行政院國家科學委員會補助專題研究計畫成果報告

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※家禽白血病 J 亞群病毒偵測方法之建立 ※

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Establishment of Detection method for subgroup J avian leukosis virus

計畫類別：■個別型計畫　　□整合型計畫

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計畫主持人：王金和

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一、中文摘要

家禽白血病 J 亞群病毒 (Subgroup J avian leukosis virus, ALV-J) 引起家禽腫瘤性疾病，造成產業經濟甚巨。一般皆以 ELISA 偵測本病感染的情形，然而 ELISA 無法分辨外源性及內源性病毒的感染，使其應用的大受限制，本研究的目的即發展出 ELISA 方法分別雞隻受內源性或外源性病毒感染，供診斷及控制本病之應用。由臺灣三家原種雞場進行 ALV 的檢測及病毒分析，於不同週齡時採取血液樣本，以酵素免疫吸附法 (ELISA) 檢測血漿中 gs Ag (group specific antigen）、抗 subgroup J gp85 Ab，再以 RT-PCR 及核酸定序 (sequencing) 確診亞群感染。結果顯示，於 ALV-J 感染雞群早期 ALV Ag 的陽性率上升但非感染場下降；感染雞群抗 ALV-J 抗體上升較早較高。因此在 1 週齡及 6 週齡檢測雞隻血中 gs Ag 的變化情形可以分辨內源性病毒或 ALV-J 感染。此偵測方法的發現對此病的檢測及控制有莫大的助益。

關鍵詞：家禽白血病 J 亞群病毒、群特異抗原、反轉錄聚合酶連鎖反應

Abstract

Subgroup J avian leukosis virus (ALV-J) causes serious economic losses in commercial poultry industry. Measuring group-specific (gs) antigen (Ag) by ELISA has been used to identify chickens infected with this virus. However, the inability of ELISA to discriminate the gs Ags from endogenous and exogenous origin has limited its usage. The purpose of the present study was to find a method to discriminate between endogenous and exogenous ALV gs Ag by ELISA. The gs Ag and anti-ALV-J antibody in the plasma samples from chickens at different ages in three grand-parent farms were measured by ELISA. Infected flocks were confirmed by RT-PCR using different subgroup-specific primers. The results indicated that the gs Ag of ALV-J-infected flocks increased, but that of the uninfected flocks decreased in young ages. The anti-ALV-J antibody of infected flocks was higher and increased earlier than uninfected flocks. In conclusion, measuring the gs Ag in blood at the ages of 1 and 6 weeks by ELISA is suitable to discriminate between endogenous and ALV-J infection. This discovery is a valuable method for the detection and control of ALV-J infection.

Keywords: ALV-J, ELISA, gs Antigen, RT-PCR

二、緣由與目的

Avian leukosis viruses (ALVs) are prevalent in the poultry industry worldwide and cause severe economic losses from tumor mortality and condemnations (2, 13). The viruses infecting chickens are classified into 6 subgroups based on specific envelop surface glycoprotein responsible for specific viral interference patterns, virus neutralization and host range (6), which are A, B, C, D, E, and J. Subgroups A and B occur as common exogenous viruses in the field and cause principally lymphoid leukosis. Unlikely, subgroups C and D are rare (10) and subgroup E is not pathogenic for poultry. The control of subgroups A and B has been accomplished in the 1970's. However, subgroup J ALV (ALV-J) isolated in 1988 by Payne (5) has become widespread in commercial meat type during 1990's (4). It has caused severe economic loss in broiler breeders. The transmission of ALV-J is much higher than other ALV subgroups, thus making control and eradication much more difficult.

The need for a rapid sensitive, and specific test for the laboratory diagnosis of ALV-J has been recognized (2,3,6,8,11). Several methods have been developed for this diagnosis, such as virus isolation in cells, PCR for viral genome, and enzyme-linked immunosorbent assay (ELISA) for group-specific (gs) antigen (Ag) or antibody. Virus isolation is laborious and time-consuming and the proper cells are not available in many laboratories around the world.

While, the ELISA tests are the most convenient method for all laboratories and industry because they are commercialized supplied and easy to manipulate (2,12).

Most countries hold only grand-parent and their downstream stocks and want to make a diagnosis at the early time of the chickens. Serum from those chickens could be used for diagnosis because it is available during the rearing period but not cloaca or albumen from the dams. Serum is the most popular material for routine diagnosis in the poultry industry, and detecting ALV infection in serum by ELISA is the most convenient way for chickens in the world (12). In addition, Fadly (3) reported that serum was the most sensitive material for gs Ag diagnosis, although Payne (7) found that serum was not suitable for ALV infection because both exogenous and endogenous ALVs produce gs Ag. The critical point of gs Ag in serum was its non-specific false positive induced by ubiquitous endogenous ALV in chicken. Since serum is the most common sample and ELISA is the most convenient method for most laboratories in the world, the purpose of the present study is to search for a method to certify ALV-J infection by ELISA tests in serum, which could rule out the interference of endogenous virus. Indeed, the present results show that the increase of gs Ag in serum during the early ages is caused by ALV-J but not endogenous virus.

