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Identification of True Exogenous Avian Leukosis Virus Transmitters by Serological and Molecular Assays

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Exogenous Avian leukosis virus subgroup A is the major causative agent of lymphoid leukosis causing significant loss to poultry industry through subclinical conditions. Eradication of exogenous ALV A in breeding stock is affected by lack of vaccines and difficulty in identification and elimination of the shedders and transmitters birds. The present study was conducted at the farm level to simulate the existing poultry rearing. We used commonly available serological and molecular diagnostic tests to screen laying hens and their embryos for the presence of ALV A infection. Adult laying hens were tested for proviral DNA and viral RNA using PCR and RT-PCR respectively. And their antibody and viremic status were studied using gsAg based ELISA. Similarly, 21-days old embryos of the respective dams were analysed. Based on the presence of viremia, antibody against gsAg and PCR, RT-PCR results out of 16 dams tested 12 hens were categorized as transmitters. In conclusion, combination of serological and molecular methods screening dams and their embryos/chicks, will facilitate early detection of true exogenous ALV A transmitters in the breeding stock, in order to establish ALV a free flock.

Key words: Avian leukosis virus, Lymphoid leukosis, Shedders, Transmitters.

Avian leukosis virus (ALV) is widespread among commercial chickens, but the prevalence of lymphoid leukosis (LL) is comparatively very low (1 - 2 %)[1]. Even though the mortality caused by the neoplastic condition is low, it has major impact on economy due to reduction in productive traits in the poultry industry, because of subclinical infections and non-neoplastic conditions[2,3,4]. ALV has been classified into 10 subgroups *i.e.* A-J, includes both endogenous and exogenous viruses. Subgroups A, B, C, D, E and J affect domestic chickens, except subgroup E, others are exogenous viruses. The major causative agent of LL is ALV subgroup A. ALV is transmitted by three modes, *i.e.* horizontal, vertical in case of exogenous viruses and endogenous viruses spread mainly through genetic transmission[5]. In order to establish exogenous ALV free flock, the foremost preventive measure to be taken not only to break the vertical transmission from dam to progeny but also to prevent reinfection of the progeny through horizontal infection[6,7,8]. At present there is no commercial vaccines available against ALV, thus identification and elimination of hens that shed ALV to the egg albumen hence to the embryos and subsequently to chicks is the only possible way to achieve ALV free flock [4]. In the present scenario, identification and elimination of shedder and transmitter hens by screening of breeding stock at different ages, using diagnostic tests such as, molecular assays such as polymerase chain reaction (PCR), reverse transcriptase-PCR (RT-PCR) and 'gold' standard gsAg based serological assays [8]. Our study was envisioned to identify the

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true transmitters of exogenous ALV A through combination of serology and molecular techniques to screen the dam and progeny in order to establish a definitive method to eradicate ALV from breeding flock.

MATERIALS AND METHODS

Experimental birds

Sixteen Chickens of Central Avian Research Institute Variety, Colored Synthetic Male Line (CSML) were used in this study. Chickens were artificially inseminated and fertile eggs were kept for incubation and embryos were sacrificed before hatching on 21st day. Tissue and sera samples were collected separately from each embryo and stored at -20°C until further use.

DNA isolation and PCR

Genomic DNA was isolated from whole blood, using phenol:chloroform:isoamyl alcohol (PCI) extraction methodwith certain modifications[9]. For standardization of thermocyclic conditions for ALV E, ALV A and ALV B & D were done using RAV 0, RAV 1, and RAV 2 DNA respectively and the same RAV DNA were used as positive controls for respective PCR reactions.

DNA isolate was tested for presence of ALV A infection using specific primer[10] by PCR under following conditions, *viz*., initial denaturation 95 °C for 3 min followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 57 °C for 30 sec, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. Other subgroups *i.e*. ALV B, D and E, were screened using specific primers. In case of ALV E, ALV B & D[11] and the presence of ALV without subgroup discrimination using ALV A-E[12] with similar PCR conditions except annealing at 60 °C for 45 sec for ALV B & D and E, 60 °C for 30 sec for ALV A-E. Sequences of different primers were mentioned in the table 1.

