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Article

Identification of Avian Leukosis Virus from layer chicken by serological test and embryo inoculation technique

Mst. Deloara Begum[1], Md. Mostafizer Rahman[1], Mir Rowshan Akter[1], Md. Atiqul Haque[1]*, Md. Khalesur Rahman[1], Md. Mobarak Hossain[1] and Md. Nurul Amin[2]

[1]Department of Microbiology, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh
[2]Department of General Animal Science and Nutrition, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh

*Corresponding Author: Dr. Md. Atiqul Haque, Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh. E-mail: atique@hstu.ac.bd

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Abstract: The present study was designed with a view to identify the Avian Leukosis Virus (ALV) by direct Enzyme Linked Immunosorbent Assay (ELISA) as well as to know the prevalence of ALV in different age groups of layer birds at Dinajpur district of Bangladesh. The birds were categorized into three groups, namely group A (brooder) included bird aged 13 days, group B (grower) included bird aged 16 weeks and lastly group C (layer) included bird aged 32 weeks. In this study a total of 92 cloacal swab samples were examined and 13 positive cases of ALV was found among which 5, 4 and 4 positive samples were in grower, brooding and layer chickens respectively. This study showed that ALV positive cases were 14.13% in layer birds whereas 14.5% positive cases in Sonali chicken and 13.3% in case of Hy-Line brown chicken. The inoculation of cloacal swab was done into five 10 days old embryonated chicken eggs through yolk sac route. After 5 days of inoculation death of the all embryos with haemorrhage was observed indicating the prevalence of Avian Leukosis Virus in the study area of Bangladesh. Since Avian Leukosis Virus transmitted both horizontally and vertically therefore measures to prevent spread are more demanding.

Keywords: Avian Leukosis Virus (ALV); direct enzyme linked immunosorbent assay (ELISA); p27 antigen, layer birds

1. Introduction

Poultry farming is emerging as a strong agro-based industry from the backyard poultry rearing system to commercial intensive rearing systems during the last two decades in Bangladesh. This rapid growth supplement their income with the fast development of poultry industry, the occurrence of diseases has increased many folds which remain the major problem affecting its economy (Uddin *et al*, 2011). Poultry industry is a rising sector in Bangladesh. Poultry eggs and meat provide approximately 38% of total animal protein in the Bangladesh (FAO, 1999). But, the advancement of poultry industry is being hampered seriously by some fatal infectious and noninfectious diseases.

Viral diseases have been reported to be the major problem in poultry industry worldwide including Bangladesh (Samad, 2000). Avian leukosis virus (ALV) infection has been regarded as one of the important causes of economic damage in the poultry industry (Spencer, 1984). ALV cause neoplasms in chickens, commonly known as lymphoid leukosis (big liver disease, lymphatic leukosis, visceral lymphoma, lymphocytoma, lymphomatosis, and visceral lymphomatosis) (Payne and Fadly, 1997). The leukosis/sarcoma group of diseases comprises a variety of transmissible benign and malignant neoplasms of chickens caused by members of avian retroviruses

belonging to the family Retrovirida (Coffin, 1992). There are six subgroups- A, B, C, D, E and J (Calnek, 1968). ALV spread by either vertical or horizontal transmission (Smith and Fadly, 1988).

Exogenous type of ALV has been shown to cause several neoplastic diseases in infected chickens (Coffin *et al*, 1983). Affected birds showed inappetence, abnormal feathering, paleness of comb and wattles, loss of weight, decrease in egg production, depression, paralysis and death (Latif and Khalafalla, 2005).

There is no vaccines available and no specific treatment of avian leukosis. Control has been based on the elimination of positive chickens identified by a specific test, management practices and biosecurity program in hatchery and farm, aiming to reduce the spread of ALV and clinical disease (Silva, 1999). So rapid and confirmatory diagnosis is essential to overcome the avian leukosis problem in eradication from breeding stock has been accomplished by a variety of method (Spencer, 1984) including virus isolation, complement fixation and antigen capture Enzyme Linked Immuno Sorbent Assays (AC-ELISA) (Crittenden and Smith, 1984). More recently, molecular based diagnostic methods including in situ hybridization (Arshad *et al*., 1998) and the polymerase chain reaction (PCR) have been developed for the detection exogenous ALV from clinical samples (Garcia *et al*, 2003). The incidence of lymphoproliferative disease in Bangladesh is 2.77% and higher in aged layers (5.04%). The incidence of ALV in Bangladesh is 9.4% and 12.8% of semi-scavenging chickens ageing below or equal and over 60 days were categorized for harboring the antigen in soft organ but not in eggs (Biswas *et al.*, 2005).

