

Potency test

At the age of 20 days, the chicks in group B were divided into five sub-groups, each having 30 chicks, named k, l, m, n, and o and vaccinated with ADVac-I, ADVac-II, ADVac-III and ADVac-IV, respectively, except chicks in subgroup o which were kept as non-vaccinated control. Route and dose of vaccination was adopted according to the manufacturer's instructions (Table 1). Vaccine potency was tested by challenge protection. Ten chicks of subgroup k, l, m, n and o were subjected to challenge protection by 2 ml of 10% infectious virus suspension. These chicks were observed for five days post challenge (Ahmed and Hasan, 2004).

Histopathology

All survived chicks of sub group k, l, m, n and o were subjected to post mortem examination after slaughtering at the age of day 42. The tissue block of liver (~ 4 to 5 mm size) representing the pathological lesion, periphery, line of demarcation and normal area were incised and obtained for routine histopathology. Each tissue block was placed in 10% formalin (8 to 12 h) for hardening and fixation/preservation, and dehydrated in series of ascending grades of alcohol, that is, 30 (2 h), 50 (2 h), 70 (2 h), 80 (1 h), 95 (2 h), 95 (1 h), 100 (1 h) and 100% (1 h). After dehydration, the tissue blocks were passed from the two fresh changes of xylene I (1 h) and xylene II (2 h) for dealcoholization or clearing. Then, blocks were placed in the melted paraffin (62°C) in the paraffin oven (6 h) for infiltration. The embedding/blocking of tissue blocks were done in paraffin wax and sectioned using microtome. Before staining, specimen tissue section was deparaffinized with xylene I (5 min) and xylene II (5 min) and rehydrated through decreasing grade of alcohol, that is, absolute (3 min), 90 (3 min), 80 (3 min), 70% (3 min), distilled water (few dips), washed in tap water and rinsed in distilled water. After that, it was stained with the routine procedure of Haematoxyline and Eosin staining method.

RESULTS AND DISCUSSION

Pathogenicity

Determination of lethal dose 50 (LD_{50}) of Angara disease virus suspension

Biological titre (LD_{50}) of the viral suspension was

Table 2. Determination of lethal dose (LD_{50}) of angara disease (AD) virus.

Sub-group	Dilution	Number of chicks inoculated	Number of dead chick	Number of survived chick	Cumulative proportion death rate	Mortality (%)
a	10^{-1}	5	5	0	12/12	100
b	10^{-2}	5	3	2	7/9	60
c	10^{-3}	5	2	3	4/7	40
d	10^{-4}	5	1	4	2/6	20
e	10^{-5}	5	1	4	1/5	20

Table 3. Gross pathological lesions recorded after challenge dose of viral suspension inoculated in chicks.

Sub-groups of chick	Vaccines administered	Postmortem lesion
k	AD Vac-I	Five birds with lesions on liver, hydronephrosis and water around heart (yellow colour).
l	AD Vac-II	Three birds with lesions on liver, hydronephrosis and water around heart (yellow colour).
m	AD Vac-III	Four birds with lesions on liver, hydronephrosis and water around heart (yellow colour).
n	AD Vac-IV	Onebird with lesions on liver, hydronephrosis and water around heart (yellow colour).
O	Non-vaccinated	Seven birds with lesions on liver, hydronephrosis and water around heart (yellow colour).