RNA isolation and RT-PCR

RNA was isolated from whole blood of adult chicken and embryos using TRIzol LS (Intvirogen) and Ribozol™ (Amresco) method respectively following manufacturer's protocol. After isolation of RNA, cDNA was synthesized employing RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific), following manufacturer's protocol. Then RT-PCR was performed using similar conditions as PCR to screen for presence of various subgroups of ALV.

ELISA

Each serum sample collected from adult chicken and embryos were screened for the presence of group specific antigen (gsAg) and anti-gsAg antibody by Avian Leukosis Virus Antigen and Antibody Test Kits (FlockChek*, IDEXX Lab) respectively, following manufacturer's protocol.

RESULTS AND DISCUSSION

At present the poultry breeding industry follows different ALV eradication procedures. Different paradigms have been followed to achieve ALV free flock in broiler and layer breeding stocks. A five stage testing protocol performed till 40 weeks of age followed in breeding stocks to eradicate ALV J infection [8]. The present study was aimed to identify true transmitters of exogenous ALV A subgroup which is the major causative agent of LL. In our study we have used most sensitive molecular techniques such as PCR and RT-PCR for genome level detection of various ALV subgroups and also 'gold' standard gsAg based ELISA for screening of both gsAg and anti-gsAg antibody to determine the birds viremic and antibody status against ALV. In the past 4 decades after the discovery of the ALV, various studies were conducted to demarcate shedders and transmitters and they have classified those based on different criteria, but none of them were conclusive in defining the true transmitters. Infected birds that shed virions or gsAg into cloacae or egg albumen have been classified as 'shedders'[13]. ALV infected birds that transmit virions to progeny are referred to as 'transmitters'. More than 60% ALV shedders virtually transmitters, since they transmit the virus vertically to their progeny[14]. ALV infected birds present a complex pattern of shedders and non-shedders. Based on presence or absence of viremia, antibody and shedding of virions or gsAg into egg albumen or cloacae, shedders and non-shedders may be classified as: i) Viremic, antibody-positive, shedders (V+A+S+); ii) Viremic, antibody-negative, shedder (V+A-S+); iii) Non-viremic, antibody-positive, shedders (V-A+ S+); iv) Non-viremic, antibody-positive, non-shedders (V-A+S) and; v) Non-viremic, antibody-negative, non-shedders (V-A-S-) [8].

Adult CSML hen screening

Whole blood genomic DNA isolates of all 16 adult CSML laying hens revealed PCR amplification products for various subgroup specific primers, *viz*., 326 bp for ALV A-E, 1250 bp for ALV E and 229 bp for ALV A and there was no specific amplification product for ALV B & D, observed at 1100 bp. RT-PCR results were consistent with PCR findings revealed the presence of viral RNA in the blood (Figure 1 and 2).Sera collected from all 16 adult CSML laying hens tested for the presence of gsAg and anti-gsAg antibody

Table 1. Oligonucleotide primers used in PCR and RT-PCR assays to detect different ALV subgroups

S. No.	ALV subgroup	Primer	Sequence	Product size
1.	ALV A-E	Forward	5'-GGATGAGGTGACTAAGAAAG-3'	326 bp
		Reverse	5'-GGGAGGTGGCTGACTGTGT-3'	
2.	ALV-E	Forward	5'-CGAGAGTGGCTCGCGAGATGG-3'	1.25 kb
		Reverse	5'-GGCCCCACCCGTAGACACCACTT-3'	
3.	ALV-B&D	Forward	5'-CGAGAGTGGCTCGCGAGATGG-3'	1.1 kb
		Reverse	5'-AGCCGGACTATCGTATGGGGTAA-3'	
4.	ALV-A	Forward	5'-CTACAGCTGTTAGGTTCCCAGT-3'	229 bp
		Reverse	5'-GCCTATCCGCTGTCACCACTG-3'	

Table 2. gsAg based detection of ALV infection in adult CSML laying hens using Flock Chek* ALV Antigen and Antibody Test Kits

