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Research Article

Detection of Avian Leukosis Viruses in commercial poultry flocks by Immunohistochemistry and PCR

G. Sathish[1], M. Parthiban[1], Kurunchi C. Divya[2] and, K.Kumanan[1] and Stalin Vedamanickam[1]

[1]Department of Animal Biotechnology, Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai, Tamil Nadu, India
[2]Department of Biotechnology, Faculty of Allied Health Sciences, Chettinad Academy of Research and Education.
*Corresponding author

Abstract

Avian Leukosis Viruses (ALVs) are placed in the *Alpharetrovirus* genus of the family *Retroviridae* and divided into six subgroups based on viral envelope antigens. Subgroups A, B, C, D and J are classified as exogenous viruses and E is endogenous. ALV A-D can induce B cell lymphomas, lymphoid leukosis in susceptible chickens and ALV-J causes myeloid leukosis. A total of 203 suspected tissue samples viz liver, spleen were collected from chicken at Namakkal, Tamil Nadu and initially screened for histopathological examination by using paraffin fixed sections. Based on histopathology results, the samples were further analyzed using immunohistochemistry using polyclonal serum. Avian leukosis viruses (pol flanked region – 295-326bp) were checked by using the template DNA extracted from immunohistochemistry positive tissue sections and tissues. Sensitivity of these primers was checked by using different dilutions of positive tissue DNA samples. The purified PCR products of ALV were sequenced, the phylogenetic tree was constructed using the *pol flanked sequence* (268bp) sequence of reference strains present in the NCBI database and Tamil Nadu field strain. The positive samples were used for ALV subgroup specific PCR for identification of ALV subgroups. Twenty six samples showed positive amplicon size of 2.4kbp by using primers for ALV all subgroups. Out of 26 samples, 25 samples showed amplicon size of 1.25kbp for ALV subgroup E and one sample showed amplicon size of 1.1kbp for ALV subgroups B and D. The PCR positive samples were used as inoculums for isolation in Chicken embryo fibroblast culture (CEF). CEF infected with 5^{th} passage ALV produced no cytopathic effect after 5^{th} day of infection. Hence, presence of virus in the infected culture was confirmed by Immunofluorescence using virus specific serum (polyclonal serum against RSV-A, US biologicals) of infected culture fluid in comparison with uninfected culture fluid clearly indicated the multiplication of virus in cell culture system.

Keywords: Avian Leukosis Viruses, *Alpharetrovirus,* histopathological examination, *pol flanked sequence* .

Introduction

Ellerman and Bang (1) in Copenhagen and Rous (1910) in New York, showed thatleukemia (leukosis) and sarcomas in the domestic fowl have a viral etiology. Many medical workers worked with Rous sarcoma virus (RSV) and the avian leukosis viruses (ALV) as model systems with which to study the role of viruses in the causation of tumors. These viruses in chickens are grouped into the *Alpharetrovirus* genus and are classified into five pathogenic subgroups: A, B, C, D, E and most recently J (2). Avian leukosis viruses, called also as leukosis/sarcoma viruses, the L/S group, cause a group of leukoses, sarcomas and related neoplasms (3). ALV is a lymphoproliferative disease of chickens affecting primarily the bursa of Fabricius and visceral organs, transforming the B-type lymphocytes (4). However, with the recognition of subgroup J ALV infection, myelocytomatosis was frequently diagnosed during the 1990s, particularly in

meat type chicken breeders, as ALV-J transforms myeloid cells (5). Dougherty *et al.* (6) explained the immunohistochemistry using peroxidase-labelled antibody to study the distribution of virus antigens in chicken cells infected with two subgroups ALV-A and B. Later Arshad *et al*. (7) designed *in situ* hybridization for detecting the virus-specific *gag* and *envelope* sequences as an adjunct to the immunochemical detection of viral proteins in the tissues of infected birds.

