Detection of avian leukosis virus subgroup J in albumen of commercial and native fowl eggs using RT-PCR in Fars province of Iran

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Summary

The subgroup J of ALV (ALV-J) has emerged as an important pathogen of meat-type chickens since 1989. This virus is responsible for economic losses due to both mortality and depressed performance in chickens. So, the objective of this study is the detection of ALV-J in the albumen of commercial and native fowl eggs using RT-PCR. Three hundred and seventy egg albumens were randomly selected from different farms of Fars province, Iran. These eggs were obtained from the flocks of two research centers on native fowl production (70 eggs), a broiler grandparent farm (60 eggs), three broiler breeder farms (180 eggs), and a commercial layer flock (60 eggs). RT-PCR was undertaken on isolated RNA from egg samples using a pair of ALV-J specific primers H5/H7 that produced a 545 basepair fragment. RT-PCR analyses detected ALV-J in 15 of 180 (8.33%) samples from three broiler breeders farms, 17 of 70 (24.28%) samples from flocks of two research centers of native fowls production, and none of the samples of commercial layer and broiler grandparent farms. Direct sequencing using primers specific for subgroup ALV-J verified the viral subgroup in the RT-PCR amplification products. This is the first report of the ALV-J in egg albumen in Iran which indicates the necessity to apply eradication programs for ALV-J in the poultry industry and native fowls in Iran.

Key words: ALV-J, RT-PCR, Egg albumen, Fars province, Iran

Introduction

Avian leukosis viruses (ALV) are the most common avian retroviruses associated with a variety of neoplasms including lymphoid and myeloid leukosis (Fenton *et al*., 2005). Avian leukosis viruses of chickens have been classified into six well defined subgroups A, B, C, D, E and J based on differences in viral envelop glycoprotein antigens, which determine virus neutralization properties, virus interference patterns, and host range (Zhang *et al*., 2005). The exogenous ALV subgroups A, B, C, D and J are horizontally and vertically transmitted in chicken (Fadly and Payne, 2003), but the endogenous subgroup E are transmitted by Mendelian inheritance (Pham *et al*., 1999a).

The subgroup J of ALV (ALV-J) has emerged as an important pathogen of meat-type chickens since 1989 (Venugopal *et al*., 1998; Thapa *et al*., 2004). ALV-J is believed to have evolved by recombination between an exogenous ALV and the endogenous avian viruses (Benson *et al*., 1998; Silva *et al*., 2000; Fenton *et al*., 2005). This virus spread worldwide and became ubiquitous in commercial broiler breeder chickens (Kim and Brown, 2004). ALV-J is responsible for serious economic losses in the broiler industry due to myeloid leukosis, reduced growth rate, decreased quantity and quality of egg production, and increased tumor-induced mortality and possibly by immunosupression (Spencer *et al*., 2000; Hair-Bejo *et al*., 2004; Kim and Brown, 2004). Since there are no treatments and

vaccines for this viral infection (Venugopal, 1999; Haiti *et al*., 2005), the best way to control ALV-J infection mainly is the prevention of transmission (Haiti *et al*., 2005) through culling of infected chicken (Wang *et al*., 2007). However, for this purpose the poultry industry must identify infected eggs and chickens (Pham *et al*., 1999a; Wang *et al*., 2007).

Conventional methods for detection of ALV-J include virus isolation in tissue culture, immunofluorescence assay (IFA), and enzyme-linked immunosorbent assay (ELISA) (Pham *et al*., 1999b; Venugopal, 1999), which are very time-consuming (Pham *et al*., 1999a; Silva *et al*., 2007). Unlike the methods relying on the biological amplification of ALV, molecular technology is a more rapid, specific and sensitive test for detection of ALV-J during the early stages of infection (Abdel-Latif and Khalafalla, 2005; Haiti *et al*., 2005). PCR methods have been previously developed for diagnosis of ALV-J from many different sample types including serum, whole blood, meconium, and feather tips (Bai *et al*., 1995).

In the present study, the presence of ALV-J in albumen of commercial and native fowl eggs in Iran was investigated with a developed RT-PCR method (Smith *et al*., 1998) and direct sequencing of RT-PCR products.