S. No.	Adult CSML Hen No.	Serum gsAg levels * Mean A(650) ± SE #	S/P ratio	Anti-gsAg antibody levels ** Mean A(650) ± SE	Mean S/P ratio ± SE
1.	M3752	0.617 ± 0.007	1.182	0.248 ± 0.006	1.909 ± 0.002
2.	M4569	0.897 ± 0.057	1.640	0.281 ± 0.012	2.282 ± 0.118
3.	M3301	1.306 ± 0.052	2.471	0.177 ± 0.011	1.117 ± 0.107
4.	M3275	0.116 ± 0.001	0.056	0.387 ± 0.018	3.473 ± 0.094
5.	M1355	1.266 ± 0.006	2.390	0.330 ± 0.009	2.834 ± 0.016
6.	M4499	0.979 ± 0.005	1.807	0.359 ± 0.031	3.159 ± 0.287
7.	M4341	0.444 ± 0.001	0.102	0.293 ± 0.032	2.417 ± 0.298
8.	M5878	0.355 ± 0.049	0.721	0.271 ± 0.022	2.170 ± 0.207
9.	M3487	0.133 ± 0.001	0.541	0.290 ± 0.021	2.386 ± 0.175
10.	M3201	0.276 ± 0.007	0.090	0.331 ± 0.020	2.840 ± 0.220
11.	M3277	0.173 ± 0.004	0.381	0.290 ± 0.014	2.377 ± 0.050
12.	M4578	0.268 ± 0.003	0.172	0.232 ± 0.020	1.736 ± 0.199
13.	M4425	0.367 ± 0.011	0.365	0.404 ± 0.036	3.658 ± 0.392
14.	M3822	1.083 ± 0.057	0.565	0.178 ± 0.009	1.122 ± 0.067
15.	M4321	0.625 ± 0.024	2.019	0.254 ± 0.003	1.982 ± 0.014
16.	M4374	0.157 ± 0.004	1.089	0.391 ± 0.029	3.518 ± 0.302
i. Positive Control Serum		0.581 ± 0.024		0.167 ± 0.002	
ii. Negative Control Serum		0.088 ± 0.003		0.077 ± 0.004	
iii. Blank (Antigen Control)		0.068 ± 0.001		0.042 ± 0.001	

* : Single test (Sandwich ELISA, S/P ratio > 0.2, indicates positive)
FlockChek* ALV Antigen Test Kits (M/S IDEXX Lab, The Netherlands)
** : Mean of 2 tests (Indirect ELISA, S/P ratio > 0.4, indicates positive)
FlockChek* ALV Antibody Test Kits (M/S IDEXX Lab, The Netherlands)
#:Mean of 2 wells

using ELISA. The ELISA results revealed only 12 hens were positive out of 16 hens screened, (S/P ratio > 0.2) for gsAg, while all hens had anti-gsAg antibody (S/P ratio > 0.4) (Table 2). All the 16 adult CSML hens had anti-gsAg antibody in their serum, indicated all were antibody-positive (A+), exposure to exogenous ALV infection or presence of endogenous ALV E. While only 12 birds except M3275, M4341, M3201 and M4578 had gsAg in their serum indicated presence of viremia (V+). But this gsAg based ELISA did not differentiate between endogenous and exogenous subgroups, since the gsAg is common for all ALV subgroups [8]. Since gsAg based ELISA cannot differentiate between different subgroups of exogenous ALV, genome level detection tests like PCR and RT-PCR were used to screen the presence of different exogenous and endogenous subgroups. Both PCR and RT-PCR revealed presence of proviral DNA and viral RNA of exogenous ALV A respectively in all the 16 birds screened. All the birds were also positive for endogenous ALV E but none of them had subgroup ALV B and D. Endogenous ALV E is found in almost all the birds and ALV A found to be the most common one in ALV infections; the occurrence of subgroup ALV B and D is uncommon our findings also supported the previous reports[1].

Screening of embryos

Genomic DNA and RNA isolates were isolated from 21-days embryonic fibroblasts of an embryo respective of each hen. Screening of embryos, before hatching into chicks will avoid horizontal transmission. Vertical transmission of ALV from dam to chick was screened by PCR and RT-PCR using genomic DNA and RNA isolates of 21-days embryonic fibroblasts respectively. All the embryos of the respective dams showed positive amplification products specific for ALV A-E and ALV E in both PCR and RT-PCR indicating genetic transmission of endogenous viruses. While PCR and RT-PCR results revealed only 12 embryos were positive for exogenous ALV A, indicating vertical transmission of exogenous ALV A. None of the embryos were found positive for ALV B & D in