三、研究報告應含的內容

Plasma samples and ALV detection

Ninety-two to 276 plasma samples were taken each time from chickens in 3 grand parent farms at the ages of 1, 6, 16, 26, 46, 49 or 69. A, B, C, D lines in Farms 1 and 2 were male of male line, female of male line, male of female line and female of female line, respectively. In Farms 3, only parent stocks were taken, which were male line and female line (Table 1). Blood samples were put immediately into tube containing EDTA as anticoagulant (Becton Dickinson, Franklin Lakes, NJ) in ice box. Plasma was separated for ELISA test, PCR and isolation. The gs Ag of ALV and antibody to ALV-J were detected in plasma by ELISA using commercial kits (Idexx, Westbook, ME) according the manufacturer's instructions.

RT-PCR, sequencing and phylogenetic analysis

Plasma showing positive for gs Ag were performed RT-PCR with primer H5/AD1, H5/H7 (11) and PE1-PE2 (8), which were specific for subgroup A-E, subgroup J and subgroup E, respectively. RNA in plasma was extracted with TRIzol (Gibco BRL, Life Technol, Rockville, MD). The procedures of RT-PCR using each primer was performed according the method described by the primers designers. The PCR products were sent for direct sequence using ABI prism Cycle Sequencing Kit (Perkin Elmer, Branchburg, NJ). Forward and reverse sequences were performed for the insurance of correction. The nucleotide sequences of our samples were compared with the protoviruses of subgroup A, B, C, D, E, and J from GenBank. The sequences were aligned using the Clustal multiple alignment algorithm in MegAlign program (DNASTAR Inc., Madison, WI). Phylogenetic trees were established with the computer program Grow tree in SeqWeb of Taiwan (http://gcg.nhri.org.tw). The accession numbers of each subgroup were sub A: M37980; sub B: AF052428, sub C: J02342, sub D: M22730, sub E: M12172, *Gallus gallus*: Y12574, and HPRS- 103: Z46390.

Virus isolation and pathological examination

The plasma samples showing H5/H7 PCR positive, were selected for virus isolation in DF1 cell (ATCC, CRL-12203, ATCC, Manassas, VA), which was chicken embryo fibroblast and resistant to subgroup E ALV. The samples were co-culture with DF1 cell at 39 C in a CO_2 incubator. The supernatant was taken for the present of ALV-J genome by RT-PCR described above using H5/H7 primer.

Affected chickens were sacrificed on the farms for necropsy. Tissues from dead and diseased chickens were fixed in 10% buffered formalin, stained with hematoxylin and eosin, and examined for microscopic lesions. ALV-J-induced myeloid leukosis was diagnosed on the basis of characteristic gross and microscopic lesions.

Statistical analysis

Differences of percentages in different flocks were analyzed by Chi-square test at the significant level of 95% (9).

Results

Confirmation of ALV-J infection by RT-PCR

The expected sizes of RT-PCR products were obtained with respective primers, which were 325 bp with H6/AD1 to be subgroup A to E, 265 bp with PE1/PE2 to be subgroup E, and 545 bp with H5/H7 to be subgroup J. From the detection of viral genome by RT-PCR, the line B flock in Farm 1 was infected with ALV-J. This was reconfirmed by histological examination showing myeloid leukosis and by virus isolation in some dead chickens. Plasma samples and isolated viruses were confirmed to be ALV-J by specific RT-PCR using H5/H7 primer. In Farm 1, line B was confirmed to be infected by ALV-J from H5/H7 PCR (Table 2) and histopathological lesions in dead chickens but not lines A, C, and D. In Farm 2, lines A and B were confirmed to be infected but not lines C and D. In Farm 3, only male line was infected but not female line. Almost all samples showed positive with AD1/H5 primer (subgroup A to E) were positive with PE1/PE2 primer (subgroup E). So those chickens containing gs Ag were due to endogenous ALV. Samples showing positive with PE1/PE2 were

negative with H5/H7 primer except male line in Farm 3. One of the two H7/H5 positive samples showed positive with PE1/PE2 primer. This chicken might be due to dual infection.

