Identification of two novel multiple recombinant avian leukemia viruses in two different lines of layer chicken

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Avian leukemia virus (ALV) is the most common oncogenetic retrovirus that emerges spontaneously as a result of recombination between exogenous viruses, exogenous viruses and endogenous viruses, and exogenous viruses and non-homologous cellular genes. In the present study, two natural recombinant avian leukemia viruses (rALVs) (LC110515-5 and LC110803-5) carrying a subgroup C gp85 gene, a subgroup E gp37 gene, and a subgroup J 3'UTR and 3'LTR were isolated from two different lines of layer flocks, Black-bone silky fowl (BSF) and commercial layer chicken, that suffered from myeloid leukosis. Although tumours were not observed in rALV-infected individual chickens, other non-neoplastic inflammatory lesions were evident. The two rALVs were cultured on DF-1 cells and identified by PCR, immunofluorescence assay and gene sequencing. The gp85 nucleotide sequence in the two isolates displayed a high identity (>95%) with that of the gp85 gene in ALV-C, but the identity was less than 90% with ALV-A/B/D/E and only 51% with ALV-J. Phylogenetic analysis of the nucleotide and amino acid sequences confirmed that the two isolates were recombinant between ALV-C, ALV-E and ALV-J. Subgroup C ALV is rarely found in field cases. This report is the first to provide evidence that ALV-C has recombined with ALV-E and ALV-J in two different chicken lines. The source and characteristics of the two rALVs and ALV-C need to be further investigated.

INTRODUCTION

Avian leukosis viruses (ALVs) are a group of oncogenetic retroviruses that cause neoplastic and production problems in chickens (Fadly & Payne, 2003). ALVs are the most common avian retroviruses in the field. Based on host range, antibody neutralization and receptor interference studies (Fadly & Payne, 2003), ALV was classified into six subgroups (A–E and J) in chickens. Subgroups A and B are classic and common pathogenic exogenous viruses that induce lymphoid leukosis. Subgroups C and D have rarely been reported in the field (Sandelini & Estola, 1974). Subgroup E is an endogenous leukemia virus with no pathogenicity (Crittenden, 1991). Subgroup J (ALV-J) was first reported in the UK (Payne et al., 1991) and was found to be associated with myeloid leukemia and other tumours in meat-type and layer-type chickens (Cheng et al., 2010; Fadly & Smith, 1999; Payne et al., 1991; Stedman & Brown, 1999; Xu et al., 2004).

The high level of genetic variation characteristic of ALVs indicates that they have a high potential to undergo recombination in any suitable host or environment. These recombinant strains allow themselves to escape the host defences, present an extended host range and form new pathogenicities. Recombination has been observed between exogenous viruses, such as an ALV-J virus encoding an ALV-A/B envelope (Gingerich et al., 2002; Lupiani et al., 2000, 2006), exogenous and endogenous viruses, such as ALV-J that arose by recombination between a Rous sarcoma virus and an endogenous avian retroviral (EAV) sequence (Bai et al., 1995), and between exogenous viruses and non-homologous cellular genes, such as an acutely transforming isolate of ALV-J (strain 966) that induces rapid-onset tumours (Chesters et al., 2001).

The present paper describes the background, histopathology and gene sequence characterization of the two recombinant ALVs (rALVs) that were isolated from a
Black-bone silky fowl (BSF) flock and a commercial layer (Hy-line) flock.

RESULTS

Gross and histopathology findings

Grossly, two of five BSF birds and three of six commercial layer birds showed tumour modules in liver, spleen, lung, duodenum and kidney. Microscopically, tumours were identified as single and concurrent myelocytomas with haemangiomomas and histiocytic sarcoma (data not shown). However, tumours were not found in the rALV-infected individual chickens. Non-neoplastic lesions that presented in the two rALV-infected chickens included inflammatory infiltration of lymphocytes in liver, kidney, lung and heart; fatty degeneration of liver; vascular disorder in lung, kidney and spleen; and depletion of lymphocytes in the spleen.

