



## SHORT COMMUNICATION

### PCR Based Evidence of Reticuloendotheliosis Virus Infection in Chickens from Turkey

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#### ABSTRACT

In this study, presence of avian leukosis virus (ALV) and reticuloendotheliosis virus (REV) was investigated in neoplastic cases observed in breeder hens older than 20 weeks in commercial broiler breeders. Tumor samples were examined by PCR combined with primer sets specific for ALV and REV. It was found that the tumors were REV-originated. This is the first report showing the presence of REV infection in Turkey.

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#### INTRODUCTION

Etiology of neoplastic diseases in avian species has not been fully enlightened. However, some viruses have been definitely shown to cause neoplastic diseases in avian species. Marek's disease caused by herpes viruses, avian leukosis and reticuloendotheliosis caused by some retroviruses are the most prevalent neoplastic diseases in poultry (Fadly, 2000; Payne and Venugopal, 2000; Witter *et al.*, 2005; Hafez, 2011). Avian leukosis virus (ALV), an *Alpharetrovirus*, causes 2-20% tumor-associated deaths and also subclinical infection results in significant production losses in chicken flocks (Bacon *et al.*, 2004). Avian leukosis viruses (ALVs) are classified into six subgroups (A, B, C, D, E and J) based on virus neutralization, receptor binding, viral infectivity, interference patterns and envelope protein, gp85, encoded by envelope (*env*) gene. Subgroup A ALV and subgroup J ALV (ALV-J) are the most encountered subgroups in the field and cause large economical losses (Witter *et al.*, 2005; Silva *et al.*, 2007; Gao *et al.*, 2010).

Reticuloendotheliosis virus (REV) is a *Gammaretrovirus* and causes lymphoma, runting disease, immunosuppression etc. in chicken, turkey, geese, duck and pigeon. This virus has strains such as defective REV-T, non-defective REV-A, chick syncytial virus, duck infectious anemia virus and named as REVs (Payne and Venugopal, 2000; Lin *et al.*, 2009; Deng *et al.*, 2010).

Retrovirus-suspected lymphoreticular cases in chicken have been notified from time to time in Turkey. However, their etiology has not been completely understood. The present study was conducted to

investigate ALV and REV viruses in neoplastic cases with increased mortality as time advanced observed in layer hens older than 20 weeks in a commercial layer farm.

#### MATERIALS AND METHODS

##### Case history

In a commercial layer farm, there were five pens, each containing 10,000 White Leghorn at different ages. In one pen with hens older than 20 weeks, 1% deaths and 10% decline in egg production was reported. Liver samples were collected from 40 chickens died at the age of 20-24 weeks and from randomly selected 10 apparently healthy chickens in this pen. In addition, liver samples were obtained from five chickens in each of the remaining four pens where the disease were not observed.

For microscopic examination; the liver samples were fixed in 10% neutral formalin, processed routinely, cut in 5 µm thickness, and stained with hematoxylin-eosin (H&E). A portion of each liver samples were stored at -80°C for PCR analysis.

##### DNA extraction, PCR and Sequencing

A commercial NucleoSpin®Tissue kit was used to extract DNA from liver samples. The extraction procedure recommended by the manufacturer (Macherey-Nagel Inc., USA) was performed. Initially, PCR amplification was carried out using general primer sets for five subgroups (ALV A-E) and a primer set specific for ALV-J (Smith *et al.*, 1998). Then, DNAs were analyzed by PCR with different primer sets for each of subgroups A, B-D and C (Silva *et al.*, 2007).

**Table I:** The primers used for detection of avian leukosis virus subgroups.

Subgroup	Oligonucleotide sequences (5'-3')	Fragment Size (bp)	Reference
ALV A-E	GGATGAGGTGACTAAGAAAG GGAGGTGGCTGACTGTGT	295-326*	Smith et al. (1998)
ALV-J	GGATGAGGTGACTAAGAAAG CGAACCAAAGGTAAACACACACG	545	Smith et al. (1998)
ALV-A	CGAGAGTGGCTCGCGAGATGG CCCATTGCTCCTCTCCTGTA	1300	Silva et al. (2007)
ALV-B and D	CGAGAGTGGCTCGCGAGATGG AGCCGGAATATCGTATGGGGTAA	1100	Silva et al. (2007)

\*Depends on the subgroups.

For the detection of REV by PCR, the primers (F; 5'-GAAGCAGACAATAGGACTGG-3' and R; 5'-TTGACCTAGGGTATCCATCTC-3') were designed using PerPrimer 1.1.18 and produced (Iontec Co., Ist., TURKEY). The primers were directed to the conserved sequences of the envelope glycoprotein (*env*) gene of REV. Amplifications were carried out at 94°C for 5 min followed by 32 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Furthermore, A final extension step was performed at 72°C for 5 min. The amplified PCR products were run on 1% agarose gel, stained with ethidium bromide and visualized under ultraviolet light. The randomly selected four PCR positive products for REV were purified by using DNA purification system (Promega). Then, purified DNAs were sequenced by using the ABI 310 Genetic Analysis System (Iontec Co., Ist., TURKEY).