Occurrence of visceral form Avian leukosis was found to be significant among the exotic breeds of poultry raised in poultry farm, but the local indigenous birds seemed to be resistant to the disease (Uddin *et al.*,1972). Diagnosis of lymphoid leukosis in our country is made by only on gross neoplastic changes recorded during postmortem examinations and such changes sometime are confused with other lymphoproliferative disease like Marek's disease (Payne and Venugopal 2000).

The use of ELISA for the detection of group-specific antigens of avian sarcoma and Leucosis viruses have been described by Smith *et al.* (1979) and Clark and Dougherty (1980). Antigen ELISA has been applied for detection of acute infection and in old laying hens shedding of ALV in egg albumen, vaginal and cloacal swabs were more rapid and useful for eradication programmes (Spencer *et al.*, 1977) and in identifying classes of hens which do not congenitally transmit ALV (Payne *et al.*, 1991).

In this study, a direct ELISA was used for detection of the ALV virus in congenitally infected birds (shedders) in their cloacal swabs. In view of above consideration, the present research program was undertaken with the following objectives:

a) To detect the avian leukosis virus from different age group of laying birds.
b) To detect the prevalence of avian leukosis virus among the different flocks of laying birds.

2. Materials and Methods

The research work was conducted in the Virology Laboratory of the Department of Microbiology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh during the period of July to December, 2013.

2.1. Collection and preparation of samples

For detection of viral antigen, a total of 92 cloacal samples were collected from the selected layer bird of different small scale layer farm having average population 1000 that were situated at Sadar upazilla under Dinajpur district of Bangladesh. The birds were categorized into three age groups. Group A included birds aged 13 days (brooding), group B included aged 16 weeks (grower), group C included aged 32 weeks (layer). At brooding stage 31 samples and at grower stage 31 samples were collected both of them from Sonali chicken whereas at layer stage 30 samples were collected which from Hy-line brown chicken. The cloacal swab samples were collected aseptically from chicken by previously sterilized cotton bud and were harvested in clean sterilized Eppendorf tube containing 1 ml of PBS and stored at -20°C for performing the direct ELISA.

2.2. Antigen detection by direct ELISA

The ALV-Ag (p27) test kit manufactured by Biochek, Holland was used for the estimation of amount of antigen. The direct enzyme-linked immunosorbent assay was performed according to the manufacturer's instruction using ALV pre coated plates and pre-diluted, ready to use reagents and buffer. To make substrate reagent, 1 tablet was added to 5.5 ml substrate buffer and was allowed to mix for 3 minutes or until fully dissolved. The prepared reagent was made on day of use. One wash buffer sachet was emptied and mixed into one liter of distilled water and allowed to dissolve fully by mixing. All other kit components were ready to use but were allowed to adjust the room temperature. Cloacal swab sample was taken into 1 ml of PBS buffer and

shake well before testing (freezing and thawing expose more antigen). A fresh pipette tip was used for each separate sample.