The latest and most rapidly advancing form of diagnosing ALV infection involves molecular technology. Unlike the methods relying on the biological amplification of ALV, this technology allows the rapid detection of ALV during early stages of infection (8). The use of PCR-based diagnostic technique for the detection ALV has been investigated (9; 10; 11; 12). Primers developed for these assays have been targeted towards the sequences of the *env* gene or LTR regions of the ALV genome (11 and 12). Poultry oncogenic viruses MDV and ALV, from different parts of India were reported namely Punjab, Haryana, Delhi, Tamil Nadu (13), Assam, Andhra pradhesh, Karnataka and Gujarat. However, there is not much molecular screening about the prevalence of ALV in India. Here in this study immunohistochemistry and PCR based molecular screening of tissue samples for ALV was done.

Materials and Methods

Sample Collection

A total of 203 suspected tissue samples viz liver, spleen were collected from chicken at Namakkal, Tamil Nadu, India and the same tissue pieces were stored in formalin for histopathological studies.

Histopathological examination

All the samples were initially screened for histopathological examination by using paraffin fixed sections.

Immunohistochemistry

Based on histopathology results, the samples were further analyzed using immunohistochemistry using polyclonal antibodies against ALV (US biologicals).

DNA isolation

All the suspected tissue samples were subjected to DNA extraction using previously described method (14). Due to absence of positive control, immunohistochemistry positive tissue section was utilized as positive and DNA extraction from this section was done as per the method described by coombs *et al.* (15).

Polymerase chain reaction

The sequences of the oligonucleotide primers used in this study were derived from the publishd sequences (5) and used according to smith et al (11). Primer H5 was designed that flanked the 3’ region of *Pol* gene, which is conserved across several ALV groups. Primer AD1when used with H5 is expected to give 292 – 326 bp, which is conserved among ALV subgroups (A- B- C- D- E). The amplification of the specific target DNA is carried out by Touchdown PCR technique for this process. It involves the use of an annealing temperature that is higher than the target optimum in early PCR cycles.

Sequencing

The purified PCR products were subjected to sequencing and the sequencing datas were analyzed using NCBI Blast analysis.

Phylogenetic analysis

The forward and reverse sequences obtained from cycle sequencing were aligned as single PCR product sequence using GENE TOOL 1.0 software. The similar sequences of different strains were obtained from NCBI database and used for phylogenetic tree construction using MEGA 5.0 software.

Subgroup specific PCR
Primer Selection for PCR

PCR primers are taken from published sequences (16).

Virus subgroup	Primer	Target sequence	Size
All ALV subgroups	Fp:5’CGAGAGTGGCTCGCGAGATGG3’ Rp:5’ACACTACATTTCCCCCTCCCTAT3’	*pol+gp37*	2.4kbp
ALV-J	Fp:5’CTTGCTGCCATCGAGAGGTTACT3’ Rp:5’AGTTGTCAGGGAATCGAC3’	*gp85+LTR*	2.3kbp

Virus subgroup	Primer	Target sequence	Size
ALV- A	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'CCCATTTGCCTCCTCTCCTTGTA3'	*pol+gp85*	1.3kbp
ALV- B and D	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'AGCCGGACTATCGTATGGGGTAA3'	*pol+gp85*	1.1kbp
ALV- C	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'CCCATATACCTCCTTTTCCTCTG3'	*pol+gp85*	1.4kbp
ALV- E	Fp:5'CGAGAGTGGCTCGCGAGATGG3' Rp:5'GGCCCCACCCGTAGACACCACTT3'	*pol+gp85*	1.25kbp

Isolation and identification of virus

Chicken embryo fibroblast culture (CEF) was used for isolation of ALV. The PCR positive samples were used as inoculum. The virus in cell culture was identified by ELISA using virus specific polyclonal antibodies (US biologicals).

