

Detection of avian leukosis virus (ALV) in albumen of Shiraz commercial and local layer flocks using ELISA and RT-PCR

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(Received 10 Oct 2007; revised version 24 Dec 2007; accepted 15 Jan 2008)

Summary

Avian leukosis viruses (ALVs) cause different types of tumours in poultry and can affect the health and egg production of the birds. To investigate the presence of the virus in chicken layer flocks in Shiraz, 222 egg albumen from local layer breeder (25 eggs), local layer grand parent (30 eggs), broiler breeder (60 eggs), commercial layer (46 eggs) and broiler grand parent (61 eggs) were tested by antigen-capture enzyme-linked immunosorbent assay (AC-ELISA). The results showed that 3.33, 76 and 80% of commercial broiler breeder, local layer breeder and local layer grand parent were positive, respectively. Thirty-five albumen samples were randomly selected and tested by RT-PCR using PU₁/PU₂ and PA₁/PA₂ primer sets. The samples with ELISA S/P ratio equal or more than 0.17 were positive by RT-PCR using PA₁/PA₂ primers. This is the first report of the presence of the ALV in egg albumen samples of chicken layer flocks in Shiraz.

Key words: ELISA, RT-PCR, ALV, Iran, Egg

Introduction

Avian leukosis virus (ALV) is one of the important causative agents of lymphoid and erythroid leukosis in industrial chickens. The quantity and quality of egg production are decreased in the infected laying hens (Gavora *et al.*, 1980). The chickens show tumours in different viscera, and sporadic deaths usually happen (Payne and Fadly, 2003). The virus spreads horizontally and vertically among the chickens in the infected flocks (Crittenden and Witter, 1978; Spencer *et al.*, 1983).

Avian leukosis viruses belong to the family Retroviridae and the genus *Alpharetrovirus*. These viruses are divided to six subgroups including A, B, C, D, E and J on the basis of the gp85 nucleotide sequences (Murphy *et al.*, 1999; Pham *et al.*, 1999a).

The gp85 and gp37 nucleotide sequences

“make” the gene *env* (Pham *et al.*, 1999b). In commercial poultry, subgroups A and B are the most common ALVs, while subgroups C and D have been rarely reported. Subgroup E includes the endogenous leukosis viruses of low pathogenicity. Subgroup J behaves as an exogenous virus causing mainly myeloid and nephroma in broiler chickens (Payne and Fadly, 2003).

There are several methods for detecting ALV, including detection of viral group-specific antigen (GSA) p27 by the antigen-capture enzyme-linked immunosorbent assay (AC-ELISA) and immunofluorescence assay (IFA) (Ignjatovic and Bagust, 1982; Spencer *et al.*, 1983). Virus isolation is another technique but it takes about two weeks to identify the isolated virus. Reverse transcription polymerase chain reaction (RT-PCR) is one of the rapid and sensitive tests to detect ALV genome in the samples

(Okazaki *et al.*, 1979; Smith *et al.*, 1997; Pham *et al.*, 1999a; Payne and Fadly, 2003), so it is a usual method in diagnosis of the disease. The best samples for detecting exogenous ALVs are egg albumen and cloacal swab. A reverse transcription PCR is also developed to detect ALV RNAs in the chicken egg albumen (Pham *et al.*, 1999a, b).

There is no report of serological and molecular detection of ALV in Iran; meanwhile, veterinary clinicians reported the tumour signs in different organs of chicken in the country. Although the genome of the Marek's disease virus (MDV) was detected by PCR in some tumour samples obtained from commercial chickens in Iran, there were many tumour samples that had negative results for MDV genome (Mohammadi *et al.*, 2005). The present investigation was undertaken to detect ALV in the egg albumen of chicken layer flocks in Shiraz.

Materials and Methods

Specimens

Two hundred and twenty-two egg albumen samples were obtained from the farms in Shiraz, including local layer breeder (25 eggs), local layer grand parent (30 eggs), broiler breeder (60 eggs), commercial layer (46 eggs) and broiler grand parent (61 eggs). Five pigeon and two quill eggs were also obtained as the negative control samples (Table 1). About 1 ml of egg albumen was transferred to the 1.5 ml tubes and stored at -70°C until used.

ELISA: The AC-ELISA test was performed by IDEXX Kit procedure. Briefly, 100 μl of egg albumen was dispensed into the wells coated with the antigen p27 antibody in

duplicate. After 1 h incubation at room temperature and washing, 100 μl of rabbit anti-p27 HRPO conjugate was added into each well. The TMB substrate reaction was stopped by the addition of stop solution and the absorbance values were measured at 650 nm by ELISA reader (BDSL Immunoskan PLUS). Each sample with S/P ratio equal to 0.2 or more was considered positive.

