Diagnosis and sequence analysis of avian leukosis virus subgroup J isolated from Chinese Partridge Shank chickens

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ABSTRACT The diagnosis of avian leukosis virus subgroup J (ALV-J) infection in Chinese Partridge Shank chickens was confirmed by necropsy, histopathological examinations, antibody tests, viral isolation, immunofluorescence assays, and sequence analysis. Myelocytoma, myeloma, and fibrosarcoma were simultaneously found in Partridge Shank flock with ALV-J infection. Sequence analysis of the env genes of ALV-J demonstrated that both gp85 and gp37 were highly homologous among the three strains from local chickens of those among ALV-J strains isolated from white meat-type chickens. The phylogenetic trees indicated that the three strains isolated in this study were closely related to reference strains isolated in so-called Chinese yellow chickens and some strains isolated from white meat-type chickens, both from the USA and China. The observed ALV-J infection was the first report on Partridge Shank chickens, and myelocytoma, myeloma, and fibrosarcoma were found at the same time in this batch of local chickens.

Key words: Avian leukemia virus subgroup J, Partridge Shank chickens, comprehensive diagnosis, sequence analysis

INTRODUCTION

Avian leukemia virus subgroup J (ALV-J) is a distinct RNA virus that causes immune suppression and tissue tumors in infected fowls (Payne et al., 1991; Payne and Nair, 2012). ALV-J was first isolated in 1988 from meat-type chickens in Great Britain (Payne et al., 1991). This virus mainly induces myelocytomatosis and nephromas in meat-type chickens. During the past decade, ALV-J has been reported in numerous areas of the world (Cui et al., 2006; Lai et al., 2011; Malkinson et al., 2004; Payne and Nair, 2012; Thapa et al., 2004). In China, ALV-J induced myelocytomatosis mainly occurs in white meat-type chickens (Cui et al., 2003; Du et al., 2000), similar to other parts of the world. Several cases of myelocytomatosis and hemangiommas have been reported in commercial layer flocks (Gao et al., 2012; Gao et al., 2010; Xu et al., 2004). Given the success of eradication programs worldwide, ALV-J related tumor cases have been decreasing in white meat-type chicken farms and layer chickens in China. However, ALV-J infections have recently become increasingly devastating on Chinese local chicken farms. (Cui et al., 2009; Mao et al., 2013; Sun and Cui, 2007).

The ALV-J infection observed in this work is the first report on Partridge Shank chickens. We also found the simultaneous incidence of myelocytoma, myeloma, and fibrosarcoma in the same flock. Thus, effective prevention and elimination measures must be developed.

MATERIALS AND METHODS

Flock Background

In May 2013, a heightened incidence of tumors was observed in 130-d-old meat-type local chickens raised on a farm in Shandong Province, China. Necropsies of the afflicted chickens revealed enlarged livers and spleens with miliary white spots.

Virus Culture and p27 Assay

Four diseased chickens were selected from the flock. From these chickens, 1mL of blood was aseptically collected in sodium citrate and centrifuged at 2,000 rpm for 3 min and the plasma was used to inoculate a monolayer of DF-1 cells that are resistant to ALV subgroup E (American Type Culture Collection, Manassas, VA, USA). Uninfected DF-1 cells were used as a blank control. After inoculation, the DF-1 cells were
incubated at 37°C. After 2 h, the culture medium was discarded and replaced with fresh Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% fetal bovine serum (FBS). The medium was further cultured for 7 d in an incubator at 37°C. One blind passage was performed with sterilized cover slips placed onto culture dishes in advance. After 7 d, the supernatant was collected and frozen at −80°C for future use. The cover slips carrying the cells were fixed with acetone/ethanol (3:2) for 5 min.

The culture supernatant was collected, and an ALV p27 antigen enzyme-linked immunosorbent assay kit (IDEXX Laboratories, Inc., Westbrook, ME, USA) was used in accordance with the manufacturer’s instructions to test for the presence of exogenous ALV p27 antigen.

**Immunofluorescence Assay**

The cells fixed on the cover slips were subjected to an indirect immunofluorescence assay (IFA) to detect REV monoclonal antibodies 11B154 and 11B118 (Qin et al., 1986), ALV-J monoclonal antibodies JE9 (Qin et al., 2001), ALV-A/B monospecific serum (Wang et al., 2011), and Marek’s disease virus (MDV) monoclonal antibodies BA4 and H19 (Lee et al., 1983). After the primary antibodies were washed away with phosphate-buffered saline, the cells were stained with fluorescein isothiocyanate-labeled antimouse or antirabbit immunoglobulins (Sigma, Shanghai, China), mounted in buffered glycerol (50%) and observed using a fluorescence microscope.

**Antibody Test**

Venous blood was collected from the wings of the 4 chickens, and the serum antibodies were detected using an Avian Leukosis Virus Antibody Test Kit (ALV-A/B), Avian Leukosis Virus Antibody Test Kit Subgroup J (ALV-J), and Reticuloendotheliosis Virus Antibody Test Kit (IDEXX Laboratories, Inc.) in accordance with the manufacturer’s instructions. Detection was performed twice for each serum sample.