Materials and Methods

Flocks and eggs

To investigate the presence of ALV-J in chickens, 370 eggs were randomly selected from one broiler grandparent farm (n=60), three broiler breederflocks (n=180), one commercial layer farm (n=60), and flocks of two research centers of native fowls production (n=70) in Fars province, Iran (Table 1). After disinfection of the egg shells, 1 ml of albumen from each egg was drawn within the day of oviposition and stored at -70°C until used.

RNA extraction

RNXTM (-Plus) kit (CinnaGen, Iran) was used to isolate RNA from the egg albumen. Approximately 100 µl of albumen was added to 1 ml of RNX solution in a 1.5-ml Eppendorf tube and mixed well, then incubated at room temperature for 5 min. Next, 200 µl of chloroform was added to the mixture and mixed well for 15 sec by shaking (not vortexing). The tube was incubated on ice for 5 min and then centrifuged at 12000 RPM at 4°C for 15 min. After that the upper phase was transferred to an RNase-free tube and an equal volume of isopropanol was added to it. The tube was mixed gently and incubated on ice for 15 min and then was centrifuged at 12000 RPM at 4°C for 15 min. Next, the supernatant was discarded and 1 ml of 75% ethanol was added to the tube and vortexed to dissolve the pellet and centrifuged at 7500 RPM at 4°C for 8 min. After centrifugation, the supernatant was discarded and the pellet was allowed to dry at room temperature for a few minutes (it should not dry completely). Then the pellet was dissolved in 50 µl of double distilled water containing 1 mM EDTA, and stored at -70°C for later analyses.

Table 1: Percentage of positive samples in RT-PCR analysis using primer set PH5/PH7

Farm	Number of samples	Positive (%)
Broiler grandparent	60	0/100 (0%)
Broiler breeders[a]	180	15/180 (8.33%)
Commercial layer	60	0/100 (0%)
Native fowls[b]	70	17/70 (24.28%)
Total	370	32/370 (8.65%)

[a]: The samples were selected from three broiler breeder flocks. [b]: The samples were selected from two flocks of native fowls

Oligonucleotide primers

A specific primer set (H5/H7) for ALV-J was used for PCR detection. Primer H5 was designed against the 3' region of the pole gene and containing nucleotides 5258 to 5277 (5´-GGATGAGGTGACTA AGAAA G-3´) and primer H7 was designed from a well conserved region of the gp85 sequence of the variant viruses containing nucleotides 5783 to 5802 (5´-CGAACCAAAGG TAACACACG-3´) (Smith *et al*., 1998).

Reverse transcription

Synthesis of first-strand cDNA was performed using AccuPower RT PreMix (Bioneer, Korea) in a 20 µl reaction. The reverse transcription included the following

protocol: 10 µl of the template RNA with 10 pmol forward and reverse primers was mixed in a sterile tube and incubated at 70°C for 5 min and then placed on ice. Next, the incubated mixture was transferred to an AccuPower RT PreMix tube, and then the reaction volume was filled with DEPC treated water. The tube was vortexed and briefly spun down to dissolve the lyophilized pellet. Mineral oil was added to the tube and then incubated at 42°C for 60 min for synthesis of cDNA. For inactivation RTase, the tube was then incubated at 94°C for 5 min.

Polymerase chain reaction

The reaction volume for second strand cDNA synthesis and PCR amplification was 25 µl including 5 µl of cDNA, 2.5 µl of 10 × PCR buffer, 0.5 µl of dNTPs with a final concentration of 0.2 mM, 0.5 µl of 50 mM $MgCl_2$ with a final concentration of 0.5 mM, 10 pmol of forward and reverse primer, and 1 U Taq DNA polymerase. The amplification was performed in a thermal cycling system (Eppendorf) with a "touch-down" PCR program (Smith *et al*., 1998). The following steps were used for the amplification: denaturation at 93°C for 1 min, annealing at 60°C for 1 min decreasing by 1°C in each cycle and extension at 72°C for 90 s for 13 cycles, followed by 30 cycles of 93°C for 1 min, 48°C for 1 min, 72°C for 1 min 30 s, with a final extension at 72°C for 10 min. The PCR product was loaded onto a 1.5% agarose gel containing 10 ng/ml of ethidium bromide and was run at 100 V/cm for 45 min and then visualized with an UV transiluminator. The strain HPRS-103, as prototype for subgroup J ALV and egg albumen without ALV-J which was tested many times were used as positive and negative controls, respectively. Also, double distilled water was used as a blank in the tests.

