

## DETECTION OF AVIAN LEUKOSIS VIRUS USING POLYMERASE CHAIN REACTION AND ENZYME LINKED IMMUNOSORBENT ASSAY

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### SUMMARY

The applications of polymerase chain reaction (PCR) and enzyme linked immunosorbent assay (ELISA) in detecting avian leukosis virus (ALV) subgroups A and J were studied in a flock of breeder chickens. Out of 74 chickens tested 9, 36, 13 and 16 were found positive for both p27/gp85, negative for both, positive for p27 and positive for gp85, respectively. All the chickens that were found positive for p27 antigen were also positive for subgroup A proviral DNA. Although 25 chickens were positive for subgroup J gp85 antibody, none of the chickens were found positive for subgroup J proviral DNA. Hence, detection of p27 antigen from cloacal swab was found to correlate more with detection of proviral DNA. However, none of the chickens were found positive for viral RNA and did not shed infectious viruses. In conclusion, accurate diagnosis of ALV infection requires more than one laboratory test for confirmation.

Keywords : avian leukosis virus subgroup A and J, PCR, ELISA and virus isolation

### INTRODUCTION

Avian leukosis virus (ALV) subgroup is a C-type retrovirus causing varieties of tumour in chickens. ALV can be further classified into six subgroups: A, B, C, D, E and J based on virus neutralisation, virus interference assay and host range (Bova *et al.*, 1986). Subgroup A is widespread and easily detected in chickens followed by subgroup B, whereas subgroups C and D are rarely found (Spencer, 1984). However, subgroup E is abundant in chicken genome, mostly integrated with host genome and transmitted to chicks by Mendelian fashion (Austrin *et al.*, 1979). The newly identified subgroup J is associated with myeloid leukosis mainly in meat type chicken (Payne, 1992) and also in layer chickens (Gingerich *et al.*, 2002).

Several diagnostic tests are available for the diagnosis of ALV. The most common practice of diagnosis of ALV-J is virus isolation in endogenous virus resistant-specific cell culture, followed by ELISA for screening and PCR for molecular level confirmation (Smith *et al.*, 1998a; 1998b; Venugopal, 1999; Fadly, 2000; Silva *et al.*, 2000; Zavala *et al.*, 2002). The most commonly used test for screening of flocks for ALV is p27 based ELISA. p27 is a group specific antigen commonly secreted from chickens infected with ALV (Fadly, 2000). Meanwhile a subgroup specific ELISA based on gp85 antigen is for the detection of ALV-J specific antibody. Both test kits are manufactured commercially by Idexx Laboratories, USA. PCR technique has been found to be rapid, specific and more sensitive than the available conventional diagnostic

tests (Smith *et al.*, 1998b). Primers specific to the various subgroups of ALV have been developed and tested on various samples (Smith *et al.*, 1998a; 1998b; Silva *et al.*, 2000; Pham *et al.*, 1999). Virus isolation in specific chicken embryo fibroblast (CEF), which is resistant to endogenous virus, is also an important diagnostic tool for diagnosis of ALV-J. Since most ALV do not produce visible morphologic changes in the cell culture, it should be followed by other biological assays such as PCR, ELISA, immunofluorescent and/or immunoperoxidase tests.

Previously it has been proposed that chickens exposed to subgroup J ALV are associated with immunosuppression that lead to the outbreak of Newcastle disease (Asiah *et al.*, 2001; Thapa *et al.*, 2004). However, no virus was isolated following inoculation into DF-1 cells (Thapa *et al.*, 2004). Hence, this study explores the applications of several laboratory techniques in detecting ALV in a flock of clinically healthy broiler breeder chickens that have the history of ALV-J infection.

### MATERIALS AND METHODS

#### Chickens

Seventy-four culled commercial broiler breeder chickens age > 65 weeks were used in this study. The chickens were obtained from different flocks of broiler breeder chickens with a history of exposure to ALV-J. The chickens were kept in an experimental isolation unit. Feed and water were provided *ad libitum*. After 7 to 10

**Table 1: Various samples collected from broiler breeder chickens used for detection of ALV**

Samples	Detection methods	Application
Whole blood	PCR Virus isolation	Proviral DNA of subgroup J and A ALV Subgroup J and A ALV
Serum	ELISA	gp85 specific antibody of subgroup J ALV
Cloacal swab	ELISA	p27 common antigen for ALV
	PCR	Proviral DNA of subgroup J and A ALV
	RT-PCR	Viral RNA of subgroup A ALV
	Virus isolation	Subgroup J and A ALV
Tissues	PCR	Proviral DNA of subgroup J and A ALV

days, the chickens were killed and samples such as whole blood, cloacal swab and visceral organs were collected and tested by different detection techniques (Table 1).