Virus isolation and identification

The two isolates grow well in cultured DF-1 cells, and no cytopathogenicity was observed in them. The ELISA results showed that the two isolates were positive for groupspecific antigen (gsa) (p27) of ALV, and the PCR was positive for the primers of ALV-J. The immunofluorescence assay (IFA) results were negative for ALV-J or ALV-A/B mAb (Fig. 1). These data indicated that the envelope protein of ALV isolates might be another subgroup of ALV that could be amplified by the ALV-J primers. To determine the subgroup of the two isolates, the PCR products were cloned and sequenced.

Virus sequence alignments and phylogenetic analysis

The PCR products were successfully cloned and sequenced. The proviral genes of the two isolates were determined after producing contiguous sequences for each of the viruses. The lengths of the LC110515-5 (BSF) and LC110803-5 (commercial layer chicken) isolates were 2571 bp and 2511 bp, respectively. The two virus isolates have 99.3 % identity, suggesting that they share a common ancestor.

A BLASTN search of the entire nucleotide sequence of the gp85 and gp37 genes of the two isolates revealed high similarity to the gp85 and gp37 sequences of viruses in the ALV-C (Pr-RSV-C) and ALV-E (ev1) subgroups. The percentage of nucleotide and amino acid identities between the two isolates and ALV-A, ALV-B, ALV-C, ALV-D, ALV-E and ALV-J are shown in Table 1 and Figs 2–4.

The gp85 nucleotide sequence in the two isolates displayed a high identity (>95 %) with gp85 in ALV-C, but the identity was less than 90 % with ALV-A/B/D/E and only 51 % with ALV-J (Table 1). Both hypervariable regions (hr) within gp85 of the isolates are more similar to ALV-C (Fig. 2), whereas the variable regions (vr) vr1, vr2 and vr3, showed amino acid residues similar to those in ALV-D/E, ALV-C/D/E and ALV-E/C, respectively.

The gp37 nucleotide sequences of the two isolates are almost identical (>97 % identity) with the equivalent sequences in endogenous viruses. The same region is only 94–96 % similar to ALV-A, -B, -C and -D, but less than 60 % similar to ALV-J (Fig. 3). Thus, within the envelope gene of the two isolates, gp85 is most similar to the equivalent ALV-C sequences, whereas gp37 is most similar to ALV-E (ev1).

The 3′UTR of the two isolates had the highest identity with ALV-J (HPRS-103), including the direct repeat 1 (DR1, 93.2 % and 93.4 % identity), E element (both 96.6 % identity), unique region 3 (U3, 91.6 % and 92.5 % identity), direct repeat region (R, both 100 % identity) and 3′ unique region 5 (U5, both 100 % identity) (Fig. 5). The non-functional redundant transmembrane (rTM) region has been deleted in the two isolates, indicating that they may have been from layer ALV-J origin (most ALV-J strains isolated from layer-type chickens have deleted rTM) (Gao et al., 2012). Similar to a previous report, the E elements of the two isolates have undergone substantial deletions in the 3′UTR. Part of the U3 of the two isolates was deleted within the 3′LTR, whereas the transcriptional regulatory elements typical of avian retroviruses (CAAT, CARG, PRE, TATA and Y boxes) were highly conserved. The R and the 3′U5 of the LTR were well conserved. Thus, the 3′UTR and 3′LTR of the two isolates are very similar to the equivalent ALV-J sequences.

Furthermore, phylogenetic analysis of the amino acid sequence of the surface (SU) glycoprotein, the transmembrane (TM) glycoprotein and 3′UTR+3′LTR demonstrated that they are closely related to ALV-C, ALV-E and ALV-J (HPRS-103), respectively (Fig. 5).