RNA extraction: RNA extraction from the egg albumen had been performed previously using Promega RNA isolation kit but with some modifications because egg albumen coagulates into a hard protein mass in the RNA extraction process (Pham *et al.*, 1999a). In this study, Cinnagene RNA isolation kit was used without any modification.

Thirty-five albumen samples were randomly selected to extract RNA by RNA isolation kit (Cinnagene, Iran). Fourteen out of 35 samples had S/P ratio equal to or more than 0.2 and considered positive in ELISA and 21 samples were negative (S/P<0.2). Briefly, 100 μl of albumen was added to 1 ml of RNX solution provided by the kit. After vigorous shaking, 200 μl of chloroform was added and mixed by vortex. The aqueous phase obtained by spin down at 15 000 g at 4°C for 15 min. The RNAs were precipitated with equal volume of isopropanol and the washing step was performed by 75% ethanol. The isolated RNAs were eluted in double distilled water containing 1 mM EDTA and 0.1% DEPC, then stored at -70°C .

RT-PCR

For cDNA synthesis, RT premix (BIONEER, South Korea) was applied in a 50 μl reaction. The template RNAs with the volume of 25 μl were used with 50 pmol

Table 1: ELISA results for GSA (p27) presence in egg albumen of chicken layer flocks in Shiraz

Poultry	Source	NO.	S/P Mean	SD	Min.	Max.	Positives*	Percentage
Local layer breeder	Marvdasht	25	0.79	1.11	0.02	5.27	19	76
Broiler breeder	Shiraz	60	0.05	0.04	-0.008	0.2	2	3.33
Local layer grand parent	Kharameh	30	1.3	1.59	0.04	5.2	24	80
Commercial layer	Shiraz	46	0.05	0.03	0.01	0.16	0	0
Broiler grand parent	Sepidan	61	0.04	0.03	0	0.14	0	0
Pigeon	Shiraz	5	0.03	0.03	0	0.07	0	0
Quill	Shiraz	2	0.03	0.01	0.025	0.04	0	0
Total		229						

*S/P ratio more than 0.2

Table 2: ALV oligonucleotide primers used in RT-PCR assays

Subgroup	Primer	Sequence (5'-3') ^a	Orientation	Product (bp)
A, B, C, D, E	PU ₁	CTRCARCTGYTAGGGYTCCCAGT	Forward	229
	PU ₂	GYCAYCACTGTGCGCCTRTCCG	Reverse	
A	PA ₁	CTACAGCTGTTAGGTTCCCAGT	Forward	229
	PA ₂	GTCACCACTGTGCGCCTATCCG	Reverse	

^a R = A/G and Y = T/C

of the primers PU₁ and PU₂ for the detection of all ALV subgroups and PA₁ and PA₂ primers for the specific detection of subgroup A (Table 2). The cDNAs were synthesized on the basis of the manufacturer's procedure. 10 µl of cDNAs were used in PCR using PCR premix (BIONEER, South Korea) containing 1 U of Taq DNA polymerase, 250 µM of each dNTP, 10 mM tris-HCl (pH = 9), 40 mM KCl and 1.5 mM MgCl₂ in 20 µl of reaction. Twenty pmol of the same primers were used in the reactions. The program used in the thermal cycler (Eppendorf) is as follows: 94°C for 5 min and 40 cycles of 94°C for 30 s, 60°C for 1 min and 68°C for 2 min. The strain LR-9 was used as the positive control. Gel electrophoresis was conducted on the amplified RT-PCR products using 1% agarose (Merck) with 2.5 mg/ml of ethidium bromide and visualized using an UV transilluminator.

Results

ELISA

The assays showed that most of the tested commercial chicken eggs did not have GSA in their albumen. Only two egg albumens from a broiler breeder farm had S/P ratio equal to 0.2, but 76 and 80% of the tested chicken eggs from the local layer breeder and local layer grand parent farms had S/P ratio more than 0.2, respectively (Table 1). The tested eggs of these two flocks had a mean S/P ratio of 0.79 and 1.3 in a range of 0.02 to 5.27 and 0.04 to 5.2 levels of GSA in the albumen, respectively (Table 1). The maximum absorbance value (OD) was 0.95.

RT-PCR

Molecular analysis on egg albumen samples from the commercial and local farms was performed using two pairs of

oligonucleotide primers including PU₁ and PU₂ for all subgroups of ALV_S and PA₁ and PA₂ specific for subgroup A (Table 3; Fig. 1).