#### *Enzyme linked immunosorbent assay*

The ELISA procedures were carried out as recommended by the manufacturer (Idexx Laboratories, USA). A total of 74 samples of cloacal swabs were collected in sample diluent provided in the assay kit and examined for p27 antigen. Likewise, serum samples were also obtained from those 74 chickens for the detection of subgroup J gp85-specific antibody.

#### *Extraction of nucleic acids*

DNA from whole blood, cloacal swabs and tissue samples were extracted using the Wizard Genomic DNA Purification Kit (Promega, USA). The extraction procedures were performed as recommended by the manufacturers. The concentration and purity of the extracted DNA were determined by spectrophotometer (Beckman, USA) according to the method described by Sambrook *et al.* (1989).

#### *PCR amplification of proviral DNA*

PCR was carried out directly from the DNA extracted from various samples using primers specific to subgroup J: H5 (5'-GGATGAGGTGACTAAGAAAG-3'), H7 (5'-CGAACCAAAGGTAACACACG-3') and subgroup A: PA1 (5'-CTACAGCTGTTA GGTTCACAGT-3'), PA2 (5'-GTCACCACTGTCGCCTATCCG-3'). Briefly, PCR was carried out in 50 µl reaction mixture containing 1 µg DNA, 5 µl 10x PCR Buffer (Mg free), 1 µl 10mM dNTP mixture, 4 µl 25 mM MgCl<sub>2</sub>, 0.5 µl Taq Polymerase (2.5u/µl) (Promega, USA), 1 µl (25 pmole) each of specific forward and reverse primers of each subgroup J and A and nuclease-free distilled water was added and made to a total volume. The primer sequences and PCR cycle conditions were followed as described by Smith *et al.* (1998b) for subgroup J and Pham *et al.* (1999) for subgroup A.

#### *Reverse transcription-polymerase chain reaction*

Samples that were found positive for proviral DNA were tested for viral RNA using One Shot Access RT-PCR (Promega, USA). Briefly, a total reaction mixture of 50 µl was prepared in 0.2 ml PCR tube by adding: 10 µl 5X AMV Reverse Transcriptase buffer, 2 µl 25 mM MgSO<sub>4</sub>, 2 µl 10 mM dNTP, 1 µl each 25 pmole of the forward and reverse primers, 1 µl RT-AMV enzyme, 1 µl Taq polymerase, 2 µl Rnase Inhibitor Promega, USA), 0.5 µg of RNA and RNase free water. The reaction mixtures were then incubated in thermal cycler (MJ Research, USA) at 48°C for 45 min for cDNA synthesis followed by 94°C for 2 min to inactivate the RT enzyme. The cDNA was amplified with the following cycle conditions: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 68°C for 2 min for 39 cycles and with a final extension at 68°C for 7 min and then 4°C to stabilise PCR product.

#### *Agarose gel electrophoresis*

The amplified products were run on 1 to 1.7% agarose gel electrophoresis at 60 volts for 1 to 1.5 hours. The gel was then stained with ethidium bromide (0.5 µg/ml) and photographed under UV illumination.

#### *Virus isolation*

An attempt was carried out to isolate subgroup A and J viruses in endogenous resistant CEF, DF-1 cells (ATCC, USA). The cell line was derived from a fibroblast of 10 day old East Lansing Line (ELL-0) chicken embryo, which is resistant to subgroup E endogenous virus but susceptible to all other exogenous viruses: A, B, C, D, and J.