To confirm that the isolates are recombinant viruses and not coinfections of ALV-C and ALV-J, we designed three additional sets of differentiable primers that cover the recombinant site (Table 2) to amplify the two rALVs in various tissues. The positive PCR results (Fig. 6)
Table 1. Percentage nucleotide identities

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<th>Gene</th>
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<th>ALV subgroup (%)</th>
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<td>A</td>
</tr>
<tr>
<td>gp85</td>
<td>LC110515-5</td>
<td>85.9</td>
</tr>
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<td></td>
<td>LC110803-5</td>
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<tr>
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<td></td>
<td>LC110803-5</td>
<td>94.7</td>
</tr>
<tr>
<td>3’UTR</td>
<td>DR1</td>
<td>87.2</td>
</tr>
<tr>
<td></td>
<td>LC110515-5</td>
<td>86.5</td>
</tr>
<tr>
<td></td>
<td>LC110803-5</td>
<td>54.5</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>54.5</td>
</tr>
<tr>
<td>3’LTR</td>
<td>U3</td>
<td>43.8</td>
</tr>
<tr>
<td></td>
<td>LC110515-5</td>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>U5</td>
<td>0</td>
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<td></td>
<td>LC110803-5</td>
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</table>

Determined that the two isolates were real recombinant ALVs in their natural host rather than a mixture of ALV-C and ALV-J. Further sequencing showed that the PCR products have 100 % identity with primary PCR sequencing.

**DISCUSSION**

ALV-C is rarely isolated in field flocks. The strains of ALV-C include RAV-7, RAV-49, B77 and Pr RSV-C. Sandelini & Estola (1974) isolated ALV-C in Finnish breeding flocks and reported that the antibody against ALV-C was widespread in Finland at that time. In natural or experimental infection, RAV-7 and RAV-49 mainly cause stunting, obesity, hypothyroidism, hyperinsulinaemia, alterations in lipid metabolism, meningoencephalomyelitis and a low incidence of lymphoid leukosis (Carter & Smith, 1983, 1984; Carter et al., 1983; Heidrich et al., 1987; Whalen et al., 1988). The severity of these isolates, however, appears to be strain dependent (Karakoz et al., 1980; Smith & Schmidt, 1982; Stepanets et al., 2003). Bozhkov et al. (1987) described the pathomorphological lesions caused by experimental inoculation of Pr RSV-C, which were characterized by erythroblastosis, lymphoid leukosis and a proliferative lymphosarcoma-like tumour. ALV-C has cytopathogenicity in cultured DF-1 cells (Himly et al., 1998). In recent years, ALV-C has often been used as a comparative system for establishing pathways leading to immunodeficiency syndrome in both homologous and heterologous hosts (Stepanets et al., 2001, 2003).

The potential of ALV for undergoing recombination is no surprise as the emergence of the original ALV-J isolate has been attributed to recombination occurring between an exogenous virus (gag/pol of ALV-C, Pr RSV-C strain) and an endogenous retroviral sequence (EAV-E51, ev/J and EAV-HP) (Bai et al., 1995; Benson et al., 1998; Silva et al., 2000; Smith et al., 1999). The novel recombinant virus of ALV-J extended its host range and developed new pathogenicity.

In the present paper, two recombinant ALVs were isolated from BSF and commercial egg-type chickens suffering from stunting diseases. The viruses were initially thought to be ALV-J isolates based on histopathology and positive PCR amplification with primers for ALV-J (the primers covered part of the pol gene, all of the env gene, the 3’UTR and the 3’LTR of ALV-J). Furthermore, ELISA, IFA, PCR and sequence analysis of the isolated viruses demonstrated that the two isolates were recombinant viruses that carried subgroup C gp85, subgroup E (ev1) gp37 and subgroup J 3’UTR and 3’LTR. The sequence characterization of the SU region (gp85) of the viral glycoprotein indicated the virus belongs to subgroup C (＞95 %), whereas the TM portion (gp37) of the viral envelope protein presented the highest identity (＞97 %) with that of subgroup E and the 3’UTR + 3’LTR presented the highest identity (＞95 %) with that of subgroup J.