calculated, and the results are depicted in Table 2. Chicks in sub groups a, b, c, d and e (5, 3, 2, 1 and 1, respectively) died and 0, 2, 3, 4 and 4, respectively survived. The LD_{50} titre of the 10% viral suspension prepared from liver filtrate was found to be $10^{-2..5}$ per ml in 26 days old broiler chicks. Lesions recorded in the dead chicks were representative of typical field cases of Angara disease (hydropericardium syndrome). However, the result of this findings is comparatively different from previously reported findings, that is, 1×10^5 LD_{50}/ml (Ahmed et al., 1989) and 4×10^4 LD_{50}/ml (Ahmed, 1989). The reduction in virulence of virus may be due to extensive vaccination in the field as (Ahmed and Hasan, 2004) determined; the biological titre, that is, LD_{50} of the 20% viral filtrate as $1 \times 10^{1.1}$/ ml inoculated subcutaneously in 25 days old broiler chicks. Biological titre, that is, LD_{50} of viral suspension as $1 \times 10^{1.1}$/ml inoculated sub-cutaneously in 28 days old broiler chicks was reported by Ahmed (1999). However, Mashkoor et al. (1994) reported the LD_{50}/ml as 2.5×10^4 LD_{50}/ml.

Vaccine potency testing

Vaccine potency testing was performed by challenge protection test and antibody titration after vaccination of chicks of sub-groups k, l, m and n. Chicks of sub-group o were kept as non-vaccinated control. All the vaccinated (sub-groups k, l, m and n) and non-vaccinated (group o) chicks were subjected to challenge protection test 17 days post vaccination. No bird (vaccinated and/or non-vaccinated) died or showed clinical manifestation of disease up to five days post challenge. In previously reported works, the challenge protection test was performed in the chicks aged between 25 and 30 days (Ahmed, 1989; Ahmed and Hasan, 2004; Mashkoor et al., 1994), but in this study, the chicks were aged 37 days at the time of challenge protection. Moreover, the biological titre, that is, LD_{50} of the viral suspension was determined in the chicks at the age of 25 days in the study. This may be one of the reasons for failure of mortality in control sub-group o as well as in vaccinated sub-groups k, l, m and n. On the 6th day post challenge, all chicks of sub-groups k, l, m, n and o were slaughtered and subjected to post mortem. Some chicks from sub-group k, l, m, n and o showed lesions of hydropericardium, which are detailed in Table 3. These findings proved that the injected virus suspension was biologically active.

Pathological findings

On day 6th post challenge, the survived chicks of sub-groups of k, l, m, n and o were slaughtered to observe the postmortem lesions as already described. The tissues were collected for histopathological observations. The histopathological findings in liver tissue of the chicks of sub-group k, l, m, n and o are as follows:

Table 4. Number of inclusion bodies histopathologically recorded in different sub-groups of chicks post challenge.

Treatment group	Inclusion bodies per high field (PHF)	Mean number of inclusion body
	90	
	77	
k	67	77
	100	
	51	
	66	
	99	
l	33	66
	44	
	88	
	68	
	60	
m	52	60
	90	
	30	
	52	
	72	
n	32	52
	27	
	79	
	135	
	120	
O	105	120
	100	
	140	

Histopathological findings

Hepatocytes of liver tissue sub-group k showed basophilic intranuclear inclusion bodies of varied sizes and numbers depending upon the intensity of inclusion bodies produced in hepatocytes. Hepatocytes showed varied stages of necrosis (N), cloudy swelling (S) and liquefactive necrosis. Necrotic cells showed free nuclei (F), cytoplasmic blabing, and fatty degeneration as compared with the number of inclusion bodies present in hepatocytes of this sub-group as shown in Table 4 and Figure 1.

The number of inclusion bodies produced in sub-group I is shown in Table 4. However, the main pathological changes observed were basophilic intranuclear inclusion bodies of varied sizes and numbers depending upon the intensity of inclusion bodies produced in hepatocytes. Hepatocytes showed varied stages of necrosis (N), cloudy swelling (S) and liquefactive necrosis. Necrotic cells showed free nuclei (F), cytoplasmic blabing and fatty degeneration (Table 4 and Figure 2).

The number of inclusion bodies produced in sub-group m is shown in Table 4 and Figure 3. The main pathological changes observed were basophilic intranuclear inclusion bodies of varied sizes and numbers depending upon the intensity of inclusion bodies produced in hepatocytes. Hepatocytes showed varied stages of necrosis (N), cloudy swelling (S) and liquefactive necrosis. Necrotic cells showed free nuclei (F), cytoplasmic blabing and fatty degeneration.