Results

Gross pathology

In lymphoid leukosis, liver showed diffused enlargement, grayish white in colour, soft and friable. In some cases discrete variable sized grayish white nodules were distributed in liver parenchyma. Spleen showed two to three times enlargement than normal and also grayish white, diffused or nodular lesions. Bursa of Fabricius six times larger than normal was showing multiple grayish white nodules (Fig 1).

Histopathology

Avian leukosis virus affected tissue section showed homogenous monomorphic lymphoid infiltration (Fig 2).

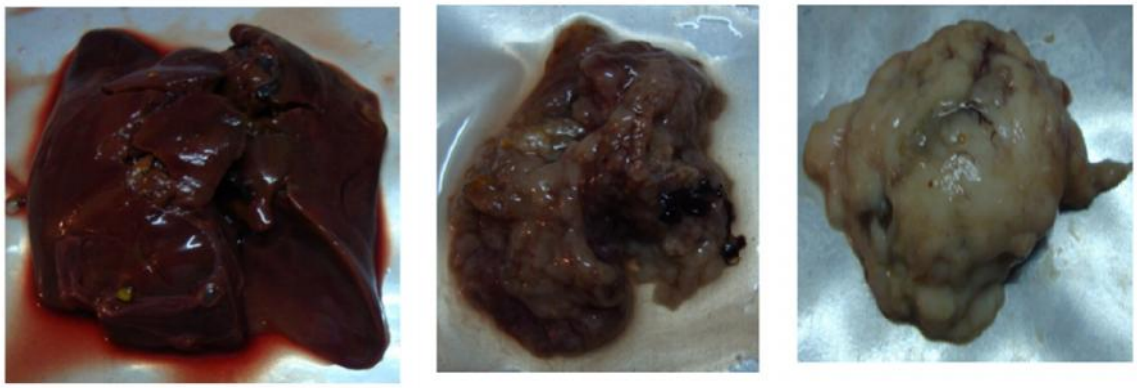


Fig. 1: Susceptible samples (liver- diffused enlargement and bursa - six times larger than normal) were collected from chicken and turkey farms.

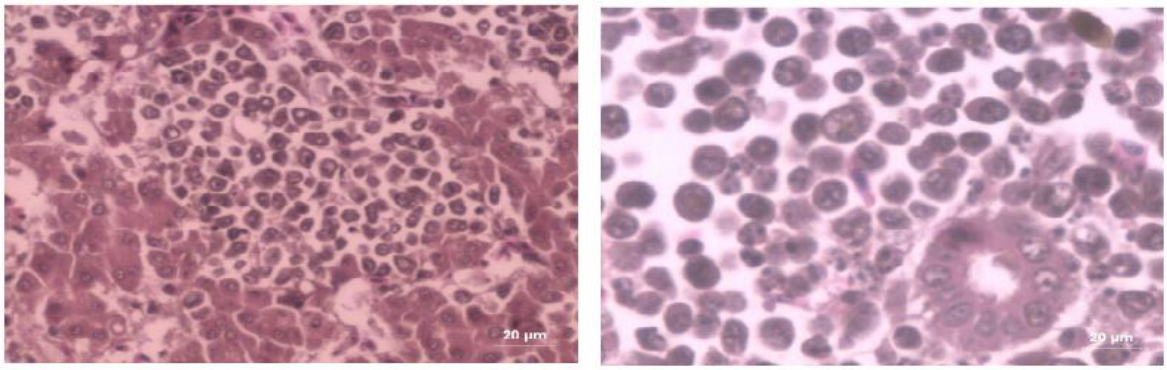


Fig. 2.1: Liver- monomorphic infiltration of Lymphoid cells; Fig. 2.2: Kidney – monomorphic infiltration of Lymphoid cells

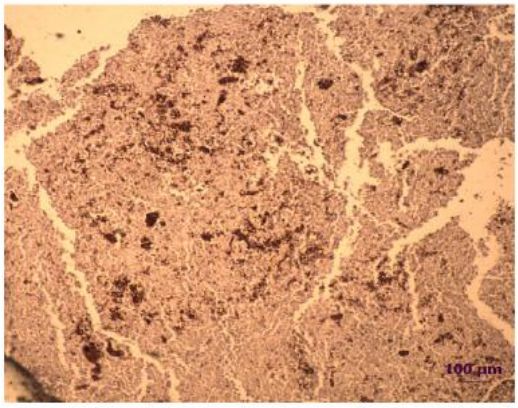


Fig.3.3: Spleen-Avian leukosis virus particles seen extensively in lymphoid cells stained by immunohistochemistry with virus specific antibodies and heamatoxylin as counter stain.