Table 3. gsAg based Detection of Transmission of ALV Infection to Embryos

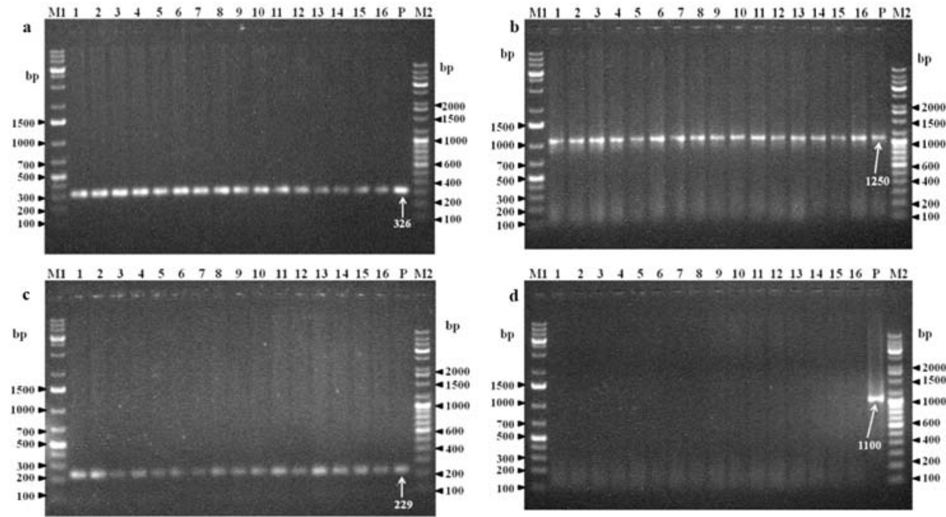
S. No.	21-days embryo of Adult CSML Laying Hen No.	Serum gsAg levels *		Anti-gsAg maternal antibody levels**	
		Mean A (650) ± SE	S/P ratio	Mean A (650) ± SE	Mean S/P ratio ± SE
1.	M3752	0.262 ± 0.003	0.353	0.217 ± 0.001	1.559 ± 0.009
2.	M4569	0.361 ± 0.018	0.553	0.241 ± 0.019	1.831 ± 0.186
3.	M3301	1.515 ± 0.037	2.894	0.045 ± 0.013	-0.363 ± 0.036
4.	M3275	0.097 ± 0.001	0.018	0.312 ± 0.003	2.632 ± 0.012
5.	M1355	0.197 ± 0.004	0.221	0.068 ± 0.005	-0.111 ± 0.018
6.	M4499	0.180 ± 0.001	0.186	0.054 ± 0.004	-0.262 ± 0.006
7.	M4341	0.253 ± 0.010	0.334	0.044 ± 0.003	-0.371 ± 0.006
8.	M5878	0.173 ± 0.005	0.171	0.179 ± 0.014	1.133 ± 0.122
9.	M3487	0.170 ± 0.008	0.165	0.217 ± 0.019	1.562 ± 0.138
10.	M3201	0.111 ± 0.002	0.046	0.189 ± 0.018	1.254 ± 0.161
11.	M3277	0.147 ± 0.006	0.118	0.148 ± 0.003	0.789 ± 0.018
12.	M4578	0.138 ± 0.001	0.100	0.176 ± 0.008	1.108 ± 0.042
13.	M4425	0.140 ± 0.006	0.104	0.212 ± 0.019	1.506 ± 0.177
14.	M3822	0.172 ± 0.010	0.169	0.105 ± 0.006	0.310 ± 0.031
15.	M4321	0.519 ± 0.063	0.874	0.213 ± 0.020	1.514 ± 0.144
16.	M4374	0.289 ± 0.037	0.332	0.157 ± 0.004	0.887 ± 0.028
i. Positive Control Serum		0.581 ± 0.024		0.167 ± 0.002	
ii. Negative Control Serum		0.088 ± 0.003		0.077 ± 0.004	
iii. Blank (Serum Control)		0.068 ± 0.001		0.042 ± 0.001	