The number of inclusion bodies produced in sub-group n is shown in Table 4. The main pathological changes observed were basophilic intranuclear inclusion bodies of varied sizes and numbers depending upon the intensity of inclusion bodies produced in hepatocytes. Hepatocytes showed varying stages of necrosis (N) and cloudy

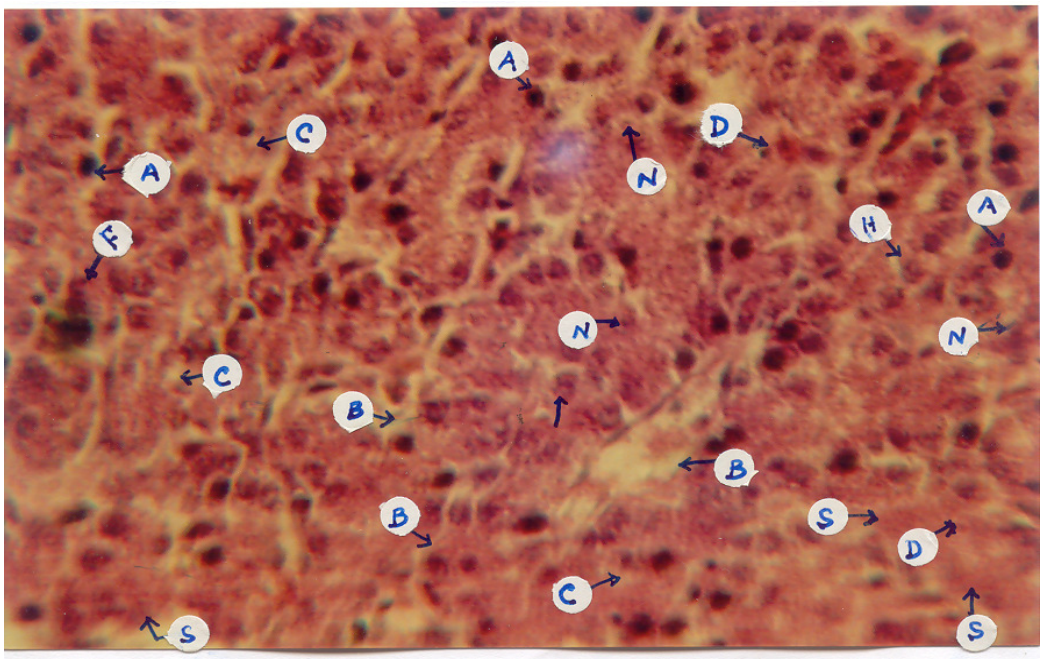

Figure 1. Histopathological findings of the vaccinated subgroup-k. Liver hepatocytes showing basophilic intranuclear inclusion bodies (A), of experimental inoculated avian adeno virus in the vaccinated 37 days old commercial broilers. Various sizes of inclusion bodies were noted depending on intensity of inclusion bodies produced, sinusoidal space dilated (B), cytoplasmic blabing (C), fatty degeneration (D), hepatocytes showing various stages of necrosis (N), cloudy swelling (S), liquefactive necrosis. Necrosed cells showing free nuclei (F) (Stained with H&E stain 6902x).