RT-PCR products sequencing and alignments

The PCR products were submitted to Millegen Co. in France for direct sequencing. Randomly 10 samples of PCR products were chosen and sequenced using both sense (H5) and antisense (H7) primers, which resulted in 100% similarity between the sense and antisense nucleotide sequences. The nucleotide sequences generated from the sequenced samples were aligned with the published sequence of HPRS-103 as the prototype for subgroup J using the computer program the Clustal W method, as implemented in MegAlign (DNASTAR Inc., Madison, WI) and Multiple sequence alignment with hierarchical clustering (F. CORPET, 1988, Nucl. Acids Res., 16 (22), 10881-10890). The accession number of the sequence of HPRS-103 was Z46390.1.

Results

Positive cases were detected in 15 of 180 (8.33%) samples from three broiler breeder farms, 17 of 70 (24.28%) samples of native fowls, and none of the samples of commercial layer and broiler grandparent farms (Table 1 and Fig. 1). No viral

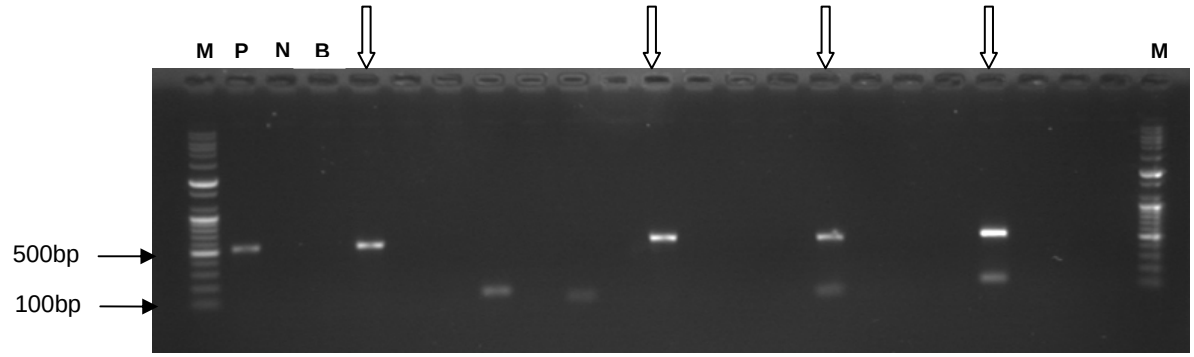

Fig. 1: RT-PCR amplifications of RNA extracted from egg albumen samples using primer set PH5/PH7. The electrophoresis was performed on ethidium bromide stained agarose gel (1.5%). Lane M: 100 bp DNA marker, Lane P: positive control, Lane N: negative control, and Lane B: Blank sample. The arrows indicate positive samples and the rest of the samples are negative. PCR product is 545 bp

Table 2: Sequencing results of RT-PCR products

Farm	Number of samples	Samples name	Percentage of homology of samples to HPRS-103	
Native fowls[a]	5	L 1-5	L1 and L4 (98%)	L2, L3 and L5 (100%)
Broiler breeders[b]	5	L 6-10	L6 and L8 (98%)	L7, L9 and L10 (100%)
Total	10		4 of 10 (40%)	6 of 10 (60%)

[a]: The samples were selected from two flocks of native fowls. [b]: The samples were selected from three broiler breeder flocks

amplification was detected in the negative control sample.

Randomly 10 RT-PCR products were chosen from positive samples from flocks of native fowls production (n=5, referred to as L1-5), and broiler breeders (n=5 referred to as L6-10) (Table 2). These RT-PCR products were sequenced using both sense (H5) and antisense (H7) primers which resulted in 100% similarity between the sense and antisense nucleotide sequences. Comparing the sequence data of the samples together showed that the samples L1, L4, L6, and L8 and samples L2, L3, L5, L7, L9 and L10 were completely similar to each other. Samples L1 and L2 showed a 98% homology to each other. Comparing the samples L1 and L2 with HPRS-103 showed 98 and 100% similarity to it, respectively (Fig. 2). Analysis of the deduced amino acid sequences of L1 and L2 in comparison to that of HPRS-103 showed 98 and 100% similarity to it, respectively (Fig. 3).

