

Molecular characterization of avian leukosis virus subgroup J in Chinese local chickens between 2013 and 2018

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ABSTRACT Avian leukosis virus subgroup J (ALV-J) was first isolated from broiler chickens in China in 1999; subsequently, it was rapidly introduced into layer chickens and Chinese local chickens. Recently, the incidence of ALV-J in broiler and layer chickens has significantly decreased. However, it has caused substantial damage to Chinese local chickens, resulting in immense challenges to their production performance and breeding safety. To systematically analyze the molecular characteristics and the epidemic trend of ALV-J in Chinese local chickens, 260 clinical samples were collected for the period of 2013–2018; 18 ALV-J local chicken isolates were identified by antigen-capture enzyme-linked immunosorbent assay and subgroup A-, B-, and J-specific multiplex PCR. The whole genomic sequences of 18 isolates were amplified with PCR and submitted to GenBank. Approximately, 55.5% (10/18) of the 18 isolates demonstrated a relatively high homology

(92.3–95.4%) with 20 ALV-J early-isolated local strains (genome sequences obtained from GenBank) in *gp85* genes clustering in a separated branch. The 3' untranslated region (3' UTR) of the 18 isolates showed a 195–210 and 16–28 base pair deletion in the redundant transmembrane region and in direct repeat 1, respectively; 55.5% (10/18) of the 18 isolates retained the 147 residue E element. The U3 gene of 61.1% (11/18) of the 18 isolates shared high identity (94.6–97.3%) with ALV-J early-isolated local strains. These results implied that the *gp85* and U3 of ALV-J local chicken isolates have rapidly evolved and formed a unique local chicken branch. In addition, it was determined that the gene deletion in the 3'UTR region currently serves as a unique molecular characteristic of ALV-J in China. Hence, the obtained results built on the existing ALV-J molecular epidemiological data and further elucidated the genetic evolution trend of ALV-J in Chinese local chickens.

Key words: subgroup J avian leukosis virus, Chinese local chicken, molecular characterization, molecular epidemiology, genetic evolution

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INTRODUCTION

Avian leukosis is induced by avian leukosis viruses (ALV), which belong to a group in the genus Alpharetrovirus of the family Retroviridae. These viruses can cause malignant or benign tumorigenic diseases in poultry. Avian leukosis viruses can be differentiated into 11 subgroups, namely A–K, based on viral envelope

interference, host range, and cross-reactivity with neutralizing antibodies (Payne et al., 1991; Weiss, 1992; Coffin et al., 1997; Payne and Nair, 2012; Zhao et al., 2018). Subgroups A, B, C, D, J, and K, which prevail in chickens, are considered exogenous ALV; the E subgroup is considered an endogenous ALV and is not pathogenic. Subgroups A and B are recognized as common pathogenic viruses that induce lymphoid leukosis and sarcoma, whereas subgroups C and D have only rarely been reported. However, avian leukosis virus subgroup J (ALV-J), a new exogenous ALV, is the most prevalent in chickens and has exhibited increased pathogenicity and faster transmission ability in recent years (Payne et al., 1992a; Jirong et al., 1998; Lai et al., 2011; Payne and Nair, 2012), primarily inducing myeloid

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leukosis in meat-type chickens (Venugopal et al., 2000). Since the first strain of ALV-J was isolated from meat-type breeder chickens in the UK in 1988, it has rapidly spread around the world, causing severe economic losses to the poultry industry (Payne et al., 1992b).

Since 2004, ALV-J outbreaks have occurred in chicken farms in several provinces in China, resulting in morbidity and mortality rates as high as 50% and huge economic losses to the Chinese layer and local chicken industry (Cheng et al., 2005; Sun and Cui, 2007; Gao et al., 2010; Zhang et al., 2011; Li et al., 2018). After the successful eradication programs, ALV-J has been optimally controlled, and its incidence in white meat-type chicken and layer chicken farms has decreased yearly in China. However, ALV-J infections in Chinese local chicken have become increasingly devastating in recent years (Meng et al., 2018; Zhang et al., 2018). Avian leukosis virus subgroup J causes an adverse decline in egg production and results in high mortality; additionally, it poses a grave threat to the safety of local chicken breeding sources in China.

Although there are a few prior studies on the isolation and sequencing of ALV-J strains from local chickens (Cheng et al., 2005; Sun and Cui, 2007; Wang et al., 2012b; Li et al., 2016; Zhang et al., 2018), no systematic epidemiological investigation and study of the molecular characteristics of ALV-J isolates from local chickens has been performed. Thus, in the present study, 260 samples were collected from local chickens in the 5 main local chicken breeding provinces of China (Jiangsu, Guangxi, Hunan, Shanghai, and Hebei) from 2013 to 2018. A total of 18 ALV-J strains were isolated, and their complete proviral genomes were sequenced. The molecular epidemiology and the evolutionary trend of the ALV-J isolates from local chickens were systematically analyzed.