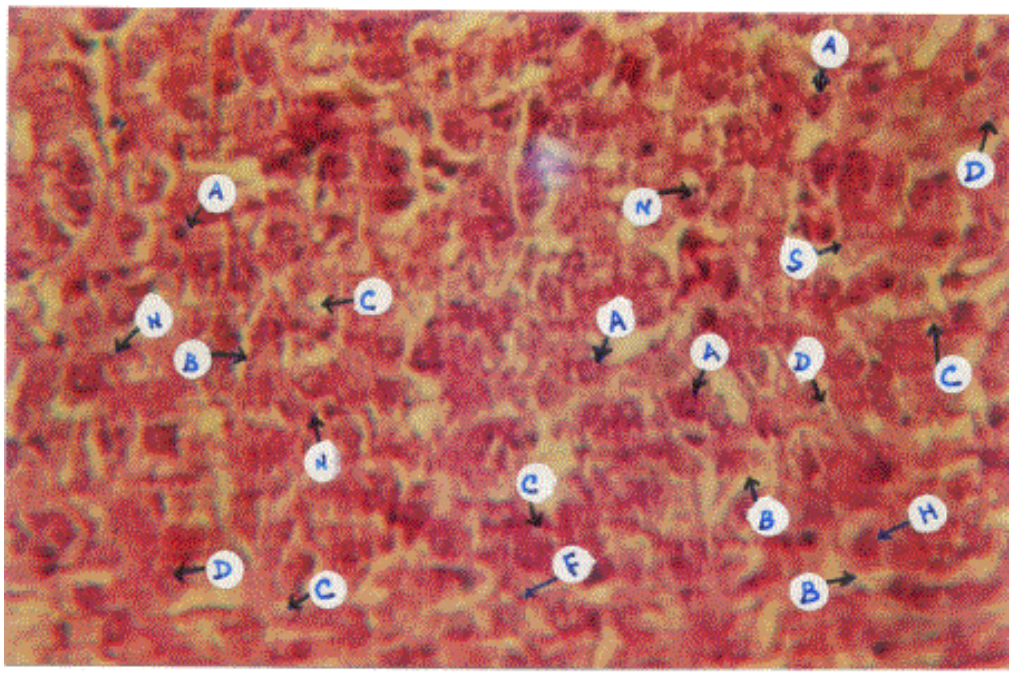

Figure 2. Histopathological findings of the vaccinated subgroup-l. Liver hepatocytes showing basophilic intranuclear inclusion bodies (A), of experimental inoculated avian adeno virus in the vaccinated 37 days old commercial broilers. Various sizes of inclusion bodies were noted depending on intensity of inclusion bodies produced, sinusoidal space dilated (B), cytoplasmic blabing (C), fatty degeneration (D), hepatocytes showing various stages of necrosis (N), cloudy swelling (S), liquefactive necrosis. Necrosed cells showing free nuclei (F) (stained with H&E stain 6902x).