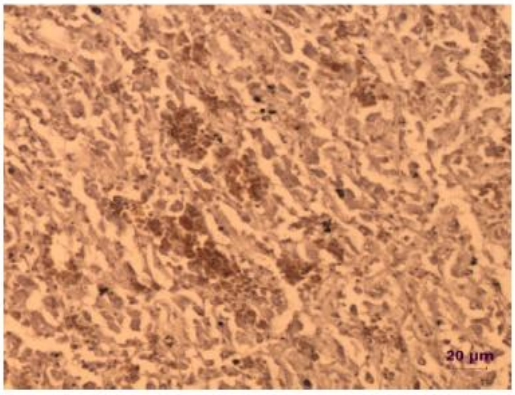
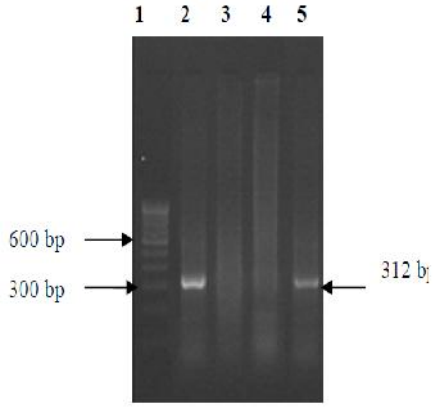


Fig.3.4: Spleen-Avian leukosis virus particles seen extensively in lymphoid cells stained by immunohistochemistry with virus specific antibodies and heamatoxylin as counter stain.

Immunohistochemistry

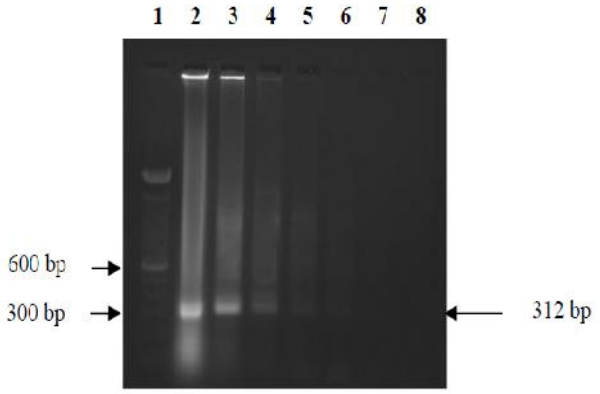
Avian leukosis virus positive signals were seen extensively in the cytoplasm an

Fig. 4: PCR amplification – H5 and AD1 primers – 295-326bp



Specificity of H5 and AD1 primers

Lane 1: DNA molecular weight marker (100 bp)
Lane 2: IHC-ALV positive tissue DNA
Lane 3: IHC-MDV positive tissue DNA
Lane 4: IHC-MDV positive tissue section DNA
Lane 5: IHC-ALV positive tissue section DNA