MATERIALS AND METHODS

Clinical Samples

A total of 260 samples (including kidney, liver, spleen, or whole blood) were collected from 5 provinces in China (Jiangsu, Guangxi, Hunan, Shanghai, and Hebei) between 2013 and 2018. A tissue sample (the liver, spleen, or kidney collected randomly) represents a chicken, and a tube of whole blood represents a chicken. The local chickens showed clinical signs such as abnormal enlarged livers, spleens, and kidneys, slow growth, and decreased fertility. The tissue samples frozen at -20°C and whole blood stored at 4°C were transported from the field to the laboratory, and virus isolation was performed immediately.

Virus Isolation and Identification

Avian leukosis virus isolation and identification procedures in cell cultures were performed as previously described (Gao et al., 2012). First, tissue samples were homogenized in 1 mL sterile phosphate-buffered saline (8.0 g NaCl, 0.2 g KCl, 1.15 g $\text{Na H}_2\text{PO}_4$, and 0.2 g KH_2PO_4 in 1 L ddH_2O) and centrifuged at $3,000 \times g$ for 10 min. Whole-blood samples were centrifuged at

$3,000 \times g$ for 5 min. Second, the supernatant from a tissue sample or the plasma from whole blood was filtered through a $0.22\text{-}\mu\text{m}$ Millipore membrane, then added 0.5 mL of filtered supernatant to monolayer DF-1 cell cultures in 12-well plates, which are known to be susceptible only to exogenous ALV (Maas et al., 2006). Lastly, following virus adsorption, the supernatant was removed, and the DF-1 cells were maintained in Dulbecco's modified Eagle's medium (Thermo Scientific, Rockford, IL), supplemented with 1% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich, St. Louis, MO) and 1% (v/v) penicillin/streptomycin (Summus, Beijing, China) solution at 38.5°C under a humid atmosphere with 5% CO_2 for 7 d. After 3 freeze-thaw cycles, the virus was serially passaged 3 times in DF-1 cells according to the virus isolation method described above.

The supernatant of infected DF-1 cells was harvested and tested for the presence of the ALV group-specific antigen (p27) by antigen-capture enzyme-linked immunosorbent assay (ELISA) using an ALV antigen test kit (Yun et al., 2013). Proviral DNA was directly extracted from positive cultured DF-1 cells using a Tissue DNA Extract Kit (Axygen Scientific, Inc., Union City, CA) according to the manufacturer's instructions. To further identify the subgroup of isolates, the genomic DNA was detected using subgroup A-, B-, and J-specific multiplex PCR with specific primers (Table 1) (Gao et al., 2014). The PCR was performed in a $25\text{-}\mu\text{L}$ system containing 2 μL DNA, 12.5 μL Premix Taq DNA polymerase (TaKaRa, Dalian, China), 2 μL PF (10 pM), 0.8 μL AR (10 pM), 1 μL BR, 1 μL CR (10 pM), and 5.7 μL double-distilled water (ddH_2O). The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min; and a final elongation step at 72°C for 7 min. The PCR products were evaluated by 1.0% agarose gel electrophoresis.

PCR Amplification, Cloning, and Sequencing

To obtain the genome sequences of the ALV isolates, 3 primer pairs (JAF/JAR, JBF/JBR, and JCF/JCR) were designed for amplification of the whole genomic sequences of the ALV isolates (Table 1) based on ALV-J prototype strain HPRS-103 (GenBank No. Z46390). The PCR was performed in a $50\text{-}\mu\text{L}$ system containing 2 μL DNA, 25 μL PrimeSTAR Max premix DNA high-fidelity polymerase (TaKaRa), 2 μL upstream primer (JAF, JBF, or JCF) (10 pM), 2 μL downstream primer (JAR, JBR, or JCR) (10 pM), and 19 μL of ddH_2O . The PCR conditions were as follows: initial denaturation at 98°C for 5 min; 35 cycles of 98°C for 15 s, 52°C for 15 s, and 72°C for 50 s; and a final elongation step of 72°C for 10 min. The PCR products were excised from a 1.0% agarose gel and purified using the AxyPrep DNA gel extraction kit (Axygen), cloned into the pMD18-T vector (TaKaRa) and then transformed into DH5 α cells. Three different clones of each fragment were confirmed by sequencing (Kumei, Changchun, China).

Table 1. Primers used in the present study.

Primer	Sequence (5'-3')	Size (bp)	Amplification target
PF	CGGAGAAGACACCCTTGCT		
AR	GCATTGCCACAGCGGTACTG	715	ALV-A
BR	GTAGACACCAGCCGGACTATC	515	ALV-B
JR	CGAACCAAAGGTAACACACG	422	ALV-J
JAF	TGTAGTGTATTGCAATACTCTTATGTAACG	2,758	JA
JAR	TTGAGCGGAATAGCCAGATGTAG		
JBF	AGGGAGTATCCTGGGAAGAG	2,818	JB
JBR	ACAACGGAAATAATAACCACGC		
JCF	GGAGAAGACACCCTTGCTGCC	2,460	JC
JCR	TGAAGCCATCCGCTTCATGC		