* : Single test (Sandwich ELISA, S/P ratio > 0.2, indicates positive)
FlockChek® ALV Antigen Test Kits (M/S IDEXX Lab, The Netherlands)
** : Mean of 2 tests (Indirect ELISA, S/P ratio > 0.4, indicates positive)
FlockChek® ALV Antibody Test Kits (M/S IDEXX Lab, The Netherlands)

Table 4. Comparison of molecular and serological tests for the detection of ALV A transmitters

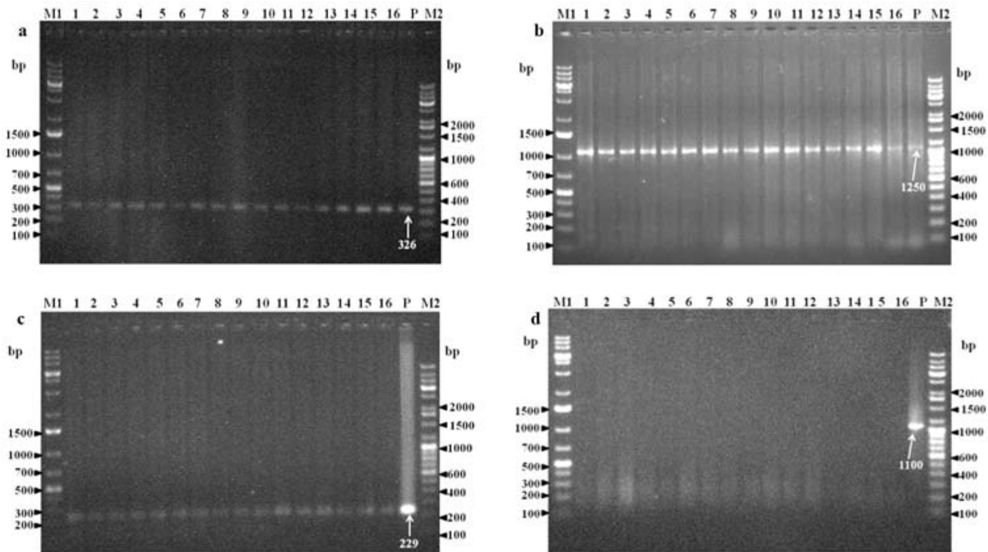
S. No.	Adult Hen CSML Laying No.	Adult CSML Hens: Molecular Assays		Adult CSML Hens: Serology ELISA based on gsAg		21-Days Embryos: Molecular Assays		21-Days Embryos: Serology ELISA based on gsAg		Transmitter Hens			
		PCR	RT-PCR	Sandwich ELISA*	iELISA**	PCR	RT-PCR	Sandwich ELISA*	iELISA**	Viremic (V)	Antibody (A)	Shedder (S)	Transmitter (T)
1.	M3752	+	+	+	+	+	+	+	+	+	+	+	+
2.	M4569	+	+	+	+	+	+	+	+	+	+	+	+
3.	M3301	+	+	+	+	-	-	+	-	+	+	+	-
4.	M3275	+	+	-	+	+	+	-	+	-	+	-	+
5.	M1355	+	+	+	+	-	-	+	-	+	+	+	-
6.	M4499	+	+	+	+	-	-	-	-	+	+	-	-
7.	M4341	+	+	-	+	-	-	+	-	-	+	+	-
8.	M5878	+	+	+	+	+	+	-	+	+	+	-	+
9.	M3487	+	+	+	+	+	+	-	+	+	+	-	+
10.	M3201	+	+	-	+	+	+	-	+	-	+	-	+
11.	M3277	+	+	+	+	+	+	-	+	+	+	-	+
12.	M4578	+	+	-	+	+	+	-	+	-	+	-	+
13.	M4425	+	+	+	+	+	+	-	+	+	+	-	+
14.	M3822	+	+	+	+	+	+	-	-	+	+	-	+
15.	M4321	+	+	+	+	+	+	+	+	+	+	+	+
16.	M4374	+	+	+	+	+	+	+	+	+	+	+	+

* : FlockChek* ALV Antigen Test Kit (M/S IDEXX Lab, The Netherlands)
** : FlockChek* ALV Antibody Test Kit (M/S IDEXX Lab, The Netherlands)
+ : Presence of ALV infection
- : Absence of ALV infection