Moreover, the analysis of some recombinant isolates, such as 5701A (Lupiani et al., 2000) that carries a subgroup A gp85 and a subgroup E gp37 gene, and AF115-4 (Lupiani et al., 2006) that carries a subgroup B gp85, a subgroup E gp37 and a subgroup J 3’UTR, revealed that both the gp37 coding region of the subgroup E viruses and the 3’UTR upstream of the DR1 region are hot spots for recombination between ALV-J and other exogenous and endogenous viruses. To determine if the samples obtained from the affected chickens had rALV or a mixture of subgroup C and J viruses, we screened all samples using PCR with three sets of differential primers for the two rALVs and sequenced them. Three PCR products that cover pol and gp85, gp85 and gp37, gp37 and 3’UTR + LTR were obtained. The PCR and sequencing results demonstrated that the two isolates were rALVs.
BSF (Gallus gallus domesticus Brisson) is a famous chicken with snow-white silky feathers and black-coloured bones, meat and skin. The bird is sourced from South China. As a well-known part of traditional Chinese medicine, it has been used for more than 1000 years in the Orient to improve the immunity of humans, treat diabetes and anaemia, and cure women’s diseases such as menoxenia and post-partum complications (Tian et al., 2007). Thus, the ALV infection first found in the flocks needs to be further assessed for its potential effects.

The two flocks belong to different farms that were located far from each other. However, the high identity of the two recombinant isolates indicated that the recombinant virus or ALV-C may be prevalent in egg-type flocks. Investigating and reducing the potential for exogenous viruses to undergo recombination with other exogenous viruses or with endogenous retroviral sequences will be necessary.

**METHODS**

**Flock background.** A total of five sick 180 day-old BSF and six 150 day-old commercial layer chickens (Hy-line) were sent to the Molecular Pathology Laboratory, College of Veterinary Medicine,
Shandong Agricultural University for diagnosis in May and August, respectively, of 2011. The five BSF chickens were from one 2000 parent layer flock that was bred for Chinese medicine production. The six commercial layer chickens were from a flock of 8000 birds. The two farms are approximately 300 km apart. The birds breeding in the two farms were from different hatchery. The birds of the two flocks were housed in three-storey cages in a separate poultry house and received routine layer feed, municipal drinking water and vaccinations. The total mortality of the BSF and commercial flocks was approximately 10% and 5%, respectively. Egg production was significantly lower than expected. Affected chickens from the two flocks showed wasting, paralysis, abdominal distension, diarrhoea and anaemia. At autopsy, two of the five BSF and three of the six commercial layer chickens had tumours of the viscera.

**Histopathology.** Tissue samples from the liver, spleen, heart, lung, kidney, proventriculus, ventriculus, intestine, muscle, ovary, brain, bone marrow and tumours 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 haematoxylin and eosin (H&E) for observation.

**Virus culture and p27 assay.** The virus was isolated from serum, liver, kidney or tumour homogenates from the sick chickens using standard techniques (Fadly, 2000). Briefly, the samples were inoculated in DF-1 cell cultures [Dulbecco’s modified Eagle’s medium; DMEM (Invitrogen)] and then incubated for two serial passages of 7 days each at 37 °C. After incubation, the virus was passaged in monolayer cultures of DF-1 cells (Dulbecco modified Eagle’s medium, DMEM, containing 5% fetal bovine serum). After incubation, the virus was passaged in monolayer cultures of DF-1 cells (Dulbecco modified Eagle’s medium, DMEM, containing 5% fetal bovine serum). After incubation, the virus was passaged in monolayer cultures of DF-1 cells (Dulbecco modified Eagle’s medium, DMEM, containing 5% fetal bovine serum). After incubation, the virus was passaged in monolayer cultures of DF-1 cells (Dulbecco modified Eagle’s medium, DMEM, containing 5% fetal bovine serum). 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incubation, cell lysates were prepared after three cycles of freezing-thawing and tested for ALV group-specific antigen (gsa) (p27) by an ELISA using anti-p27 antibody-coated plates (IDEXX Laboratories).