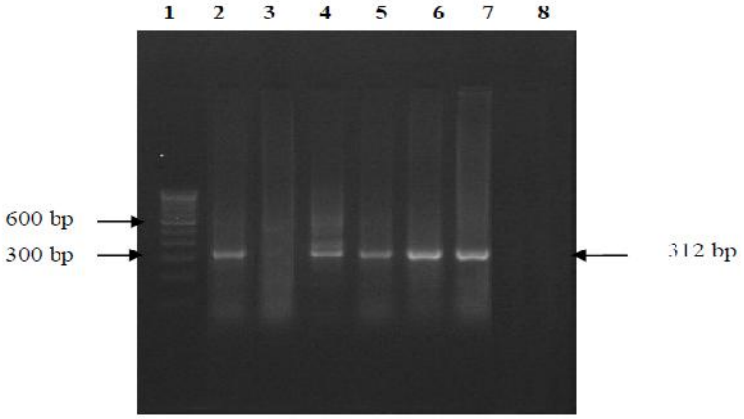


Sensitivity of H5 and AD1 primers using positive tissue DNA

Lane 1: DNA molecular weight marker (100 bp)
Lane 2: Positive tissue DNA (1236.8 ng/µl)
Lane 3-9: 10^1 to 10^6 dilutions of positive tissue DNA

Detection of ALV genome from the biological samples

Avian Leukosis viruses (pol flanked region – 295-326 bp) were checked by using the template DNA extracted from immunohistochemistry positive tissue sections and tissues. Sensitivity of these primers was checked by using different dilutions of positive tissue DNA samples (Fig. 5). A total of 203 suspected samples were screened for ALV and by PCR. The immunohistochemistry positive tissue DNA sample and uninfected tissue DNA sample were used as positive and negative controls respectively. The results were listed in Table.1.



Lane 1 : DNA molecular weight marker (100 bp)
Lane 2 : Positive control
Lane 3 : Negative tissue sample
Lane 4-7 : Positive tissue samples
Lane 8 : Nuclease free water (without DNA)

Table. 1: Screening of samples for ALV by PCR

Species	Sample type	Total number of samples screened by PCR	Number of positive samples	
			ALV	ALV-J
Chicken	Feather follicle	59	-	0
	Liver	70	15	0
	Spleen	48	10	0
	Bursa	7	0	0
	Kidney	5	1	0
	Eye	1	0	0
Turkey	Liver	5	0	0
	Spleen	5	0	0
	Intestine	3	0	0
Total		203	26	0

Sequence analysis

The purified PCR products of REV were sequenced and the sequences obtained were analyzed using BLASTn to check for the homology (www.ncbi.nlm.nig.gov/blastn). The percentage homology of our field sample sequences with other GenBank sequences were listed in table (Table. 2).

Table. 2 : **Comparison of nucleotide sequence homology for *pol flanked sequence* of ALV**

S. No.	Accession No.	Organism and strain	Homology expressed in percentage
1.	EF467236.1	Avian leukosis virus isolate SDO5O1, complete genome	99%
2.	AY013303.1	Avian leukosis virus strain ev-1, complete genome	99%
3.	D10652.1	Rous sarcoma virus - Schmidt-Ruppin D genomic RNA, complete genome	98%
4.	J02342.1	Rous sarcoma virus - Prague C, complete genome	98%
5.	EU070901.1	Avian leukosis virus strain PDRC-3246, complete genome	96%
6.	AY013304.1	Avian leukosis virus strain ev-3, complete genome	96%
7.	M37980.1	Avian leukosis virus - RSA, complete genome	97%

Phylogenetic analysis

The phylogenetic tree was constructed using the pol flanked sequence (267 bp) sequence of Tamil Nadu field strain and reference strains present in the NCBI database. When rooted by the mid-point method, the Tamil Nadu field strain formed a group with SDO5O1 (China) and ev-1 (USA) sequences (Fig. 6).