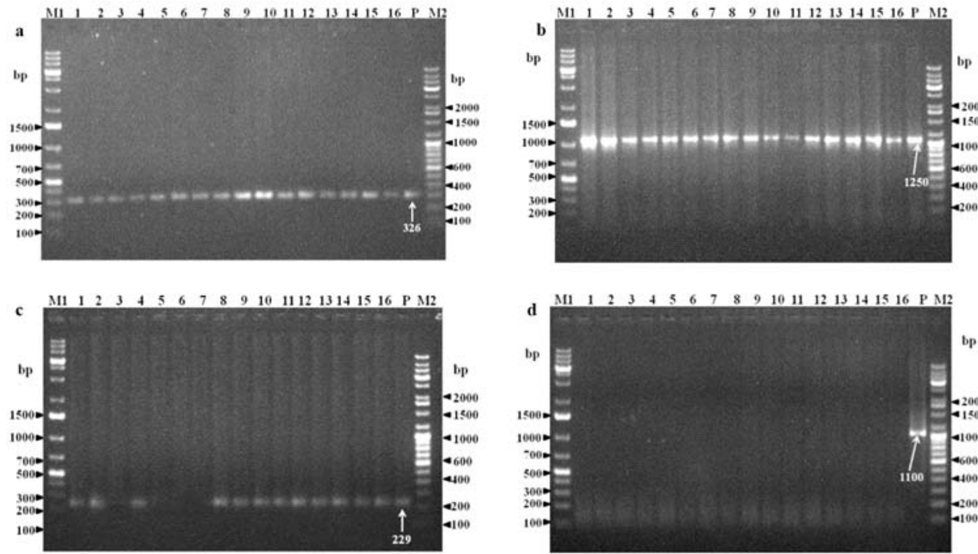
(a) ALV A-E (Lane 1-16)
(b) ALV E (Lane 1-16)
(c) ALV A (Lane 1-16)
(d) ALV B&D (Lane 1-16)
Lane M1: 1 kb DNA ladder (M/S Fermentas, USA)
Lane M2: Medium range ladder (M/S Genei, Bangaluru)
Lane P (a, b, c, & d): Positive Control

Fig. 1. PCR based amplification of whole blood genomic DNA isolates from CSML laying hens



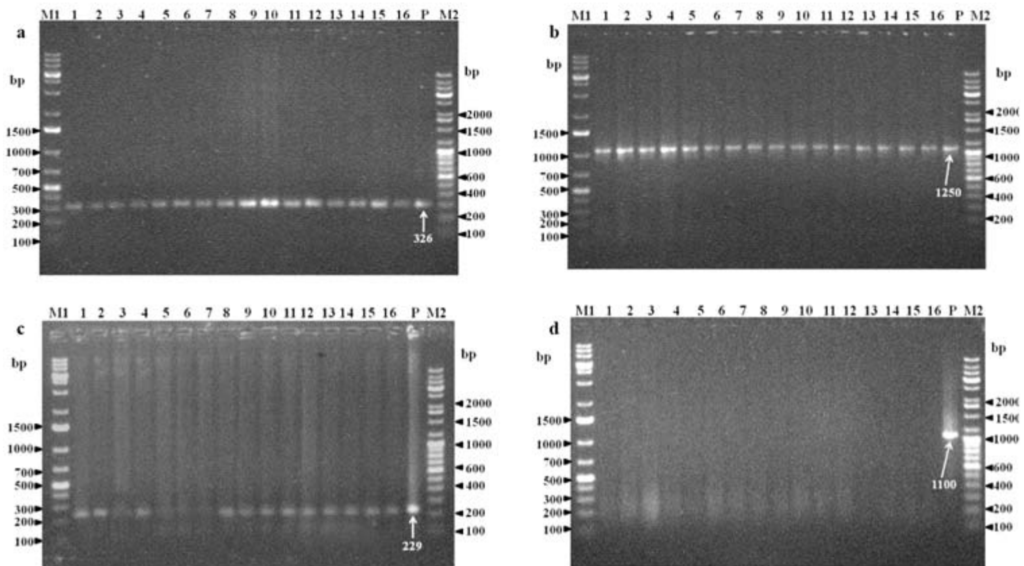
(a) ALV A-E (Lane 1-16)
(b) ALV E (Lane 1-16)
(c) ALV A (Lane 1-16)
(d) ALV B&D (Lane 1-16)
Lane M1: 1 kb DNA ladder (M/S Fermentas, USA)
Lane M2: Medium range ladder (M/S Genei, Bangaluru)
Lane P (a, b, c, & d): Positive Control