Anti-p27 coated plate was removed from sealed bag and recorded location of samples on template. 50 µl of test sample was added to each microtitre wells. 50 µl of negative control was added into wells A2 and B2. 50 µl of positive control was added into wells C2 and D2. The plate was covered with lid and incubated at room temperature (22-27°C) for 60 minutes. The contents of wells was aspirated and washed 4 times with wash buffer (350µl per well). The plate was inverted and tapped firmly on absorbent paper. Then 50 µl of Conjugate reagent was added into the appropriate wells. Then the plate was covered with lid and incubated at room temperature (22-27°C) for 60 minutes. The procedure was repeated as in previous. Then 50 µl of Substrate reagent was added into the appropriate wells and the plate was covered with lid and incubated at room temperature (22-27°C) for 30 minutes. Then 50 µl of Stop solution was added to appropriate wells to stop reaction. The ELISA plate was read by the microtiter plate reader in the Virology Laboratory, Department of Microbiology, HSTU, Dinajpur and recorded the absorbance of controls and samples by reading at 405 nm. For the test result to be valid the mean negative control absorbance should read below 0.15 and the difference between the mean negative control and the mean positive control should be greater than 0.5. The p27 positive control has been carefully standardized to represent significant amounts of p27 antigen found in chicken sample. The relative amounts of p27 in chicken samples can then be calculated by reference to the positive control. This relationship is expressed as S/P ratio (Sample to Positive Ratio). Samples with an S/P of 0.3 or greater contain p27 antigen were considered positive.

Calculation of S/P ratio:

$$\frac{\text{Mean of Test Sample - Mean of negative control}}{\text{Mean of Positive control - Mean of negative control}} = \text{S/P}$$

2.3. Inoculation of virus in chicken embryo

Inoculation of collected sample was conducted in ten 10-day-old embryonated chicken eggs. Inoculum was taken from the collected samples. Ten 10 day-old healthy embryonated eggs (moving embryos, intact blood vessels) were selected by candling. A point of inoculation on a lateral side of the egg (at the site of embryo development but free from large blood vessel) and the air sac was marked with a pencil during candling. The top of the air sac and the marked point of inoculation were disinfected with 70% alcohol. With the help of an electric dentist's drill, a hole was made at the top of the air sac. By using tuberculin syringe 0.2 cc inoculation was done by inserting the needle straight down into the yolk sac until its point is one-third to one-half the depth of the egg. The hole was sealed with molten paraffin. The eggs were incubated at 37°C up to 6 days post inoculation (d.p.i.) and examined two times daily by candling. Mortality was recorded. Five 10 day-old developing chicken embryos were kept as uninoculated control.

The embryos, which died after 5 days as well as the live embryos at 5 d.p.i. were chilled at 4°C for 1-2 hours to prevent the bleeding during the collection of samples. The embryos were open aseptically and examined for gross lesions.

2.4. Data analysis by software

As in each antibody coated plate contain 2 positive controls, 2 negative control, 92 cloacal swab samples, a total 96 well was used, the optical density (OD) value also inputted in the software layout respectively, then the result generate automatically.

3. Results and Discussion

3.1. Results of direct ELISA from the layer birds of different aged group

Collectively, 14.13% of the cloacal samples were positive for direct ELISA (Table 1).

3.2. Detection of antigen titer level from Group A

The results of the cloacal swab collected from 31 brooding chicken showed that 4 samples were positive in direct ELISA. The detailed results are shown in Table 2.

3.3. Detection of antigen titer level from Group B

The results of the cloacal swab collected from 31 grower chicken showed that 5 samples were positive in direct ELISA. The detailed results are shown in Table 3.

Table 1. Results of direct ELISA from the layer birds of different aged group.

SL. No.	Groups	Age of bird	No. of samples	No. of Positive cases	% of positive cases (total)
01	A	13 days	31	4	
02	B	16 weeks	31	5	14.13%
03	C	32 weeks	30	4	
	Total		92	13	

Table 2. Result of direct ELISA from the samples of Group A (brooding chicken).

Sample No.	Raw OD	S/P Ratio	Result
01	0.157	0.05	NEG -
02	0.177	0.09	NEG -
03	0.063	0.00	NEG -
04	0.142	0.02	NEG -
05	0.163	0.07	NEG -
06	0.159	0.06	NEG -
07	0.188	0.12	NEG -
08	0.177	0.09	NEG -
09	0.158	0.06	NEG -
10	0.163	0.07	NEG -
11	0.153	0.05	NEG -
12	0.108	0.00	NEG -
13	0.143	0.03	NEG -
14	0.153	0.05	NEG -
15	0.148	0.04	NEG -
16	0.170	0.08	NEG -
17	0.143	0.03	NEG -
18	0.163	0.07	NEG -
19	0.159	0.06	NEG -
20	0.092	0.00	NEG -
21	0.140	0.02	NEG -
22	0.188	0.12	NEG -
23	**0.468**	**0.68**	**POS +**
24	0.152	0.04	NEG -
25	**0.313**	**0.37**	**POS +**
26	**0.507**	**0.75**	**POS +**
27	0.170	0.08	NEG -
28	0.106	0.00	NEG -
29	0.156	0.05	NEG -
30	**0.352**	**0.44**	**POS +**
31	0.198	O.14	NEG -