Table 3: Comparison of RT-PCR and ELISA results of the eggs' albumen with different S/P ratios

ELISA S/P ratio	Number ^a	RT-PCR ^b	RT-PCR ^c
S/P>0.2	14	14(100%)	14(100%)
0.2>S/P>0.17	6	6(100%)	6(100%)
S/P<0.17	15	5(33.3%)	0

^a Tested albumens with both ELISA and RT-PCR, ^b Using PU₁ and PU₂ primers that detect all ALV subgroups and ^c Using PA₁ and PA₂ primers specific for subgroup A

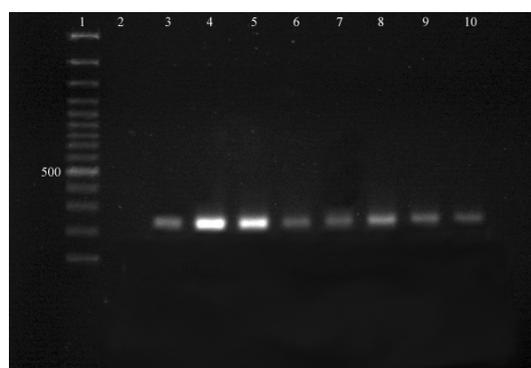


Fig. 1: RT-PCR analysis of egg albumen using PA₁ and PA₂ primers. Lane 1: 100 bp DNA marker, Lane 2: pigeon egg albumen as negative control, Lane 4: strain LR-9 as positive control and Lane 3 and Lane 5-10: positive samples. PCR product is 229 bp

All 20 egg albumen samples that had S/P ratio equal to or more than 0.17 and tested by RT-PCR using both primer pairs were positive (Table 3). From 15 egg albumens that had S/P ratio less than 0.17, five eggs which obtained from a commercial broiler grand parent were positive (5/15) with PU₁ and PU₂ primers and the rests were negative (10/15), but all of them were negative in RT-PCR analysis using PA₁ and PA₂ primers (15/15) (Table 3).

Discussion

Detection of avian leukosis virus in egg albumen is very important for studying the circulation and transmission of ALVs among chickens in an area (Smith *et al.*, 1979; Clark *et al.*, 1981; Payne *et al.*, 1983; Spencer *et al.*, 1983; DeBoer and Osterhaus, 1985; Spencer and Chambers, 1992). In this investigation, the most infections were observed in the local layer grand parent and local layer breeder eggs with 80 and 76% occurrences, respectively; while only two eggs of broiler breeder were found positive (3.33%) (Table 1). Although this study was not an epidemiological survey but the results showed that the prevalence of infection might be low in Shiraz industrial poultry farms. According to high percentage of infection in Shiraz local farms, studies on the control and eradication of the disease must be taken into consideration. Eggs screening test with ELISA and RT-PCR before chicken inbreeding could be useful in preventing the disease in the progeny by stamping out the infected breeders.

Based on the previous studies, the levels of GSA in egg albumen infected with exogenous ALV are usually higher than the eggs infected with endogenous ALVs (Spencer and Chambers, 1992). The mean S/P ratio of egg albumen obtained in ELISA were 1.3 and 0.79 for local layer grand parent and local layer breeder, respectively (Table 1). Since the manufacturer recommended the S/P ratio of 0.2 as the cut-off point, it is clear that there were a severe infection in those two farms.

The results of this study showed that each sample with ELISA S/P ratio of 0.17 or more is positive in RT-PCR using both pairs of primers, so the cut-off point of 0.17 may be more appropriate border to detect GSA produced by exogenous ALVs in the egg albumen.

On the other hand, the egg albumen with S/P ratio less than 0.17 were negative in RT-PCR test using PA₁/PA₂ primers but were positive in some eggs (5/15) using PU₁/PU₂ primers (Table 3). Therefore, it seems that RT-PCR test using the specific primers for subgroup A may detect the exogenous ALVs in the egg albumen.

It was shown that RT-PCR assay is more

sensitive than ELISA to detect ALV in the egg albumen (Pham *et al.*, 1999a, b). It was also shown that the samples with absorbance value up to 0.54 were positive for endogenous ALVs in RT-PCR but the samples that were positive for exogenous ALV had absorbance values more than 0.54 (Pham *et al.*, 1999b). In this study, the absorbance values of some samples were even more than 0.9 that shows the severity of the infection with exogenous virus.

According to the study by Pham *et al.* (1999b), RT-PCR assay along with direct sequencing of RT-PCR product is more accurate for the detection of virus and discrimination of viral subgroups. Therefore, our next research will focus on the study of molecular epidemiology of avian leukosis in chicken flocks of Fars province of Iran.

Since ELISA can not distinguish exogenous ALVs from endogenous, it seems that RT-PCR assay by using the primers specific for subgroup A is an appropriate test to show the exogenous ALVs.

This is the first report of egg infection with ALV in chicken layer flocks in Shiraz.

Acknowledgements

We thank vice dean for research affairs of Veterinary School and research chancellor of Shiraz University for financial support of this project.

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