Fig. 2. RT-PCR based amplification of plasma viral RNA isolates from CSML laying hens



(a) ALV A-E (Lane 1-16)
(b) ALV E (Lane 1-16)
(c) ALV A (Lane 1-16)
(d) ALV B&D (Lane 1-16)
Lane M1: 1 kb DNA ladder (M/S Fermentas, USA) Lane M2: Medium range ladder (M/S Genei, Bangaluru)
Lane P (a, b, c, & d): Positive Control

Fig. 3. PCR based amplification of 21-days embryos tissue genomic DNA isolates from respective adult CSML laying hens



(a) ALV A-E (Lane 1-16)
(b) ALV E (Lane 1-16)
(c) ALV A (Lane 1-16)
(d) ALV B&D (Lane 1-16)
Lane M1: 1 kb DNA ladder (M/S Fermentas, USA)
Lane M2: Medium range ladder (M/S Genei, Bangaluru)
Lane P (a, b, c, & d): Positive Control

Fig. 4. RT-PCR based amplification of 21-days embryos tissue total RNA isolates from respective adult CSML laying hens

both PCR and RT-PCR (Figure 3 and 4). Serum samples of 21-days embryos of the respective dams were tested for presence of gsAg and anti-gsAg antibody. ELISA results revealed 7 out of 16 were positive for gsAg while11 had anti-gsAg antibody. Embryos from birds M3752, M4569, M4321, M4374 had both gsAg and anti-gsAg antibodies. Three embryos from M3301, M1355 and M4341 had gsAg in the absence of antibodies. Two embryos from M4499 and M3822 had neither gsAg nor anti-gsAg antibodies. Other embryos were positive for antibodies in the absence of detectible gsAg (Table 3). Major transmitters of ALV are V+A+S+, V+A-S+ transmitting virion through egg albumen to embryo [13, 14]. However, a small proportion of V-A+S+ are intermittent transmitters since they transmit virus to progeny, rather, in an infrequent manner, and cannot be identified even by screening of embryos[13,15, 16, 17]. Exposure to ALV infection in dams can be identified indirectly, through testing of serum from day-old chicks, since infected birds will transfer maternal antibodies to chicks. Twenty one-day old embryos of the all 16 hens were sacrificed and presence of gsAg and anti-gsAg antibody in the serum were screened and presence of proviral DNA and viral RNA were also tested using PCR and RT-PCR respectively. PCR and RT-PCR results indicated the presence of proviral DNA and viral RNA which were coinciding presence of proviral DNA integration and subsequent expression of viral genes. All the sixteen 21-day embryos had both proviral and viral RNA of endogenous ALV E subgroup, suggested the Mendelian inheritance of endogenous retroviruses from dam to progeny[1]. Twelve out of 16 embryos had genome of exogenous ALV A subgroup suggested the vertical transmission of exogenous retroviruses. Even though all the 16 hens had exogenous ALV A infection, 4 birds failed transmit vertically. Most of the dams were viremic (V+) since they had gsAg in their serum and were able to transmit vertically to their progeny, these results support most of the transmitters were viremic birds despite the presence of antibodies. Three of the embryos that had exogenous ALV A were from non viremic hens (M3275, M3201 and M4578), support few transmitters are non-viremic hens. 'Gold' standard gsAg based ELISA revealed embryos that had anti-gsAg antibody, found also had exogenous ALV A except the one from dam M3822. Based on the results of embryo screening both genomically and serologically, their respective dams were classified as transmitters (Table 4).

CONCLUSION

Our present study was undertaken to detect the true exogenous ALV transmitters using standard procedure with help of currently available diagnostic tests. Results indicated testing of dams and their day-old chicks will give a better way to differentiate shedders from true transmitters. And also this will facilitate to reduce the horizontal spread of infection from the shedders apart from establishing ALV free breeding stock. Combination both molecular and serological assays could help to differentiate different subgroups especially to avoid cross reaction of gsAg of endogenous ALV E with exogenous ALV A in case of gsAg based ELISA. Here we suggest that screening of birds along with their embryos would almost clearly help to achieve an exogenous ALV free stock of birds.