Sequence Analysis

The genomic nucleotide sequences of 3 gene fragments (termed JA, JB, and JC) were edited and spliced using the SeqMan software of the DNASTAR package. Phylogenetic analysis of the ALV-J sequences was performed using the neighbor-joining method with 1,000 bootstrap replicates using the MEGA, version 6.0, program. The sequences obtained in the present study have been submitted to GenBank. The ALV reference strains used in the present study included ALV-J broiler isolates (prototype strain HPRS-103, American meat-type chickens, and broiler chickens in China) and ALV-J layer isolates. To systematically analyze the molecular characterization and genetic evolution trends of Chinese local chicken ALV-J, 20 ALV-J early-isolated local strains in China (genome sequences obtained from GenBank) were also selected as the reference strains (Meng et al., 2018).

Data Availability

The complete proviral genome sequences of all 18 isolates were deposited at GenBank with accession numbers MN735292 to MN735309.

RESULTS

Isolation and Identification of ALV-J From Chinese Local Chickens

All clinical samples (including samples from kidney, liver, spleen, or whole blood) from local chickens were inoculated into DF-1 cells; after 7 d, the culture supernatants of the infected DF-1 cells were subjected to ALV p27 antigen ELISA (Figure 1A). The results showed that 18 culture supernatants were ALV positive. To further identify the subgroup, proviral DNA was extracted from the 18 positive culture supernatants and amplified by subgroup A-, B-, and J-specific multiplex PCR (Gao et al., 2014). Only the primers specific to the J subgroup (PF and JR) amplified a specific 422-base pair (bp) sequence; no specific fragments were observed for primers specific to subgroups A (PF and AR) and B (PF and BR) (Figure 1B). These results indicated that the 18 isolates belonged to the ALV-J subgroup and were designated JS13NT01, JS14NT01, JS14NT02, GX14NN01, GX14NN02, GX15JL01, GX16NN03, GX16NN04, GX16NN05, GX16YL01, HN17ZZ01, HN17ZZ02, HB18XH01, SH18JY01, SH18JY02, JS18YZ01, JS18YZ02, and JS18YZ03, respectively.

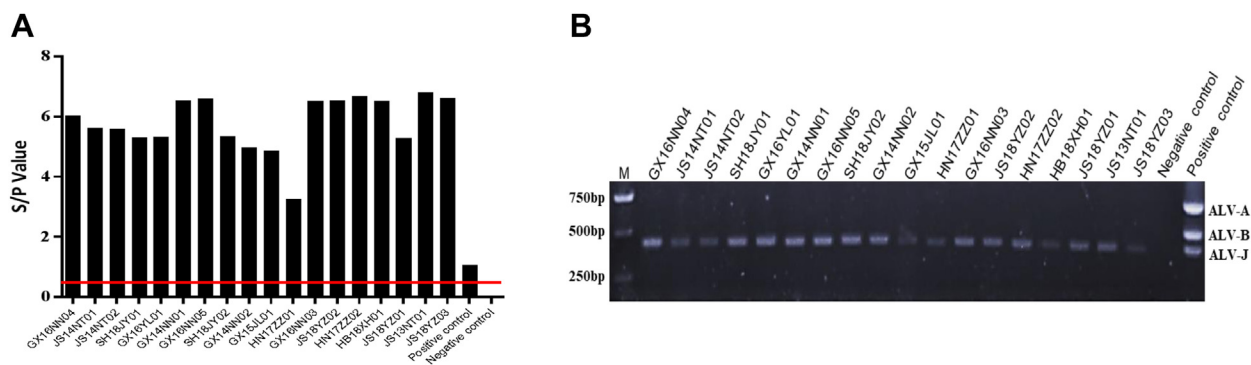


Figure 1. Isolation and identification of ALV-J local chicken isolates. (A) The supernatants of DF-1 cells were assessed using ALV p27 antigen ELISA (42). Following OD_{650} measurement, the S/P ratios were calculated and used to express the S/P ratio per sample. S/P values greater than or equal to 0.2 were considered positive. The red line indicates the cut-off value for ELISA. (B) Identification of 18 isolates by subgroup A-, B-, and J-specific multiplex PCRs. DF-1 cells infected with Rous-associated virus type 1 (subgroup A), Rous-associated virus type 2 (subgroup B), and HPRS-103 (subgroup J) were used as the positive control for PCR with primer pairs PF/AR, PF/BR, and PF/JR, respectively. Uninfected DF-1 cells served as the negative control. Abbreviation: ALV-J, Avian leukosis virus subgroup J.

Table 2. ALV-J strains used in the present study.