Blood and cloacal swab samples of four broiler breeder chickens randomly selected from each of the four groups were processed and inoculated into DF-1 cells. Briefly, 100 µl inoculum was infected in 25cm<sup>3</sup> flask and incubated at 37°C in 5% CO<sub>2</sub> humidifier incubator for an hour for virus adsorption. An additional 6 ml 2% fetal bovine serum containing DMEM media (Life Technologies, USA) was added and the flask was maintained up to 7 days. Media from the infected cell

**Table 2: Percentage of chicken grouping based on their immunological status**

Groups	p27 specific	gp85 specific	No. of chickens positive	Percentage (%)
1	+	+	9	12.2
2	-	-	36	48.7
3	+	-	13	17.6
4	-	+	16	21.6

+ = positive; - = negative

**Table 3: Comparisons between proviral PCR and ELISA for detection of ALV from whole blood samples**

Subgroups	PCR profiles	Groups of chickens based on ELISA profiles			
		p27+/gp85+	p27-/gp85-	p27+/gp85-	p27-/gp85+
J	+	0/9	0/9	0/9	0/9
	-	9/9	9/9	9/9	9/9
A	+	9/9	2/9	9/9	2/9
	-	0/9	7/9	0/9	7/9

+ = positive, - = negative

**Table 4: PCR detection of ALV from various tissue samples collected from broiler breeder chickens in group 1 (p27+/gp85+)**

Subgroup	PCR Profiles	Blood	Cloacal swab	Tissues*
J	+	0/9	0/9	0/9
	-	9/9	9/9	9/9
A	+	9/9	9/9	0/9
	-	0/9	0/9	9/9

\* Liver, lung, kidney, spleen and/or heart

culture was inoculated into fresh DF-1 cells and the cells were passed up to the third passage. Evidence of virus replication was tested by indirect immunofluorescence antibody test (IFAT). The IFAT was performed using monoclonal antibody against gp85 antigen (Thapa, 2004). The infectivity of the cell culture was also analysed by PCR detection using DNA extracted from concentrated cell suspensions.

## RESULTS

### *Enzyme linked immunosorbent assay*

The status of ALV in the broiler breeder chickens was determined by using ELISA that detects p27 antigen from cloacal swabs and gp85 ALV-J antibody. From a total of 74 chickens examined, 25 chickens were found positive for subgroup J gp85 antibody while 22 chickens that were examined had p27 ALV antigen. Based on the ELISA profiles, the broiler breeder chickens were divided into four groups; Group 1 (p27+/gp85+), group 2 (p27-/gp85-), group 3 (p27+/gp85-) and group 4 (p27-/gp85+). As shown in Table 2, only 9 out of 74 (12.2%) chickens examined were positive for both p27 antigen and gp85

antibody while 13 (17.6%) and 16 (21.6%) chickens were found positive for either p27 and gp85 respectively. A total of 36 out of 74 chickens examined (48.7%) were negative for both p27 antigen and gp85 antibody.

### *Polymerase chain reaction*

Detection of subgroups ALV-J and ALV-A proviral DNA was performed on the blood, cloacal swab and tissue samples from nine broiler breeder chickens selected from each of the four groups. As shown in Table 3 although chickens in group 1 were p27+/gp85+, none of them had ALV-J proviral DNA in their blood samples. No specific amplification of the expected size (~550 bp) was observed (data not shown). However, all the 9 chickens in group 1 had ALV-A proviral DNA in their blood and cloacal swab samples. In the case of chickens in group 2 (p27-/gp85-), 2 out of the 9 chickens had ALV-A proviral DNA whereas all chickens tested had ALV-A proviral DNA in group 3 (p27+/gp85-). Only 2 out of 9 chickens tested in group 4 (p27-/gp85+) had ALV-A proviral DNA (Table 3). The expected size PCR product for subgroup A proviral DNA is ~200 bp (data not shown). All the tissue samples from the 9 chickens from group 1 were negative

for proviral ALV-J (Table 4). Likewise, all the tissue samples tested also had no ALV-A proviral DNA (Table 4). Even though chickens from the different groups had subgroup A proviral DNA based on PCR examination of blood and cloacal swab, none of them were positive for subgroup A ALV RNA.

#### *Virus isolation*

Blood and cloacal swab samples were inoculated into DF-1 cells and maintained up to three passages. However, none of infected DF-1 cells were positive for ALV based on PCR detection using subgroup A and J specific primers. In addition, IFAT using subgroup J ALV specific monoclonal antibody were also negative (data not shown).