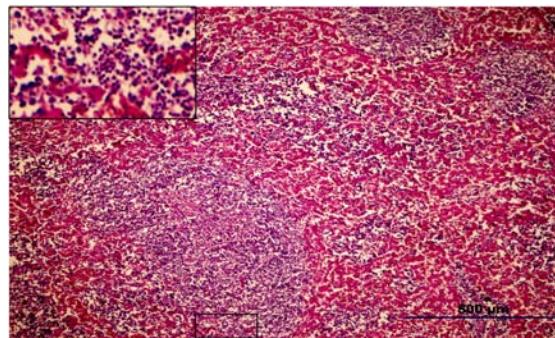
## RESULTS

Postmortem examination of dead chickens revealed neoplastic lesions of 0.6-2 cm diameter on liver, kidneys and lungs. Similar lesions were also seen in some clinically healthy chickens of pen-mates with disease. Microscopic examination revealed that lesions consisted lymphohistiocytic cells showing little pleomorphism and mitosis figures (Fig. 1). No gross and histological lesions were detected in the samples belonging to the four pens without clinical disease.

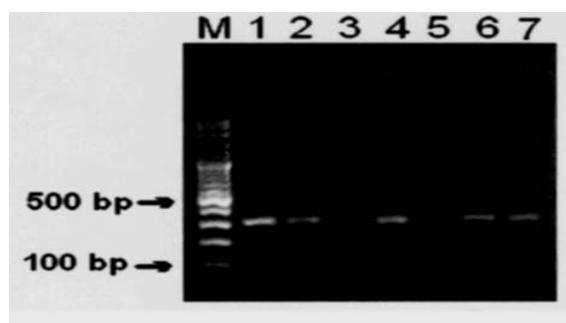
By using the general primer sets, a 310 bp long PCR product which was indicative for the presence of subgroups A-E ALV was detected in all liver samples collected from dead chickens. Interestingly, the same amplicon was detected in samples taken from clinically healthy chickens of the same pen and from the chickens of other four pens (Fig. 2). All the positive samples were also subjected to PCR combined with four different primer sets, each specific for subgroups A, B-D and C, but no amplification was detected at all. It was therefore decided that the PCR products at 310 bp belonged to subgroup E ALV (ALV-E). Also, no amplification was obtained from any of the samples for ALV-J (Data not shown).

Using the primer set for REV designed by the authors, it was found that an 850 bp long PCR product was obtained in all samples of diseased chickens but not in any samples belonging to the clinically healthy chickens (Fig. 3).

Nucleotide sequences of the partial region of the envelope glycoprotein (*env*) gene of REV amplified by



**Fig 1:** The sheets and nodules of lymphoblastic cells showing little pleomorphism and rare mitosis.



**Fig 2:** Agarose gel electrophoresis of PCR products amplified with PCR using the general primers for subgroup A-E avian leukosis viruses and the liver samples. M; 100 bp DNA ladder, Lane 1 to 5; PCR products of the clinical samples, Lane 6 and 7; the negative control samples containing the liver samples taken from chicken with no neoplasm history.



**Fig 3:** Agarose gel electrophoresis of the products amplified with PCR using the spesific primers for reticuloendotheliosis virus and the liver samples. M; 100 bp DNA ladder, Lane 1, 3, 4 and 6; PCR products of the clinical samples, Lane 2 and 5; the negative control samples containing the liver samples taken from chicken with no neoplasm history, Lane 7; the control sample (dH<sub>2</sub>O) containing no target DNA.

PCR in this study were deposited in GenBank under accession number HQ420257. Sequence analysis of four randomly selected samples revealed 100% homology. These gene sequences were compared to the sequences (GQ375848.1, FJ496333.1, FJ439120.1, DQ387450.1) obtained from EMBL database and 99-100% identity was observed (Data not shown).

## DISCUSSION

In the present study, neurological signs which are typical to Marek's disease were not observed and disease was seen in chickens at the age of above 20 weeks. The cases were thought to be due to retroviral infection at first glance. In addition, as all the sick chickens were in the same age group, a vertical contamination was suspected and samples were, therefore, analyzed to for the presence of retroviral infection.

Liver samples collected from both clinical cases and healthy chickens were analyzed by PCR for the presence of five subgroups (Subgroups A-B-C-D-E) of ALVs and only amplicons specific for ALV-E were produced. Thus, all PCR positive cases were classified as endogenous leukosis virus (ALV-E). In accordance with this finding, many previous studies reported the presence of ALV-E related *env* loci in the genome of almost all healthy chickens (Bacon *et al.*, 2004; Sacco *et al.*, 2004; Zhang *et al.*, 2008).

Samples were also analyzed for the presence of REV DNAs by PCR and an amplification product at an approximately 850 bp, indicative for the presence of REV, was detected in clinical cases but, not in any of healthy chickens. Due to the lack of positive REV samples and also to strengthen PCR results, four randomly selected REV positive PCR products were sequenced. Sequence results were matched with sequences on the European Molecular Biology Laboratory (EMBL) database and 99-100% identities were found. The cases of present study were therefore concluded to be REV virus-originated.

Clinical and pathological findings in REV infections vary significantly (Mussman and Twiehaus, 1971; Motha, 1987; Witter *et al.*, 2005). It is accepted that this variation results from differences between REV strains (Fadly, 2000). However, genomic identification for REV responsible for the cases was not performed in this study. Hence, virus strain characteristics and clinical-pathological findings could not be compared to results of other studies.

Horizontal transmission can be seen in REV infections. However, this kind of transmission was not believed to have taken place in the case of the present study because disease was occurred in only one pen but not in the other four pens. In contrast, vertical transmission seems to be more likely in the occurrence of the infection. Although it is reported to occur at a lower rate when compared with ALV, vertical contamination has been implicated in REV infection (Fadly, 2000). The present study supported this.

## Conclusions

Lymphoreticular tumors in chickens have been detected clinically from time to time in Turkey. However, there is not adequate information about the etiology of

these tumors. To the authors' knowledge, this is the first report on the presence of REV infection in Turkey. In this study, isolation, antigenic and genomic identification of REV viruses were not done, therefore, such work is suggested to be carried out in future.

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