No. ¹	Strains	Origin	Yr	Accession no.	Host ²
1	JS13NT01	Jiangsu	2013	MN735301	Local
2	JS14NT01	Jiangsu	2014	MN735308	Local
3	JS14NT02	Jiangsu	2014	MN735302	Local
4	GX14NN01	Guangxi	2014	MN735292	Local
5	GX14NN02	Guangxi	2014	MN735293	Local
6	GX15JL01	Guangxi	2015	MN735294	Local
7	GX16NN03	Guangxi	2016	MN735295	Local
8	GX16NN04	Guangxi	2016	MN735296	Local
9	GX16NN05	Guangxi	2016	MN735309	Local
10	GX16YL01	Guangxi	2016	MN735297	Local
11	HN17ZZ01	Hunan	2017	MN735299	Local
12	HN17ZZ02	Hunan	2017	MN735300	Local
13	HB18XH01	Hebei	2018	MN735298	Local
14	SH18JY01	Shanghai	2018	MN735306	Local
15	SH18JY02	Shanghai	2018	MN735307	Local
16	JS18YZ01	Jiangsu	2018	MN735303	Local
17	JS18YZ02	Jiangsu	2018	MN735304	Local
18	JS18YZ03	Jiangsu	2018	MN735305	Local
19	HPRS-103	UK	1989	Z46390	Broiler
20	ADOL-Hc1	USA	1993	AF097731	Broiler
21	UD5	USA	2000	AF307952	Broiler
22	YZ9902	Jiangsu	1999	HM235670	Broiler
23	SD9901	Shangdong	1999	AY897220	Broiler
24	SD0002	Shangdong	2000	AY897224	Broiler
25	NX0101	NingXia	2001	AY897227	Broiler
26	BJ0303	Beijing	2003	AY897232	Broiler
27	JL08CH3-1	Jilin	2008	HQ634809	Layer
28	LN09SY31	Liaoning	2008	HQ634803	Layer
29	GL09DP02	Shangdong	2009	JN378887	Layer
30	SD09DP04	Shangdong	2009	HQ634808	Layer
31	HLJ09SH01	Heilongjiang	2009	HQ634806	Layer
32	JS09GY3	Jilin	2009	GU982308	Layer
33	JS09GY6	Jilin	2009	GU982310	Layer
34	HLJ10SH04	Heilongjiang	2010	HQ634814	Layer
35	JL10HW01	Jilin	2010	HQ634800	Layer
36	JL093-1	Jilin	2012	JN624878	Layer
37	GD0510 A	Shangdong	2005	EF103132	Local
38	GD0512	Shangdong	2005	EF103133	Local
39	SCAU-0901	Guangdong	2009	FJ619190	Local
40	XG-09	Guangdong	2009	HM775332	Local
41	WN100401	Shangdong	2010	HQ271447	Local
42	WN100402	Anhui	2010	HQ271448	Local
43	WN100403	Anhui	2010	HQ332557	Local
44	SD12HN01	Guangdong	2012	KM873187	Local
45	SD12HN02	Guangdong	2012	KM873188	Local
46	WSC512	Guangdong	2012	KJ631320	Local
47	GX13QZ14	Guangxi	2013	KT598477	Local
48	GX14HG01	Guangxi	2014	KU997685	Local
49	GDKP1202	Guangdong	2012	JX453210	Local
50	GD1406-H	Guangdong	2014	KU500033	Local
51	GX14YL03	Guangxi	2014	KT598470	Local
52	GX15PP03	Guangxi	2015	KU848762	Local
53	GX16ZS01	Guangxi	2016	KY983549	Local
54	GX16ZS748	Guangxi	2016	KY983552	Local
55	GX16ZSE10	Guangxi	2016	KY983553	Local
56	GX16ZS352	Guangxi	2016	KY983550	Local

Abbreviation: ALV-J, Avian leukosis virus subgroup J.

¹Isolates 1 to 18 are ALV-J local chicken isolates identified in the present study. Isolates 19 to 26 are ALV-J broiler reference strains. Isolates 27 to 36 are ALV-J layer reference strains. Isolates 37 to 56 are ALV-J early-isolated local strains. The genome sequences of ALV reference strains are available in GenBank.

²ALV-J isolate hosts: Broiler, meat-type chicken; Layer: Layer chicken; Local: Local chicken.

Sequence Analysis of the Proviral Genomes of the 18 Isolates

The complete proviral genomes of the 18 isolates from Chinese local chickens were amplified and sequenced to systematically analyze their molecular characteristics.

The sequences of the 18 isolates have been submitted to GenBank (the accession numbers are listed in Table 2). The full-length proviral genome sequences of the 18 isolates were 7,450 to 7,635 bp in length. All genome sequences displayed a typical replication-competent type C retrovirus genetic organization (5′LTR-leader-gag-pol-env-3′LTR), lacking viral oncogenes (Ruddell, 1995). The complete genome sequences of the 18 isolates shared 93.6 to 98.9% identity with each other. The env genes of the 18 isolates were 1,512 to 1,518 bp in length, and their nucleotide sequences showed a maximum divergence of 10.7%, with nucleotide sequence identities ranging from 89.3 to 99.8%. The gag and pol nucleotide sequences of the 18 isolates also shared high sequence identity (94.3–100% and 95.8–100%, respectively).