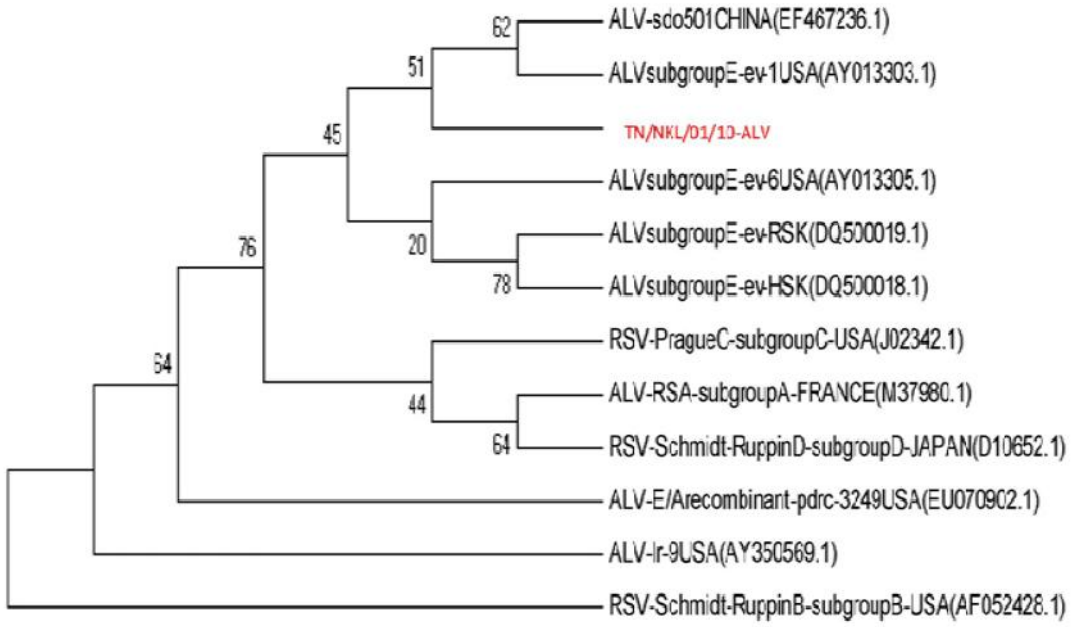
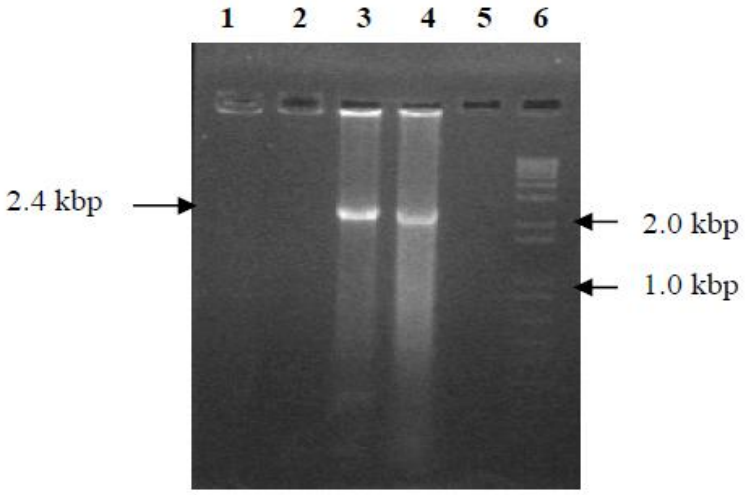