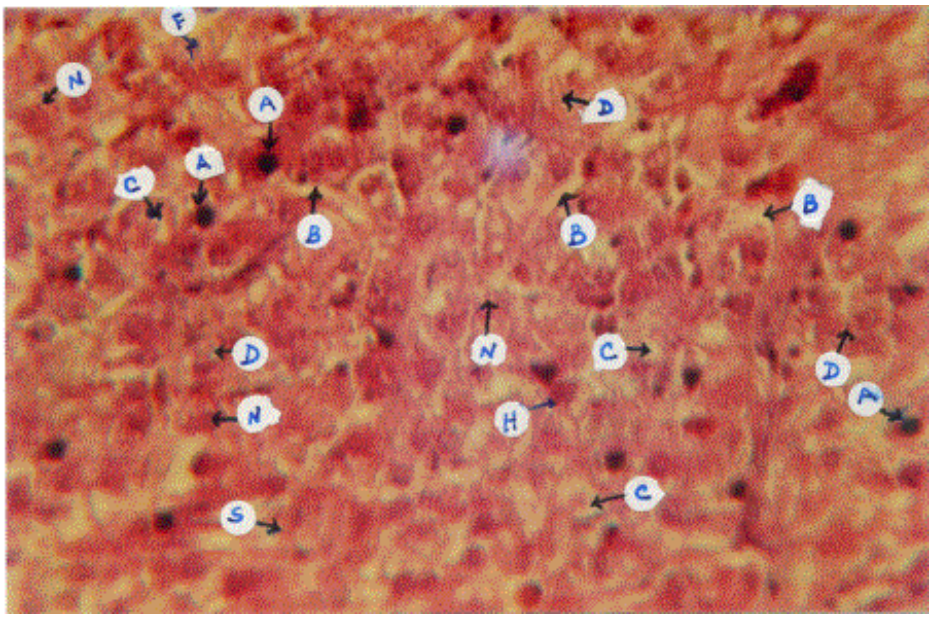

Figure 3. Histopathological findings of the vaccinated subgroup-m. Liver hepatocytes showing basophilic intranuclear inclusion bodies (A), of experimental inoculated avian adeno virus in vaccinated 37 days old commercial broilers. Various sizes of inclusion bodies were noted depending on intensity of inclusion bodies produced, sinusoidal space dilated (B), cytoplasmic blabing (C), fatty degeneration (D), hepatocytes showing various stages of necrosis (N), cloudy swelling (S), liquefactive necrosis. Necrosed cells showing free nuclei (F) (stained with H&E stain 6902x).

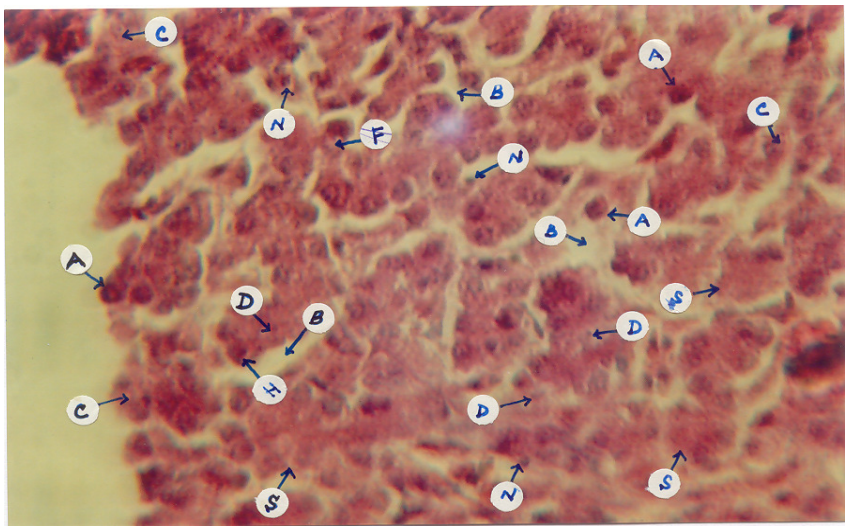

Figure 4. Histopathological findings of vaccinated sub-group n. Liver hepatocytes showing basophilic intranuclear inclusion bodies (A), of experimental inoculated avian adeno virus in vaccinated 37 days old commercial broilers. Various sizes of inclusion bodies were noted depending on intensity of inclusion bodies produced, sinusoidal space dilated (B), cytoplasmic blabing (C), fatty degeneration (D), hepatocytes showing various stages of necrosis (N), cloudy swelling (S), liquefactive necrosis. Necrosed cells showing free nuclei (F) (Stained with H&E stain 6902x).

swelling (S) (Figure 4).

Hepatocytes in the control sub-group (o) showed basophilic intranuclear bodies that is, highly significant when compared with other treatment groups (Table 4). The main pathological changes observed were basophilic intranuclear inclusion bodies of varied sizes and numbers