Immunofluorescence assay (IFA). The IFA was performed on DF-1 cells using subgroup J- or subgroup A/B-specific antibodies on either unfixed or acetone–ethanol (3:2) fixed cells. The infected DF-1 cells were stained initially with either ALV-J-specific polyclonal rabbit

Fig. 4. Sequence alignment of the 3’UTR and 3’LTR of the two recombinant ALVs and the prototype strain of ALV-J, HPRS-103.
serum or ALV-A/B-specific mAb (kindly provided by Professor Zhizhong Cui, Shandong Agriculture University, China). After the primary antibodies had been washed away with PBS, the cells were stained with FITC-labelled anti-rabbit or anti-mouse immunoglobulins (Sigma), mounted in buffered glycerol (50%) and observed in a fluorescence microscope.

Proviral DNA amplification, cloning and sequencing. Proviral genomic DNA from p27-positive DF-1 cultures was extracted, and viral genes were amplified by PCR. Normal SPF (specific pathogen-free) chicken tissue culture and the NX0101 strain of ALV-J (Cui et al., 2003) were used as negative and positive controls, respectively. These controls were incubated and processed under the same conditions as the samples. The primers designed specifically for ALV-J were as follows: forward primer, GGACATCACCCAAAAGGATGA; reverse primer, GTCAGGGAATCGACGGTC. This primer set covered part of the pol gene, all of the env gene, the 3′9 UTR and the 3′9 LTR. The cycle conditions were as follows: denaturation at 94 °C for 4 min, followed by 32 cycles of 94 °C for 30 s, 58 °C for 1 min, 72 °C for 3 min, with a final extension at 72 °C for 10 min. The amplified gene was purified using an agarose gel DNA extraction kit (E.Z.N.A. Gel Extraction Kit; Omega Bio-tek) and further cloned into the pMD18-T vector.

**Fig. 5.** Phylogenetic analysis of the two recombinant ALVs and the representative isolates of ALV-A–E: (a) gp85 envelope protein; (b) gp37 envelope protein; (c) 3′UTR and 3′LTR nucleotide sequences.

<table>
<thead>
<tr>
<th>Table 2. Differential primers of recombinant ALV</th>
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<td>Primer</td>
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</tr>
<tr>
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<td>BF2</td>
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<td>CF3</td>
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according to the manufacturer’s instructions. The recombinant plasmids were transformed into DH5α competent cells. Single colonies were selected and cultured for plasmid extraction. DNA sequences from the positive clones were determined by the Biotechnology Company. To avoid laboratory contamination of PCR, the DNA was abstracted from different tissues and the PCR amplification performed twice independently. The primers and reaction condition that were employed for confirmatory experiments are shown in Table 2.

Multiple alignments and phylogenetic analysis. The nucleotide and amino acid sequence analyses were performed on the complete genome of the ALVs. The generated consensus sequence comprised sequences of the two isolates included in the study and reference sequences. Multiple alignments were accomplished using the CLUSTAL W method (MEGALIGN sequence analysis software, DNASTAR v. 4.03; DNASTAR). A phylogenetic analysis of the sequences was accomplished with a CLUSTAL W alignment using v. 5.01 of the MEGALIGN function in the DNASTAR DNA analysis software. GenBank accession numbers for the rALVs and control reference sequences used in the phylogenetic analysis and new recombinant viruses reported in this study are as follows: rALV (LC110315-5, KC862332), rALV (LC110803-5, KC862333), ALV-A (RAV-1, M37980), ALV-B (RAV-2, K02374; RSV[S-R R], AF052428), ALV-C (Pr RSV-C, V01197), ALV-D (SR-RSV-D, D10625), ALV-E (RAV-0, M12172; ev1: AY013303), and ALV-J (HPRS-103, Z46390.1).

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REFERENCES