Phylogenetic analysis

The PCR products of plasma samples from Farms 1 to 3, using H5/AD1 primer were sequenced, and the sequences were compared with reference strains of subgroups A to E from GenBank. The samples were grouped to subgroup E by phylogenetic analysis. Multiple sequence alignment analysis was used to compared the similarity of obtained sequences with those of reference strains. They had 98.4%-98.7% nucleotide similarity with subgroup A, 96.2%-97.1% with B, 99%-99.4% with C, 98.4%-98.7% with D, 99.4%-99.7% with E, and 66.3%-66.7% with J. The phylogenetic analysis of the PCR products of our samples using PE/PE2 primer were grouped into subgroup E. The identities among our samples with subgroups A to D were low (from 48.7 to 71.3) and even lower with subgroup J (48.9-19.6). However, the homologies were high with subgroup E (98.9-99.6) and with *Gallus gallus* gene. Using H5/H7, which was specific to ALV-J, the homologies of the sequences of the recent 6 PCR samples to HPRS103 were 96.3 to 97.6. The phylogenetic analysis of our samples with HPRS103 is shown in Fig. 1. Sample number 1B48 meant the number 48 chicken in line B of Farm 1. Samples from Farm 1 were grouped together with HPRS103. Samples from Farm 2, 2B30, 2A2, and 2B21, fell into the other group, while samples from Farm 3, 3M64, 3M21, fell into another group. The sequence diversity was related to the origin of the chickens.

Serological profiles

The serological profiles of gs Ag and anti-ALV-J antibody in chickens from the 3 farms are shown in Fig. 2. The infected flocks were shown by heavy lines and the uninfected flocks were shown by dotted lines. In the infected flocks, the gs Ag positive rates increased ($P < 0.01$) from 1 week old to 6 weeks old, decreased ($P< 0.01$) at the age of 16 weeks old and then maintained the same levels (Farms 1 and 2, $P > 0.05$) or decreased later (Farm 3, $P < 0.01$). On the contrary, those of the uninfected flocks decreased during those ages. The high percentages of gs Ag at 1 week old in non-infected flocks were due to endogenous virus. The anti-ALV-J antibody in infected flocks (A line in Farm 1, A line and B line in Farm 2, male line in Farm 3) increased before laying. However, those of non-infected flocks increased after laying. The antibody at the age of 1 week in line A and line D in the Farm 1 were from maternal antibody and decreased with ages.

Discussion

All chicks embryo-injected with ALV-J show blood PCR positive and serum antibody negative from the age of 16 days to 79 days of age. Contact-infected chicks begin blood PCR positive by the age of 16 days and reach a peak from the age near 5- 6 weeks (11,14). Thus, we selected the 6 week-old samples for PCR detection. To confirm ALV-J infection by the use of PCR or RT-PCR was found to be specific and more sensitive than the other diagnostic tests for the detection of ALV (11). The primers used in the present study were specific for each subgroup (8, 11). Based on the PCR and sequence results, chicken flocks were confirmed to be infected with specific ALV subgroups. Based on the RT-PCR results, this study provides a simple method to confirm the ALV-J infection in a flock at the early stage by commercial available ELISA kits. This is, the gs Ag increases during the early life in chickens infected with ALV-J, but decreases in chickens with endogenous ALV.

In ALV-J-infected flocks, contact infection occurred during rearing ages, so the gs Ag levels increased during the early ages (Fig. 2), and then, decreased due to the elimination of ALV accompanied with the increase of anti-ALV-J antibody. The anti-ALV-J antibody in those flocks increased before laying. However, in non-infected flocks, the anti-J antibody increased after laying because male and female chickens were put together at the time of laying (A and B, C and D). All flocks except the flocks in Farm 2 (no samples available) became infected with ALV-J after laying might be due to horizontal infection.

The high percentages of gs Ag (30% to 60%) in some non-infected chicken flocks in the present study were due to the endogenous ALV because subgroup E gene but not exogenous genes was detected. According to Crittenden's report (1), the positive rates of the gs Ag in serum from endogenous ALV could be very high. So a single time of gs Ag detection is not correct for the diagnosis of ALV-J infection. On the contrary, 2 times of gs Ag in plasma is a valuable data for poultry industry to differentiate the ALV-J-infected and non-infected flocks.

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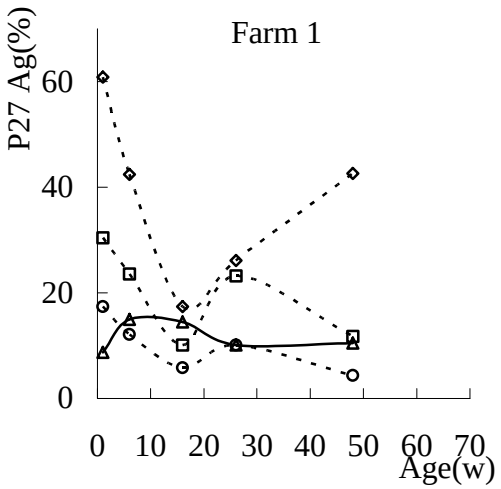