Sequence Analysis of the ALV-J gp85 Gene of the 18 Isolates

The gp85 is encoded by the env gene. The gp85 sequences of the 18 isolates were 912 to 924 bp in length, with 1 open reading frame encoding a protein of 304 to 308 amino acids, respectively. To further understand the evolutionary origins of ALV-J in local chickens, the gp85 genes of the 18 isolates were compared with sequences from reference strains (Table 2). The phylogenetic analysis of the gp85 genes indicated that 3 isolates (3/18; JS13NT01, JS14NT01, and JS14NT02) were more closely related to ALV-J broiler isolates and showed high sequence identity (95.1–97.9%) with the ALV-J prototype strain HPRS-103 (Bai, 1995), designated as group I. Two isolates (2/18; GX14NN02 and GX15JL01) shared high sequence identity (92.5–94.4%) with ALV-J layer isolates designated as group II. Ten isolates (10/18; GX14NN01, GX16NN04, HN17ZZ01, GX16NN03, JS18YZ01, HN17ZZ02, GX16NN05, GX16YL01, JS18YZ02, and JS18YZ03) shared relatively high sequence identity (92.3–95.4%) with ALV-J early-isolated local strains, designated as group III. The sequence identity of 3 isolates (3/18; SH18JY02, SH18JY01, and HB18XH01) was <90% for all reference strains; these isolates were distributed in a separate branch from groups I, II, and III; they were designated as a new branch, namely group IV (Figure 2). In addition, 12 unique amino acid substitutions (41V, 43L, 45S, 47Q, 49E, 50E, 52R, 55T, 56V, 58Y/H, 61K, and 76S) near the vr2 regions of SH18JY02, SH18JY01, and HB18XH01 in group IV were found (Figure 3). Taken together, these data indicated that the gp85 of most ALV-J local chicken isolates form a unique branch, belonging to group III.

Sequence Analysis of the 3′ Untranslated Region of the 18 ALV-J Isolates

The 3′ untranslated region (UTR) nucleotide sequences of the 18 isolates had full lengths of 125–275 bp. The redundant transmembrane region (rTM),