**Histopathology**

Two chickens among the sample flock died naturally during the time of observation. Tissue samples from the liver, spleen, kidney, bone marrow neoplasms, and tumors were removed from the birds and fixed in 10% neutral buffered formalin. The tissues were processed by standard paraffin embedding, sectioned at approximately 4 μm, and stained with hematoxylin and eosin for observation.

**Viral RNA Amplification, Cloning, and Sequencing**

Blood samples of 4 chickens were used for the purification of viral RNA using an E.Z.N.A.™ Viral RNA Kit (Omega Bio-Tek, Norcross, GA, USA). The purified RNAs were used for ALV detection by reverse transcription polymerase chain reaction (RT-PCR). Based on previous studies on representative ALV strains, a primer pair targeting the conserved region was designed to amplify all env genes. The primers are as follows: EL-F, GATGAGGCGACCGCTCTTGGTT; EL-R, TGGTTGTGGATGTAATGGCGT. The cDNA was generated with the reverse primer using a TaKaRa RNA PCR Kit (AMV) v.3.0 (Takara Bio, Shiga, Japan). The conditions were as follows: denaturation at 95°C for 5 min; 32 cycles of denaturation at 95°C for 30 s; annealing at 58°C for 50 s; and extension at 72°C for 2 min with a final elongation step at 72°C for 10 min. PCR products were run in 1% agarose gels. Bands of interest were cut from the gel and extracted using aQiQquickGel Extraction Kit (Qiagen, Hilden, Germany). The purified DNA was ligated to PMID-18T vectors [TaKaRa Biotechnology (Dalian) Co., Ltd.] and used to transform competent DH5a Escherichia coli cells. Three independent clones of each ALV isolate were sequenced by the Beijing Genomics Institute (Beijing, China), and the sequences were analyzed using DNASTAR® LaserGene Genomics Suite Software (DNASTAR, Madison, WI, USA).

**Multiple Alignments and Phylogenetic Analysis**

Nucleotide and amino acid sequence analyses were performed on the gp85 and gp37 of the ALVs. The generated consensus sequence comprised sequences of the 3 isolates included in the study, as well as reference sequences. Multiple alignments were accomplished using the Clustal W method (MegAlign sequence analysis software, DNASTAR v.7.1; DNASTAR, Inc.). A phylogenetic analysis of the sequences was accomplished with a Clustal W alignment using v.7.1 of the Megalign function in the DNASTAR DNA analysis software (DNASTAR, Inc.). Ten international reference strains and another 16 Chinese field strains isolated from white meat-type chickens were used as control reference sequences. The GenBank accession numbers of all strains are listed in Figure 3.

**RESULTS**

**Gross and Histological Lesions**

Two chickens naturally died during the time of observation. The necropsy results of one dead chicken revealed numerous small, white nodules distributed on the surface of the enlarged liver (Figure 2a). Tumors
Table 1. Antibody results from 4 chickens

<table>
<thead>
<tr>
<th>Antibody</th>
<th>SD13QJ01</th>
<th>SD13QJ02</th>
<th>SD13QJ03</th>
<th>SD13QJ04</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALV-A/B</td>
<td>− − − −</td>
<td>− − − −</td>
<td>− − − −</td>
<td>− − − −</td>
</tr>
<tr>
<td>ALV-J</td>
<td>− + − +</td>
<td>− + − +</td>
<td>− + − +</td>
<td>− + − +</td>
</tr>
<tr>
<td>REV</td>
<td>− − − −</td>
<td>− − − −</td>
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<td>− − − −</td>
</tr>
</tbody>
</table>

The serum antibodies were detected using an Avian Leukosis Virus Antibody Test Kit (ALV-Ab), Avian Leukosis Virus Antibody Test Kit-Subgroup J (ALV-J), and Reticuloendotheliosis Virus Antibody Test Kit (IDEXX Laboratories, Inc.), respectively. + = positive, − = negative.

Virus Isolation and Identification

After 14 d of culture, the p27 antigen was detected in three samples. The antibody results of 4 chickens showed that samples were REV-negative, ALV-A/B-negative, and ALV-J-positive (Table 1). The IFA results showed that all samples were MDV-negative, REV-negative, and ALV-A/B-negative. However, 3 samples were ALV-J-positive (Figure 1), and the RT-PCR results of the 3 samples were positive for ALV-J. These 3 isolates were named SD13QJ01, SD13QJ02, and SD13QJ03.

Virus Sequence Alignments and Phylogenetic Analysis

The env genes of the 3 isolates (SD13QJ01, SD13QJ02, and SD13QJ03) were compared with 10 international reference strains and another 16 Chinese field strains isolated from white meat-type chickens. Both gp85 and gp37 were highly homologous among the 3 strains from local chickens, and the identity ranged from 92.1 to 93.8% for gp85 and from 93.9 to 97.4% for gp37 at the same amino acid level. According to the phylogenetic trees for both gp85 and gp37 (Figure 3), 3 strains from Chinese local chickens were closer to the Chinese field strains isolated from Chinese local yellow chickens in 2006. Among these strains, SD13QJ03 was the most closely related to GD06SL1 (93.8%) for gp85.