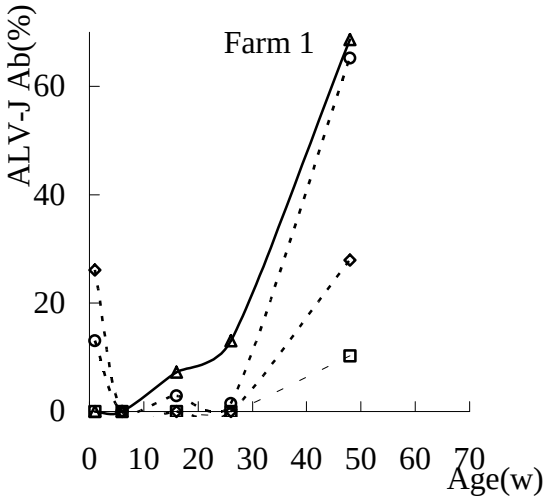

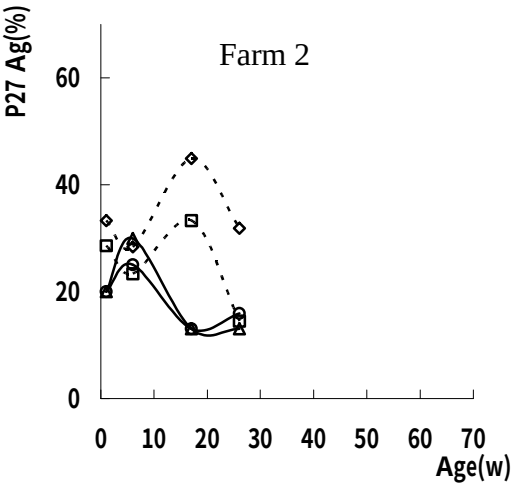

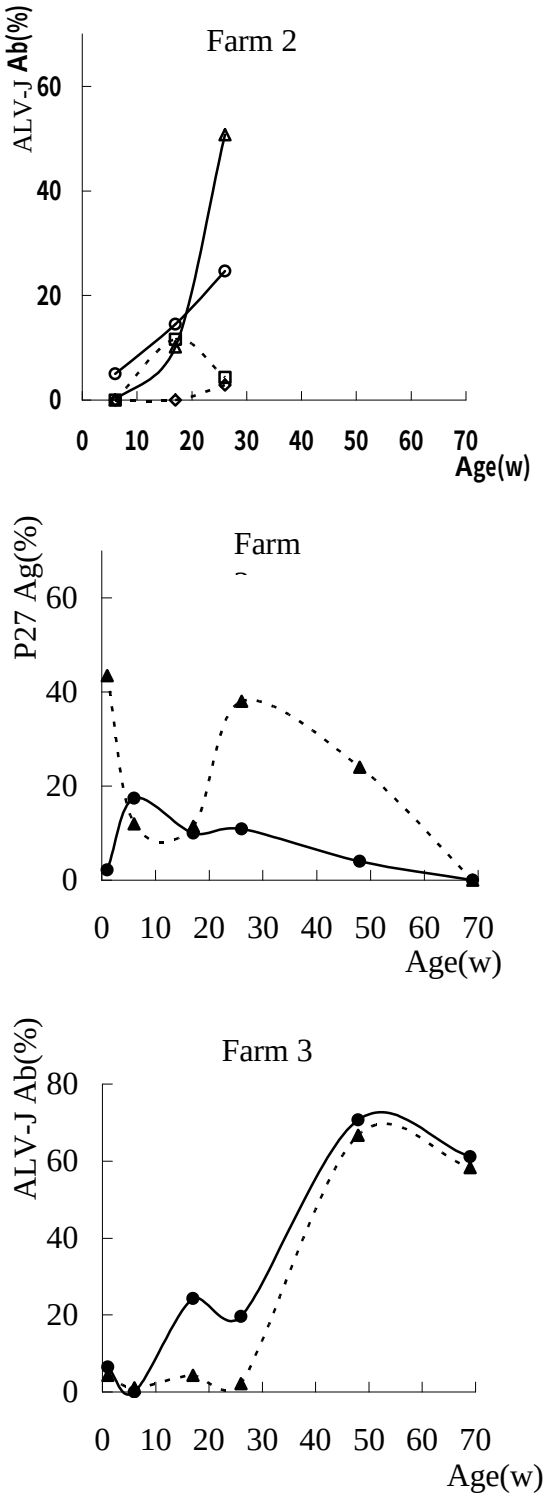

Fig. 2. Serological profiles of gs antigen and anti-ALV-J antibody in different chicken lines infected with subgroup J (heavy lines) or subgroup E (dotted lines). : line A, : line B, : line C, : line D in Farm 1 and Farm 2. : male line, : female line in Farm3.