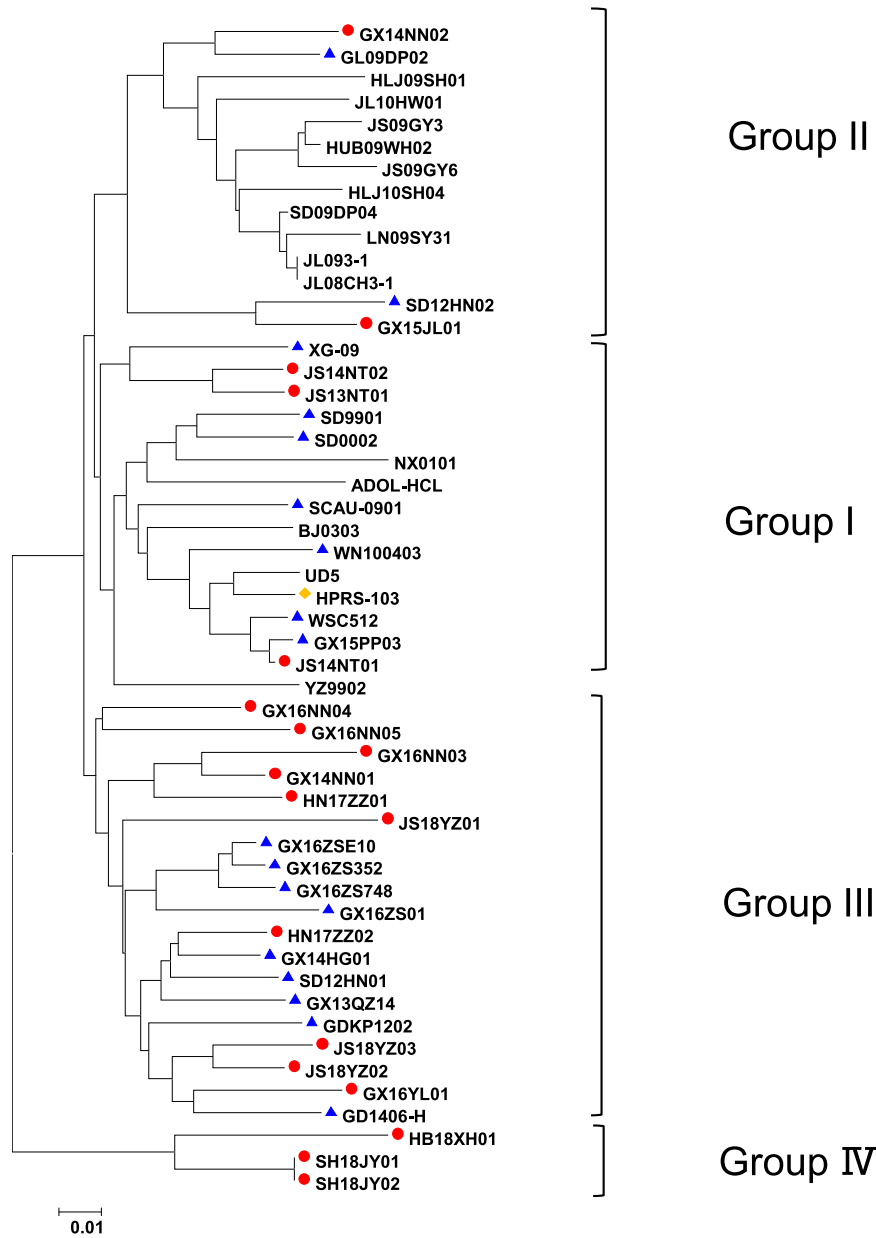


Figure 2. Phylogenetic analysis of the gp85 gene of ALV-J local chicken isolates. The tree was constructed using the neighbor-joining method with MEGA6.0 software. Bootstrap values were calculated with 1,000 replicates of the alignment. The 4 groups are marked. The red circles represent the 18 ALV-J local chicken isolates in the present study. The blue triangles represent ALV-J early-isolated local strains. The yellow diamond represents ALV-J prototype strain HPRS-103. Abbreviation: ALV-J, Avian leukosis virus subgroup J.

DR-1, and the E element are located in the 3'UTR (Tschlis et al.,1982). A comparison of the 3'UTR nucleotide sequences of all 18 isolates with the corresponding sequences in the ALV-J broiler and layer chicken isolates showed that a section of 195–210 bp was deleted from the 3' end of the rTM region, whereas a section of 16–28 bp was deleted from 5' end of the direct repeat 1 (DR-1) region in all 18 isolates and ALV-J early-isolated local strains (Figure 4). These deletions were similar to mutations in the 3'UTRs of Chinese ALV-J layer isolates.

Ten isolates (10/18; GX16YL01, GX16NN05, GX16NN03, GX15JL01 and HN17ZZ02, JS14NT02, JS14NT01, SH18JY01, SH18JY02, and HB18XH01) had a complete E element, 147 bp in length. Four isolates

(4/18; JS18YZ02, JS18YZ03, GX14NN02, and JS13NT01) contained a 16–24 bp deletion in the E element, whereas the other 4 isolates (4/18; GX16NN04, GX14NN01, JS18YZ01, and HN17ZZ01) showed a 127-bp deletion in the E element (Figure 4).

Sequence Analysis of the 3' Long Terminal Repeat of the 18 Isolates

The 3' long terminal repeat of the 18 isolates was composed of U3, R, and U5; it comprised a length of 313–325 bp. The U5 and R regions were conserved compared with those of all reference strains, sharing 95.2–96.8% sequence identity. The U3 regions of the 18