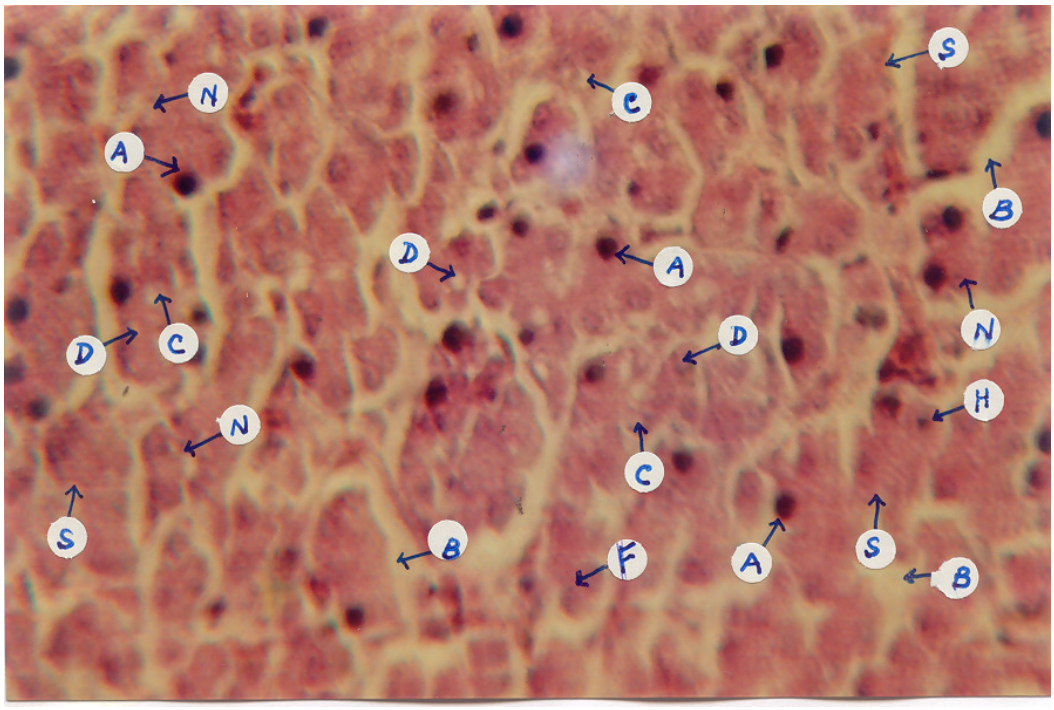

Figure 5. Histopathological findings of unvaccinated sub-group o. Liver hepatocytes showing basophilic intranuclear inclusion bodies (A), of experimental inoculated avian adeno virus in vaccinated 37 days old commercial broilers. Various sizes of inclusion bodies were noted depending on intensity of inclusion bodies produced, sinusoidal space dilated (B), cytoplasmic blabing (C), fatty degeneration (D), hepatocytes showing various stages of necrosis (N), cloudy swelling (S), liquefactive necrosis. Necrosed cells showing free nuclei (F) (stained with H&E stain 6902x).

depending upon the intensity of inclusion bodies produced in hepatocytes. Hepatocytes showed varying stages of necrosis (N), cloudy swelling (S) and liquefactive necrosis. Necrotic cells showed free nuclei (F), cytoplasmic blabing and fatty degeneration (Figure 5).

During the study, the histopathological examination of the liver tissues of the sub-group k, l, m, n and o showed the basophilic intranuclear inclusion bodies, cloudy swelling, fatty degeneration, dilated sinusoidal spaces, necrosis, cytoplasmic blabing and liquefactive necrosis. These findings are in line with the previously reported findings of Swati et al. (2000), Fadly and Winterfield (1973) and Cubillos et al. (1986) who experimentally introduced the infectious agent of hydropericardium in 28 day old broiler chicks and confirmed the presence of Fowl Adeno Virus (FAV) by observing basophilic intra-nuclear inclusion bodies in the hepatocytes of all the dead birds. However, similar study was also conducted by Fadly and Winterfield (1973) who reported that the inclusion body of hepatitis and anemia syndrome were caused by adenoviruses and these diseases were widespread in chicks. The farmers were advised to use quality vaccines against Angara disease as the vaccines can provide 100% protection against the disease if they are given at the proper age.

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