Legend:

S/P ratio	Antigen status
0.3 or greater	Positive
Less than 0.3	Negative

Table 3. Result of direct ELISA from the samples of Group B (grower chicken).

Sample No.	Raw OD	S/P Ratio	Result
01	0.149	0.04	NEG -
02	0.141	0.02	NEG -
03	0.163	0.07	NEG -
04	0.156	0.05	NEG -
05	0.254	0.25	NEG -
06	**0.511**	**0.76**	**POS +**
07	0.150	0.04	NEG -
08	0.146	0.03	NEG -
09	0.261	0.26	NEG -
10	0.164	0.07	NEG -
11	0.188	0.12	NEG -
12	0.159	0.06	NEG -
13	0.143	0.03	NEG -
14	0.148	0.04	NEG -
15	0.151	0.04	NEG -
16	**0.382**	**0.50**	**POS +**
17	0.210	0.16	NEG -
18	0.142	0.02	NEG -
19	0.147	0.03	NEG -
20	0.157	0.05	NEG -
21	0.199	0.14	NEG -
22	0.148	0.04	NEG -
23	0.152	0.04	NEG -
24	0.156	0.05	NEG -
25	0.163	0.07	NEG -
26	**0.579**	**0.90**	**POS +**
27	0.252	0.24	NEG -
28	**0.378**	**0.50**	**POS +**
29	0.181	0.10	NEG -
30	**0.378**	**0.50**	**POS +**
31	0.140	0.02	NEG -

Legend;

S/P ratio	**Antigen status**
0.3 or greater	Positive
Less than 0.3	Negative

Table 4. Result of direct ELISA from the samples of Group C (layer chicken).

Sample No.	Raw OD	S/P Ratio	Result
01	0.171	0.08	NEG -
02	0.207	0.15	NEG -
03	0.168	0.08	NEG -
04	0.155	0.05	NEG -
05	0.155	0.05	NEG -
06	0.151	0.04	NEG -
07	0.142	0.02	NEG -
08	0.151	0.04	NEG -
09	0.150	0.04	NEG -
10	0.159	0.06	NEG -
11	0.152	0.04	NEG -
12	0.146	0.03	NEG -
13	0.154	0.05	NEG -
14	0.151	0.04	NEG -
15	0.159	0.06	NEG -
16	0.144	0.03	NEG -
17	0.208	0.16	NEG -
18	**0.309**	**0.36**	**POS +**
19	0.146	0.03	NEG -
20	0.149	0.04	NEG -
21	0.205	0.15	NEG -
22	0.183	0.11	NEG -
23	**0.33**	**0.41**	**POS +**
24	**0.386**	**0.51**	**POS +**
25	0.182	0.10	NEG -
26	**0.461**	**0.66**	**POS +**
27	0.169	0.08	NEG -
28	0.147	0.03	NEG -
29	0.151	0.04	NEG -
30	0.153	0.05	NEG -

Legend:

S/P ratio	Antigen status
0.3 or greater	Positive
Less than 0.3	Negative

Table 5. Comparison of test reports of ELISA among brooding, grower and layer chicken.

SL. No.	Groups	No. of samples	No. of Positive cases	% of positive cases
01	Brooding	31	4	12.9
02	Grower	31	5	16.1
03	Layer	30	4	13.3

Table 6. Comparison of test reports of ELISA between Sonali and Hy-Line brown chicken.

SL. No.	Name of the Breed	No. of samples	No. of Positive cases	% of positive cases
01	Sonali	62	9	14.5
02	Hy-Line brown	30	4	13.3

3.4. Detection of antigen titer level from Group C

The results of the cloacal swab collected from 30 layer chicken showed that 4 samples were positive in direct ELISA.The detailed results are shown in Table 4.