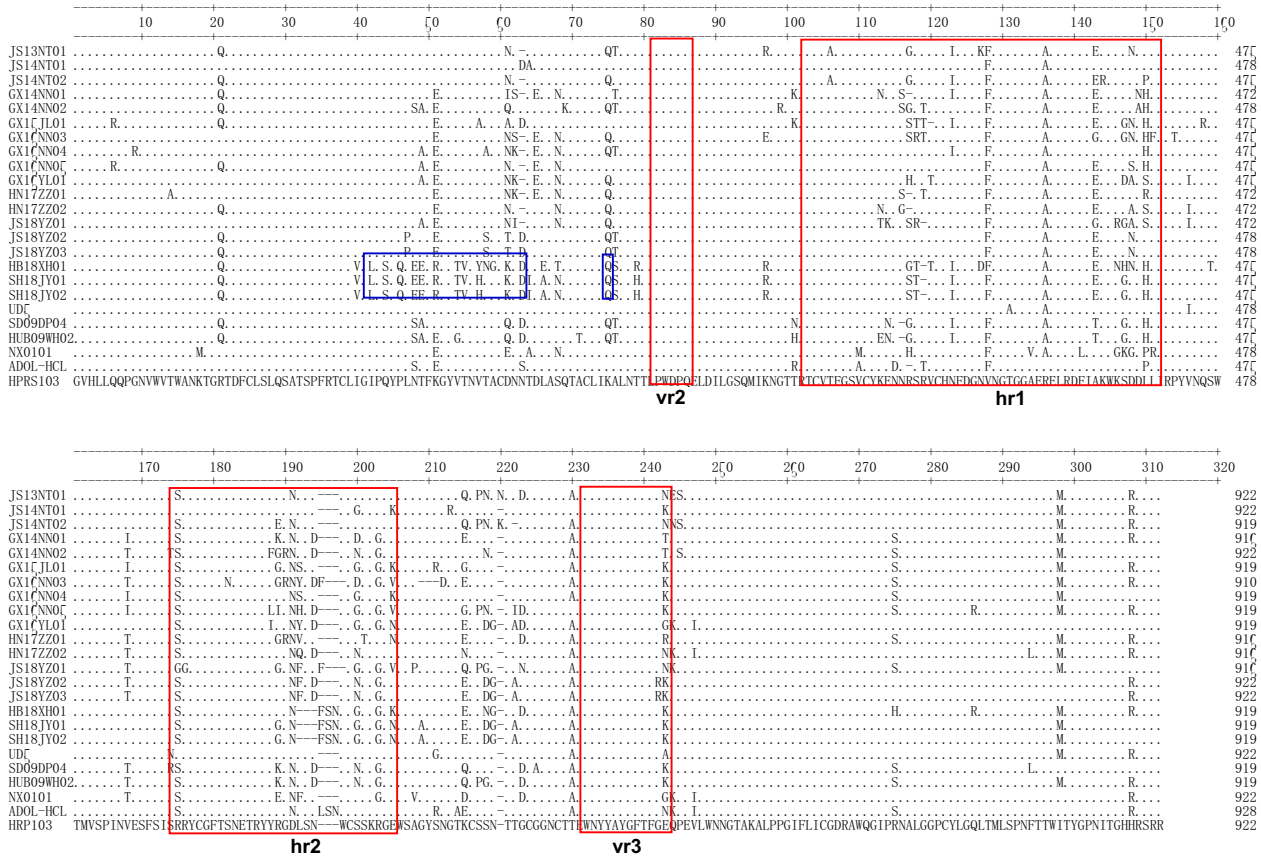


Figure 3. Sequence Analysis of the gp85 protein of ALV-J local chicken isolates. The variable domains (vr2, hr1, hr2, and vr3) are indicated in red boxes and marked. Twelve unique amino acid substitutions (SH18JY02, SH18JY01, and HB18XH01) in group IV are indicated in blue boxes. Abbreviation: ALV-J, Avian leukosis virus subgroup J.

isolates were 214–226 bp in length. Phylogenetic analysis of the U3 sequences of the 18 isolates showed that 6 isolates (6/18; JS13NT01, JS14NT01, JS14NT02, HB18XH01, SH18JY02, and SH18JY01), and the ALV-J broiler isolates belonged to a branch designated as group I (Figure 5). Eleven isolates (11/18; GX16NN03, GX16NN05, GX15JL01, HN17ZZ02, JS18YZ01, JS18YZ02, JS18YZ03, GX14NN01, GX16NN04, HN17ZZ01, and GX16YL01) exhibited the greatest sequence identity (94.6–97.3%) with ALV-J early-isolated local strains and belonged to group II (Figure 5). One isolate (1/18; GX14NN02) was closely related to ALV-J layer isolates, with a high identity of 96.8 to 99.6% and belonged to group III (Figure 5). These data confirmed that the U3 gene of most ALV-J local chicken isolates formed a unique branch.

The U3 region contains many transcriptional regulatory elements, which are also prone to nucleotide substitutions and deletions (Ruddell, 1995). Our results showed that all transcriptional regulation elements were relatively conserved in the 18 isolates, including 2 CA₂G boxes, a TATA box, a C/EBP, 2 Y boxes, and 2 PRE motifs. However, compared with the U3 region of ALV-J broiler and layer isolates, 5 unique nucleotide substitutions (T72 C, G93 A/C, A96 G, G98 A, and A130 T) were observed in ALV-J local chicken isolates belonging to group II (Figure 6).

DISCUSSION

Avian leukosis virus subgroup J was first isolated from meat chickens in China (Du et al., 2000) and has caused serious loss to the poultry industry, especially in terms of Chinese local chickens (Li et al., 2016; Zhang et al., 2018). There are several varieties of local chickens in China, which are widely distributed; these account for one-third of the domestic chicken industry (Qin, 2016). Thus, the high pathogenicity and diversity of ALV-J pose a great threat to the performance and breeding safety of local chickens in China (Meng et al., 2018; Zhang et al., 2018). To understand the molecular characteristics of ALV-J in local chicken, the full-length genome sequence of 18 isolates from local chickens in the main local chicken breeding provinces in China during the duration 2013–2018 were collected, sequenced, and analyzed. Our results show that the *gp85* and U3 genes of most ALV-J local chicken isolates formed a separate branch. The 195–210 bp deletion in the rTM and the 16–28 bp deletion in the DR-1 of the 3'UTR were found in all 18 isolates. To the best of our knowledge, this is the first systematic study of the molecular characterization of ALV-J isolates from Chinese local chickens.

The *gp85* protein forms globular structures on the surface of the virus and is closely associated with the process