Discussion

In Iran, the presence of avian leukosis viruses (ALVs) was reported (Mohammadi *et al*., 2008), but regarding the ALV subgroups, especially subgroup J, no report was available. In this investigation an RT-PCR assay along with direct sequencing were used for detection of ALV-J in albumen of commercial and native fowl eggs in Fars province, Iran. RT-PCR analysis showed that positive cases were 32 of 370 (8.65%) eggs, indicating the presence of ALV-J in these farms except broiler grandparent and commercial layer farms. None of the samples taken from the broiler grandparent farm were positive in this test, which might be due to the high level of biosecurity, good hygiene in rearing practices, good management and selection of chicks from ALV-J-free flocks.

Although egg-type chickens are experimentally susceptible to ALV-J, naturally infected commercial egg-type chickens are rare in the world. Xu *et al*. (2004) reported the infection of ALV-J in commercial brown layer flocks in northern China and also Gingerich *et al*. (2002) reported the occurrence of ALV-J in commercial White Leghorn flocks used to produce eggs for human vaccine production. In the present study, none of the samples of the commercial layer farm were positive, which was expected, but most of the infections were detected in the flocks of native fowls, and there were 17 positive samples out of 70 (24.28%). The reason for the high prevalence of ALV-J infection in flocks of native fowls is probably due to the selection of infected primary chicks used to establish these flocks. Also, inbreeding and lack of an eradication program could exacerbate this problem. In this investigation the infections were found in 8.33% of broiler breeders' samples. Whereas this virus is horizontally and vertically transmitted in chicken (Fadly and Payne, 2003), the presence of ALV-J in the broiler breeder flocks lead to infection in broiler chickens. Although ALV-J cannot develop neoplastic lesions in broiler chickens because of their short rearing period, it may induce immunosuppression (Hair-Bejo *et al*., 2004).

Analysis of direct sequencing data of 10 randomly selected RT-PCR products showed that the identity of nucleotides and predicted amino acids in samples were 98-100% homologous to HPRS-103, the prototype for subgroup J ALV.

These findings indicated that some isolates in our study demonstrate variation in their nucleotides and predicted amino acids sequences. These variations result from virus-encoded polymerase, recombination rates and selection pressure, possibly from an immune response (Venugopal, 1999). These variations among ALV-J viruses