3.5. Comparative study of ELISA among brooding, grower and layer chicken

In this study to assess the influence of age on the occurrence of ALV, birds were classified into three groups. In brooding, grower and layer chicken ALV positive was recorded 4, 5 and 4 respectively. The detailed results are shown in Table 5.

3.6. Comparative study Of ELISA between Sonali and Hyline brown chicken

In this study samples collected from Sonali and Hyline brown chicken showed that ALV positive cases were 9 and 4 respectively. The detailed results are shown in Table 6.

3.7. Propagation of virus in chicken embryo

A total number of five 10-day-old embryonated indigenous hen's egg were used to propagate the ALV with 0.2 cc collected cloacal swab sample through yolk sac route. Virus propagation was successful in the embryonated indigenous hen's eggs. After 5 days mortality and gross pathological lesions of the inoculated embryos were studied in the present experiment. It was observed that collected cloacal samples caused mortality of the chicken embryos due to hemorrhage.

The ELISA procedure proved as a highly efficient means for the detection of ALV antigen in a variety of samples (Clark and Dougherty, 1980). The present study further revealed that a total 14.13% collected samples were positive for ARV related to the findings of Bhattachajee *et al*, 1996 (6.92%), Kamal and Hossain, 1992 (1.93%) and Talha *et al*, 2001 (1.57%) which observed on layer chicken. Avian leukosis viruses are responsible for serious economic losses in the poultry industry due to reduced growth rate, decreased egg production and increased sporadic tumor-induced mortality (Rajabzadeh *et al*, 2010). ALV infection may be spread either vertically or horizontally (Rubin *et al*, 1962). Congenitally infected birds shed virus copiously, and hens regularly transmit infection to their progeny through the eggs (Weyl and Dougherty, 1977). ALV is a neoplastic disease characterized by persistent low mortality and neoplasm of the internal organ especially the liver, spleen and kidney in a flock (Mosleuddin *et al*, 1972). In this study it was observed that ALV positive cases were 12.9%, 16.1% and 13.3% in grower, brooder and layer bird respectively which supports the earlier report of Rahman and Samad (2005) whose findings were 66.67% and 33.33% in pullet and layer birds respectively. On the other hand, ALV positive cases were 14.5% and 13.3% between Sonali and Hy-Line chicken respectively. This report supports the earlier study of Hasan and Mohamed (2005) who reported that the local type birds showed highest percentage of positive ALV antigen 57% (24/42) compared to 36% (35/97) for foreign breeds. This is may be due to the role of local type chickens that do not show clinical signs and may transmit infection to foreign chickens. In most cases, mortality is often not a serious problem and, even if birds are not sick enough to die, the disease may affect bird's immune system, productivity, sexual maturity, infertility and hatchability (Gavora et al., 1980).

Embryonated chicken eggs inoculated with cloacal swab sample through yolk sac route caused mortality due to hemorrhage. This result supports the earlier report of Payne and Fadly (1997) who reported embryos inoculated by yolk sac or intravenous route with sarcoma viruses usually die from sarcoma and hemorrhage within 4 to 5 days.

4. Conclusions

From the findings of the present study ALV antigens were successfully detected from the field samples through commercially ALV-Ag (p27) test kit in the research area of Bangladesh. ALV infection may be associated with increased mortality from causes other than neoplastic diseases and with physiological dysfunctions including delayed maturity and reduced fertility. ALV positive cases may be due to the role of local type chicken that do not show clinical signs and may transmit infection to the foreign birds. This also may be due to the failure of hatchery to maintain the proper biosecurity measures and standard hygienic production procedure. Since intermittent congenital virus excretion is a common feature of avian leukosis (lymphoid leukosis) infection, frequent testing is necessary for ALV eradication program. ELISA technique is the simplest and easier to perform and have been proved useful. This study revealed that cloacal swab samples were more rapid for identifying and eradication program of ALV cases. Further investigation on detailed molecular studies such as DNA sequencing, analysis of PCR and RT-PCR products on local and wild birds could have an important role in the disease transmission and outbreak of ALV infection.

Conflict of interest

None to declare.

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