REFERENCES

1. Fadly, A.M. and Nair, V.: Neoplastic diseases-Leukosis/sarcoma group. In: *Diseases of poultry*. *12th edition*(Y.M. Saif, J.R. Glisson, A.M. Fadly, L.R. Mcdougald, L.K. Nalon and D.E. Swayne, eds). Iowa: Blackwell publishing, 2008; pp 514-556.
2. Ignjatovic, J., Fraser, R.A. and Bagust, T.J. Effect of lymphoid leukosis virus on performance of layer hens and the identification of infected chickens by tests on muconia. *Avian Pathol.,* 1986;**15**: 63-74.
3. Spencer, J.L., Chan, M. and Nadin-Davis, S. Relationship between egg size and subgroup J avian leukosis virus in eggs from broiler breeders. *Avian Pathol*., 2000; **29**: 617-622.
4. Fadly, A.M. Isolation and identification of avian leukosis viruses: A review. *Avian Pathol*., 2000; **29**: 529-535.
5. Payne, L.N. and Nair, V.K. The long review: 40 years of avian leukosis research. *Avian Pathol*., 2012; **41**(1): 11-19.
6. Spencer, J.L. Progress towards eradication of lymphoid leukosis viruses- a review. *Avian Pathol*., 1984;**13**: 599-619.
7. Chase, W.B. Eradication of avian leukosis virus by breeder companies: results, pitfalls and cost benefit analysis. In: *Proceedings in Avian Tumour Virus Symposium*. Kennett Square, PA,

American Association of Avian Pathologists, 1991; pp 5-7.

8. Payne, L.N. and Nair, V.K. Neoplastic diseases: Marek's disease, avian leukosis and reticuloendotheliosis. *Rev. Sci. Tech. Off. Int. Epiz*., 2000; **19**: 544-564.
9. Sambrook, J. and Russel. Molecular cloning: A Laboratory Manual, 2nd edn. New York: Cold Spring Harbor Laboratory Press. 2001.
10. Pham, T.D., Spencer, J.L., Traina-Dorge, V.L., Mulllin, D.A., Garry, R.F. and Johnson, E.S. Detection of exogenous and endogenous ALV in commercial chicken eggs using reverse transcription and polymerase chain reaction assay. *Avian Pathol*.,1999; **28**: 385-392.
11. Silva, R.F., Fadly, A.M. and Taylor, P.C. Development of a polymerase chain reaction to differentiate ALV subgroups**:** Detection of an ALV contaminant in commercial Marek's disease vaccines. *Avian Dis*., 2007; **51**:663-667.
12. Fenton, S.P., Reddy, M.R. and Bagust, T.J. Single and concurrent avian leukosis virus infections with avian leukosis virus-J and avian leukosis virus-A in Australian meat-type chickens. *Avian Pathol*.,2005; **34**: 48-54.
13. Tsukamoto, K., Hasebe, M., Kakita, S.,Hihara, H. and Kono, Y. Identification and characterization of hens transmitting avian leukosis virus (ALV) to their embryo by ELISAs for detecting infectious ALV, ALV antigens and antibodies to ALV. *J. Vet. Med. Sci*., 1991; **53**: 859-864.
14. Witter, R.L., Bacon, L.D., Hunt, H.D., Silva, R.E. and Fadly, A.M. Avian leukosis virus subgroup J infection profiles in broiler breeder chickens: association with virus transmission to progeny. *Avian Dis*., 2000; **44**: 913-931.
15. Rubin, B.H., Cornelius, A. and Fanshier, L. The pattern of congenital transmission of an avian leukosis virus. *Proc. Natl. Acad. Sci*., 1961; **47**: 1058-1060.
16. Rubin, B.H., Fanshier, L., Cornelius, A. and Hughes, W.F. Tolerance and immunity in chickens after congenital and contact infection with and avian leukosis virus. *Virology*, 1962; **17**: 143-156.
17. Hughes, W.F., Watanabe, D.H. and Rubin, H. The development of a chicken flock apparently free of leukosis virus. *Avian Dis*., 1963; **7**: 154-165.