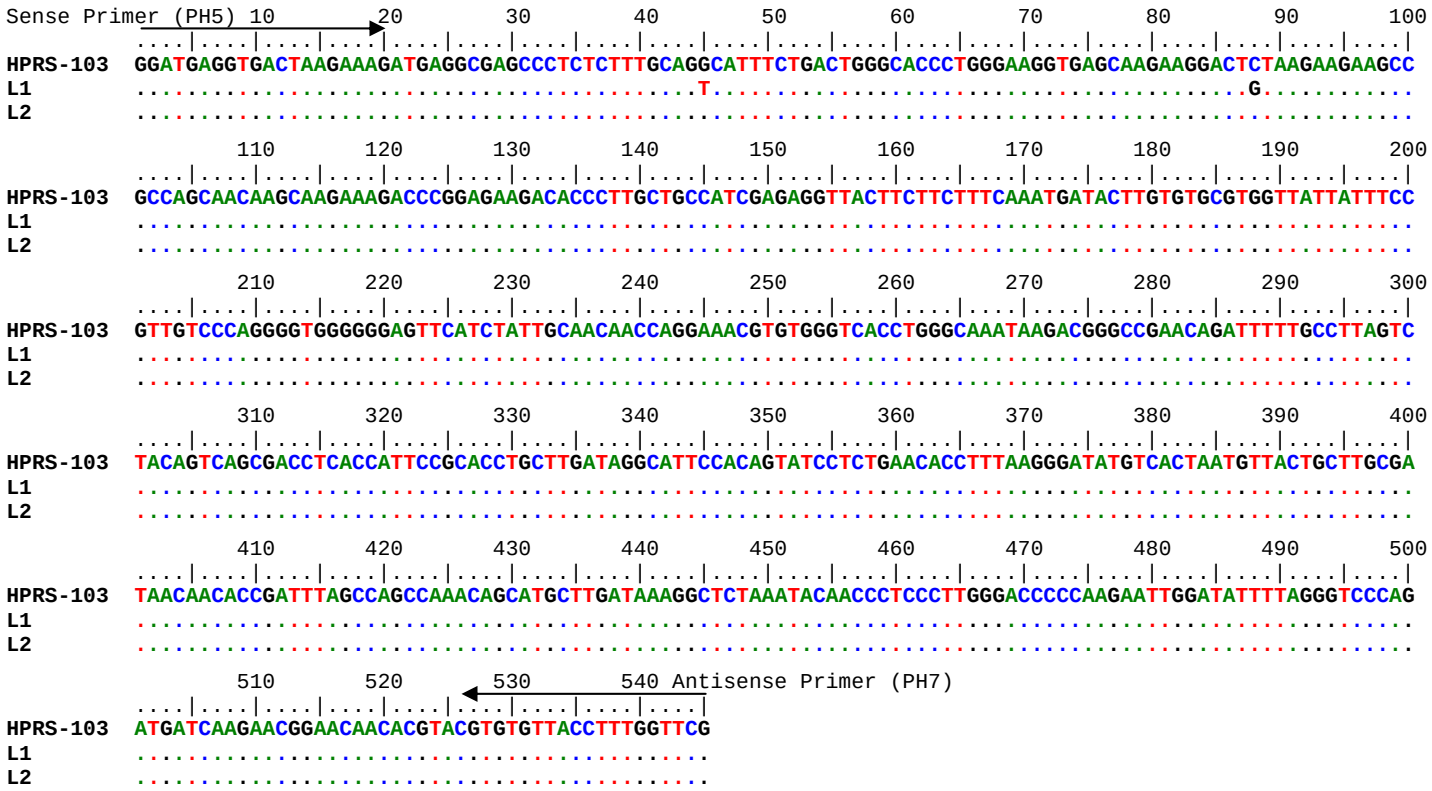

Fig. 2: Nucleotide sequence comparison of the representative samples to HPRS-103 as prototype for subgroup J. Sequences matching the HPRS-103 sequence are shown as dots. The primer set (PH5/PH7) locations that were used in RT-PCR reaction are shown with arrows. Numbers at the top represent the position of each nucleotide in the sequence

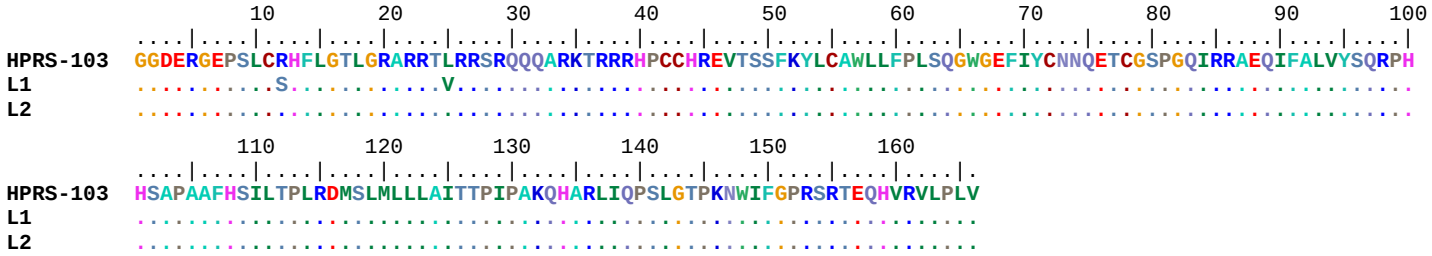

Fig. 3: Comparison of deduced amino acid sequences of representative samples to HPRS-103 as prototype for subgroup J. Sequences matching the HPRS-103 sequence are shown as dots

make it difficult to develop an effective vaccine for protection (Venugopal, 1999; Haiti *et al*., 2005).

Our results showed that the poultry industry in Iran is not free from ALV-J. This is the first report of the presence of ALV-J in egg albumen in Iran. These results indicated that subgroup J was detected only in samples from flocks of native fowls and broiler breeders, while no viruses were detected in the broiler grandparent and commercial layer farms. Since ALV-J can cause significant economic losses in the poultry industry and native fowls and there are no treatments and vaccines for these viral infections (Venugopal, 1999; Haiti *et al*., 2005), so severe quarantine is essential to prevent transmission of this virus to the broiler grandparent and commercial layer poultry. Also, much consideration should be given to eradication programs for ALV-J in the poultry industry and native fowls of Iran.

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