Figure 1. Photomicrograph of IFA test for ALV-J in second passage of DF-1 cells (200 ×) infected with chicken plasma. (a) SD13QJ01; (b) SD13QJ02; (c) SD13QJ03; (d) negative control.

Figure 2. Gross lesions and histological lesions of liver (a and b), spleen (c and d), bone marrow neoplasms (e and f), liver 2 (g and h), and kidney 2 (i and j) (200 ×). (a) Whole liver with numerous small, white tumor nodules on surface. (b) Myelocytoma cells with large nuclei and cytoplasm packed with acidophilic granules. (c) Swollen spleen with accompanying diffuse white miliary spots. (d) Myelocytoma cells with large nuclei and cytoplasm packed with acidophilic granules were present in the red pulp. (e) Bone marrow neoplasms lesions. (f) Myeloma cells. (g) Tumors in liver. (h) Original liver tissue disappears and large irregular and spindle-shaped fibroblasts are abundant. (i) Tumors in kidney. (j) Disappearance of original kidney tissue structure and replacement by large irregular and spindle-shaped fibroblasts.
Figure 3. Phylogenetic trees to demonstrate evolutionary relationships of gp85 (upper) or gp37 (lower) of ALV-J strains isolated from meat-type chickens of Chinese local breeds and from white meat-type chickens worldwide. Among the strains listed, SD13QJ01, SD13QJ02, and SD13QJ03 were isolated in this study from local breeds in Shandong Province, China. GD0512, GD0510A, GD06SL1, GD06SL2, GD06SL3, and GD06SL4 were isolated from Chinese yellow chickens in 2006. The other strains were all from white meat-type chickens.

whereas strain SD13QJ02 was the most closely related to GD06SL2 (94.4%) for gp37.

**DISCUSSION**

Although ALV-J was first isolated and characterized in the United Kingdom only a little over 20 years ago (Payne et al., 1991), the virus has rapidly evolved in both its pathogenicity and antigenicity. As the second largest broiler producer in the world, China reported no ALV-J infections until 1999 (Du et al., 1999). In the 21st century, international breeding companies have made varying degrees of progress in eradicating ALV-J. ALV-J-related tumor cases have been decreasing in white meat-type chicken farms and layer chickens in China. However, the ALV-J infection has recently become increasingly serious in Chinese local chickens. Most cases of ALV-J infection reported in Chinese local chickens involved yellow chickens (Cui et al., 2009; Sun and Cui, 2007). The present ALV-J infection was the first reported in Partridge Shank chickens, although occasional scattered cases previously occurred.

The diversity of Chinese local chickens and the differences in their growth performance provided a good environment for the appreciation and variation of
ALV-J. Meanwhile, the mixed feeding of different breeds and the sharing of the same hatchery facilitated horizontal transmission (Shen et al., 2014). These conditions led to the diversity of ALV-J, which resulted in the complexity and diversity of ALV-J infection.

Phylogenetic trees indicated that the 3 strains isolated in this study were closely related to American strains (0661, 10075-2, 10089-4, and 1022-20) isolated from white meat-type chickens, as well as to Chinese strain HN0001 isolated from a white meat-type parent breeder in the northern province of Henan in 2000 (Figure 3). Phylogenetic analysis indicates that the 3 strains isolated in this study and 6 Guangdong strains isolated from Chinese yellow chickens may have descended from a common ancestral strain. As previously reported, 8 Chinese strains isolated in China from 1999 to 2001 have a nearly identical deletion of 127 bp in the E element, which is similar to American strain 4817 isolated in 1996. Such similar deletions, which were isolated from different continents in different years, probably did not result from random mutation. American strain 4817 and the 8 Chinese isolates possibly had a common progenitor (Cui et al., 2003). A previous study found that strain GD0512 from local Chinese yellow chicken breeds was most closely related to strain HN0001, which was isolated from a white meat-type parent breeder in 2000. Another 5 strains from yellow chickens were also closer to the Chinese field strain HN0001 and the American strain 0661, which was isolated from white meat-type chickens (Sun and Cui, 2007). Thus, we speculated that ALV-J infection in Chinese local chickens may be due to certain crossbreeding practices with white meat-type breeds that caused ALV-J infection in several local companies of yellow chicken breeds in the early 1990s. In the subsequent process of breeding, selection against ALV-J infection was neglected, resulting in more virus spread. More seriously, the yellow chickens were nurtured with pure local chicken breeds on the same farm and even shared the same hatchery, which resulted in the infection of pure local chicken breeds.

Collectively, we identified the ALV-J infection in Partridge Shank chickens in China for the first time, and we also observed the simultaneous occurrence of myelocytoma, myeloma, and fibrosarcoma. All this confirms that the ALV-J infection in local chickens of China should be given more attention.

ACKNOWLEDGMENTS

This work was supported by the grant from National Natural Science Foundation of China (No. 31172330).

REFERENCES