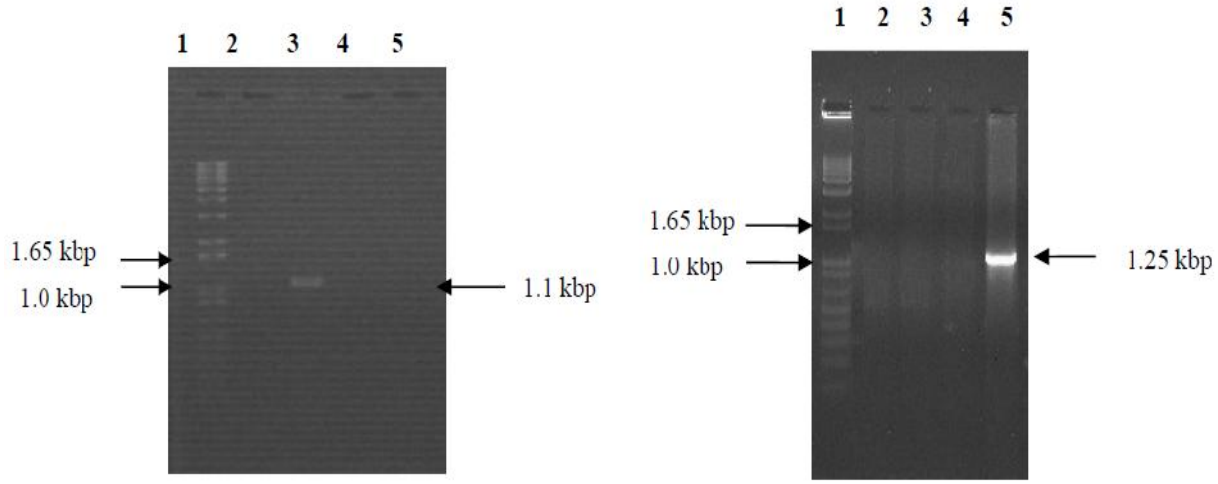
Fig. 6 : **Phylogenetic analysis of Tamil Nadu ALV field strain- *pol flanked sequence (267 bp)***

Avian leukosis virus Subgroup specific PCR

A total of 26 ALV positive DNA samples showed positive amplicon size of 2.4 kbp by using primers for ALV all subgroups (Fig. 7.1). Out of 26 samples, one sample showed amplicon size of 1.1 kbp for ALV subgroups B and D and the other samples showed amplicon size of 1.25 kbp for ALV subgroup E (Fig. 7.2 and 7.3).



Lane 1-2: Negative amplification of tissue DNA
Lane 3-4: Positive amplification of tissue DNA
Lane 5 : Nuclease free water (without DNA)
Lane 6 : DNA molecular weight marker (1kbp)



Lane 1: DNA molecular weight marker (1kbp)
Lane 2: ALV subgroup A
Lane 3: ALV subgroup B and D
Lane 4: ALV subgroup C
Lane 5: ALV subgroup E

Fig. 7: ALV Subgroup specific PCR

Isolation and identification of virus

CEF infected with 5th passage ALV produced no cytopathic effect after 5th day of infection (Fig. 8). Hence, presence of virus in the infected culture was confirmed by ELISA titre values using virus specific serum (polyclonal serum against ALV, US biologicals) (Table.3) of infected culture fluid in comparison with uninfected culture fluid clearly indicated the multiplication of virus in cell culture system.

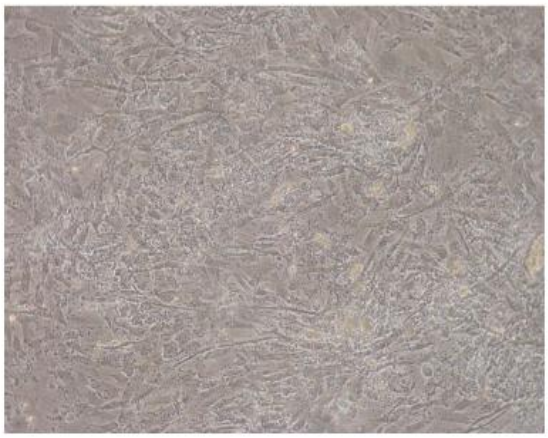


Fig. 8.1: Chicken embryo fibroblast culture - normal monolayer after 24 hrs of culture (100X); Fig. 8.2: CEF monolayer infected with ALV positive cell suspension (5 days post infection) showing no CPE (100X)

Table. 3: Presence of virus in cell culture was confirmed by ALV-ELISA based on titre values using ALV specific antibodies

Antigen	Primary antibody	ELISA OD value MEAN ± S.D
CEF infected with ALV	ALV specific polyclonal antibody	1.252 ± 0.110
uninfected CEF	ALV specific polyclonal antibody	0.186 ± 0.091
CEF infected with ALV	REV specific polyclonal antibody	0.184 ± 0.008