### **DISCUSSION**

The main objective of this study was to explore the applications of PCR and ELISA for the detection of ALV from a group of broiler breeder chickens that have history of ALV-J exposure. Out of 74 chickens tested, 25 chickens were found positive for subgroup J gp85 antibody while 22 chickens had p27 ALV antigen. However, none of the blood, cloacal swab and tissues samples from those chickens were positive for ALV-J proviral DNA. The actual explanation for this finding is not known. Probably the ELISA kit used in this study detects non-specific antibody due to endogenous expression of *env* gene. In addition, all the chickens tested were more than 60 weeks old. Another interesting observation was the average S/P ratio of sera that were positive were only 0.876 (ranging from 0.603 to 1.350) which was considered to be low to moderate. It has been shown previously that breeder flocks of age more than 55 weeks that have the history of exposure to ALV-J developed high antibody titer (Siew, 2001). A recent study by Hwang and Wang (2002) showed that the anti-ALV-J antibody of infected flock is higher than that of uninfected flock.

The ability of PCR to detect subgroup A ALV in p27-/gp85- and p27-/gp85+ groups of chickens suggests that PCR is more sensitive compared to serology and virus isolation. Similar finding was noted by Smith *et al.* (1998b) for ALV-J. In addition, the use of other primer combinations designated from LTR region (Smith *et al.*, 1998a; Garcia *et al.*, 2003) indicated that PCR is more sensitive than ELISA and virus isolation. A good correlation was found between p27 ELISA and PCR, as all the chickens that were positive for p27 were found positive by PCR for the detection of subgroup A proviral DNA from blood and cloacal swab samples. In earlier studies, it has been established that there is a good correlation between p27 based ELISA and PCR detection of ALV-J (Smith *et al.*, 1998a; 1998b). In this study, the PCR test was performed using plasmid encoding for gp85 of ALV-J as positive control. In all the samples, no specific

amplification of PCR product of the expected size was detected. The size of the expected PCR product using the H5/H7 primers is 545 bp (Thapa *et al.*, 2004). In the case of PCR detection of subgroup A proviral DNA, a clear expected size product of ~200 bp was detected from blood and cloacal swab samples from chickens that were positive for p27 antigen (Table 3 and 4). Based on the sequences of the PA1/PA2 primers, the size of the amplified product of subgroup A proviral DNA is 229 bp (Pham *et al.*, 1999).

Among the various samples tested for subgroup A, blood and cloacal swab samples were found to be more appropriate for the detection of proviral DNA. However, no subgroup A ALV RNA was detected from chickens that were positive for proviral DNA. The actual explanation is not known, but probably the chickens were not shedding the virus due to immunological responses. This may also explain the inability to isolate subgroup A virus from inoculation of proviral DNA positive samples into DF-1 cells. Alternatively, the inability to detect subgroup A viral RNA is probably due to the low level of viral RNA beyond the detection limit of the RT-PCR. Previously, Nebhya *et al.* (1990) have suggested that low copy number of viral RNA due to immune pressure might affect the transcription of viral mRNA from the integrated proviral DNA. In this study, we also failed to detect proviral DNA from gp85 positive chickens suggesting that the integrated proviral copy number may be very low or absent due to immune pressure. It has been shown that immune pressure was able to modulate the status of integrated proviral DNA in host cells (Luciw and Leung, 1992). Similar result has been observed by Baba and Humpheries (1984) where no proviral DNA was observed in thymus following RAV-1 infection. Hence, a combination of diagnostic tests should be used for routine examination of suspected cases in order to rule out false-negative findings (Malkinson *et al.*, 2004).

In conclusion, PCR from blood and cloacal swab was found to be rapid, easy and more sensitive than other conventional methods, such as ELISA and virus isolation for the detection of ALV proviral DNA. However, the application of PCR in the detection of proviral DNA and viral RNA from various tissues samples requires careful interpretation since the status of ALV may be different depending on various factors such as sensitivity of ELISA, immunotolerance, presence of neutralizing antibody and integrated site of proviral DNA. The limitation of this study was that it was carried out in clinically healthy culled broiler breeder chickens. Perhaps, the isolation of ALV viruses is best performed from chickens showing clinical signs of ALV infection due to active shedding of the virus.

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