Discussion

The chicken oncogenic retroviruses include REV (17), lymphoid leukosis, subgroups A-I (ALV) (3), and the recently described avian lymphoid leukosis virus, subgroup J (2). ALV is ubiquitous in chickens that transforms B lymphocytes and causes B-cell neoplasia. Lymphoid leukosis is characterized by diffused enlargement of liver. Other organs are also tumorous. Nodular tumors can be found in the bursa of Fabricius. ALV-J transforms myeloid cells of meat-type chickens and causes predominantly late-onset myelocytomatosis (18). The gross lesions caused by infection with avian oncogenic viruses overlap and are of a low degree of pathognomony, and diagnosis based on gross lesions is often obscure, veiled and specific laboratory diagnosis are needed (19). So, samples suspected for avian leukosis viruses were collected after post-mortem findings and analysed for further molecular diagnosis in this study.

In histopathology, ALV infection shows uniform population of lymphoid cells but there is no possibility to distinguish between ALV and REV (20). Hence the microscopic findings should be confirmed with immunological techniques. Here in this study, immunohistochemistry was used for identification of virus in suspected tissue sections using virus specific antibodies. In immunohistochemistry, heat induced antigen retrieval (HIER) in acidic buffer (Citrate buffer pH 6.0) was used for antigen retrieval. Avian leukosis virus particles were detected as surface and concentrated cytoplasmic discrete granules (6).

The PCR appears to be a method of choice for the diagnosis of avian oncogenic viruses as it overcomes the veiled aspects of differential diagnosis (21). So screening of multiple numbers of samples for Avian leukosis virus was done using PCR in this study. Due to non-availability of reference strains of ALV using the immunohistochemistry positive tissue DNA samples as positive controls, the large numbers of DNA samples were screened for ALV. The detection of ALV in the DNA from tissue samples by PCR amplification using H5 and AD1 primers yielded an amplicon of 295-326 bp which implied that the genome of the ALV was present in the sample for ALV subgroups A-E as reported by Bai et al. (5) and Smith et al. (11). The results were further confirmed by sequencing of amplicon and BLAST analysis.

The phylogenetic analysis of ALV sequence of field sample showed that the isolate was grouped with ALV endogenous virus strains SDO5O1 (China), ev-1 (USA), PDRC-3246 (USA), ev-3 (USA) and the sequence had homology of 96-99%.

The ALV positive samples were further grouped based on ALV subgroup specific PCR as reported by Silva et al. (16). These results confirmed the specific subgroups as the 25/26 samples showed positive for ALV-E but one sample showed positive for ALV B and D. In this study, ALV endogenous virus (ALV-E) was detected in most samples. Though, endogenous viruses have little or no oncogenicity (22), they can affect induction of neoplasia and other production or performance traits by their interaction with exogenous ALV. Similarly, subgroup E recombinants of endogenous and exogenous viruses have been reported to be capable of inducing neoplasm (23). ALV-B, D are exogenous viruses isolated less frequently than ALV-A from the outbreaks of lymphoid leukosis (24). Nowadays, ALV-J was reported mostly in Asian countries (25; 26). Here, in this study the samples were screened for both HPRS 103 (prototype of ALV-J) and ALV-J, but no samples showed positive.

Unlike sarcoma viruses, most ALVs produce no visible morphologic changes in culture. Thus, indirect biological assays such as complement fixation test for avian leukosis virus (COFAL), ELISA for ALV (ELISA-ALV), phenotypic mixing (PM), resistance-inducing factor (RIF), and nonproducer (NP) cell activation tests are used for the detection of ALVs. ELISA ALV is the more commonly used test for detection of ALV, because it is more sensitive and less cumbersome than other tests such as the COFAL and PM tests (27). So the presence of virus in cell culture was confirmed by ALV-ELISA based on titre values using ALV specific antibodies. The positive results were well supported by PCR amplification of P27 antigen.

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