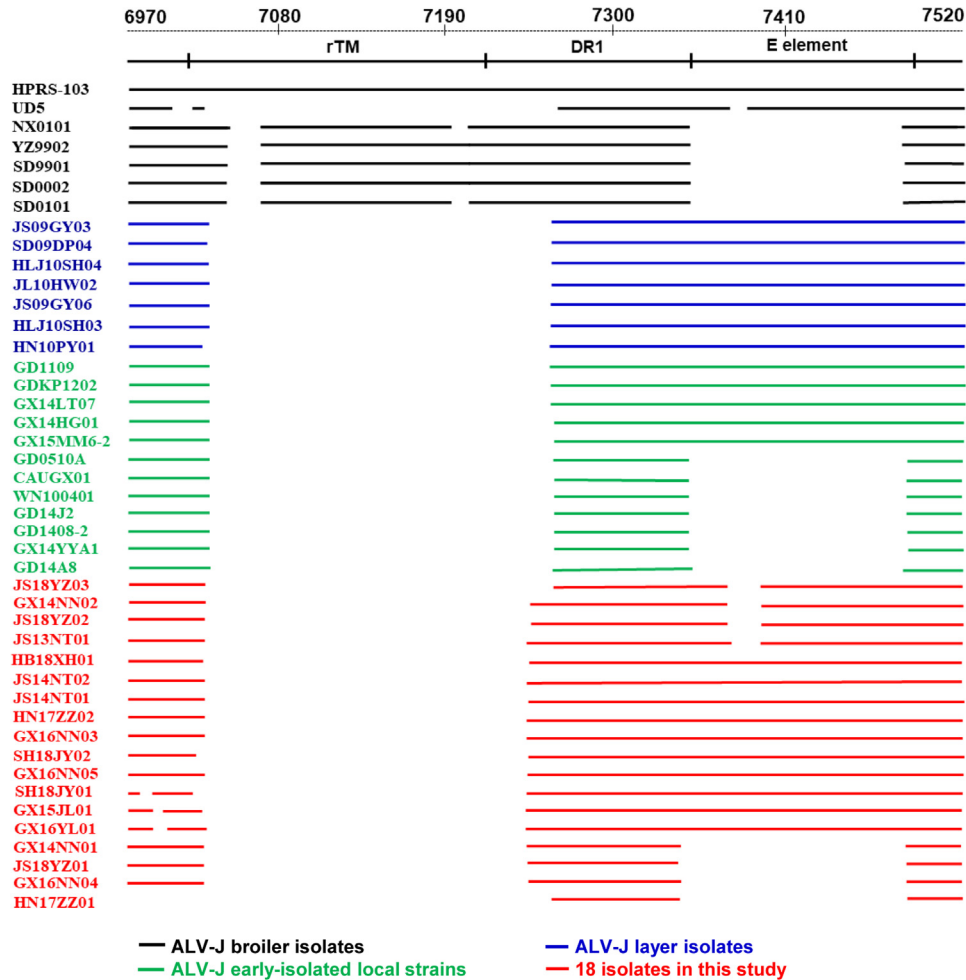


Figure 4. Diagram of the deletions in the 3'UTR of ALV-J Local Chicken, Broiler, and Layer Isolates. The top 2 lines represent the base numbers and elements in the genomic proviral DNA of HPRS-103. The deletions are indicated by empty spaces between the lines. Abbreviations: 3'UTR, 3' untranslated region; ALV-J, Avian leukosis virus subgroup J.

of viral binding and the determination of the host-specificity of each viral subgroup (Chai and Bates, 2006; Guan et al., 2017). The gp85 is the most variable of the envelope glycoproteins and exhibits high diversity in the ALV-J genome (Venugopal et al., 1998; Gao et al., 2012; Wang et al., 2017). In the present study, phylogenetic analysis demonstrated that the *gp85* genes of the 18 isolates were distributed among various genetic backgrounds and belonged to 4 different branches, indicating the complexity and diversity of variation in local chickens. Based on the phylogenetic and sequence analyses of *gp85* from the ALV-J local chicken isolates, it was hypothesized that the evolution of ALV-J in local chickens can be divided into 3 stages. First, it was determined that the *gp85* genes of ALV-J isolates from local chickens before 2014 were closely related to the corresponding genes of ALV-J broiler isolates, belonging to the first branch. Most studies indicated that certain local chicken companies practiced crossbreeding with white meat-type breeders in the past (Cui et al., 2003; Shen et al., 2014), which facilitated horizontal transmission of ALV-J. Thus, it was hypothesized that these isolates may originate from early white meat-type breeders. Second, with the introduction of ALV-J into local chickens

from 2015 to 2018, the diversity of Chinese local chickens and the differences in their growth performance provided a good environment for ALV-J variation (Meng et al., 2018). Thus, ALV-J local chicken strains continually evolved and formed a separate branch. Third, from 2018, new variations have formed in 3 isolates (SH18JY02, SH18JY01, and HB18XH01) discussed in the present study, with <90% sequence identity with all reference strains and belonging to a new branch. These findings will provide an enhanced understanding of the molecular epidemiology and evolution trend of ALV-J in Chinese local chickens.

The *gp85* contains 5 sequence variability regions, namely hr1, hr2, vr1, vr2, and vr3, based on the ALV-A to ALV-E *gp85* sequences (Dorner, 1985; Bova et al., 1988). However, the *gp85* gene of ALV-J shares only 40% sequence identity with the corresponding gene in the other subgroups (Bai, 1995). In the present study, it was found that a few mutations in ALV-J *gp85* occurred before the vr1 region and between the hr2 and vr3 regions; in contrast, the vr2 and vr3 regions of ALV-J *gp85* were relatively conserved (Figure 3). The *gp85* gene-variation characteristics of ALV-J are completely different from ALV-A to ALV-E. In

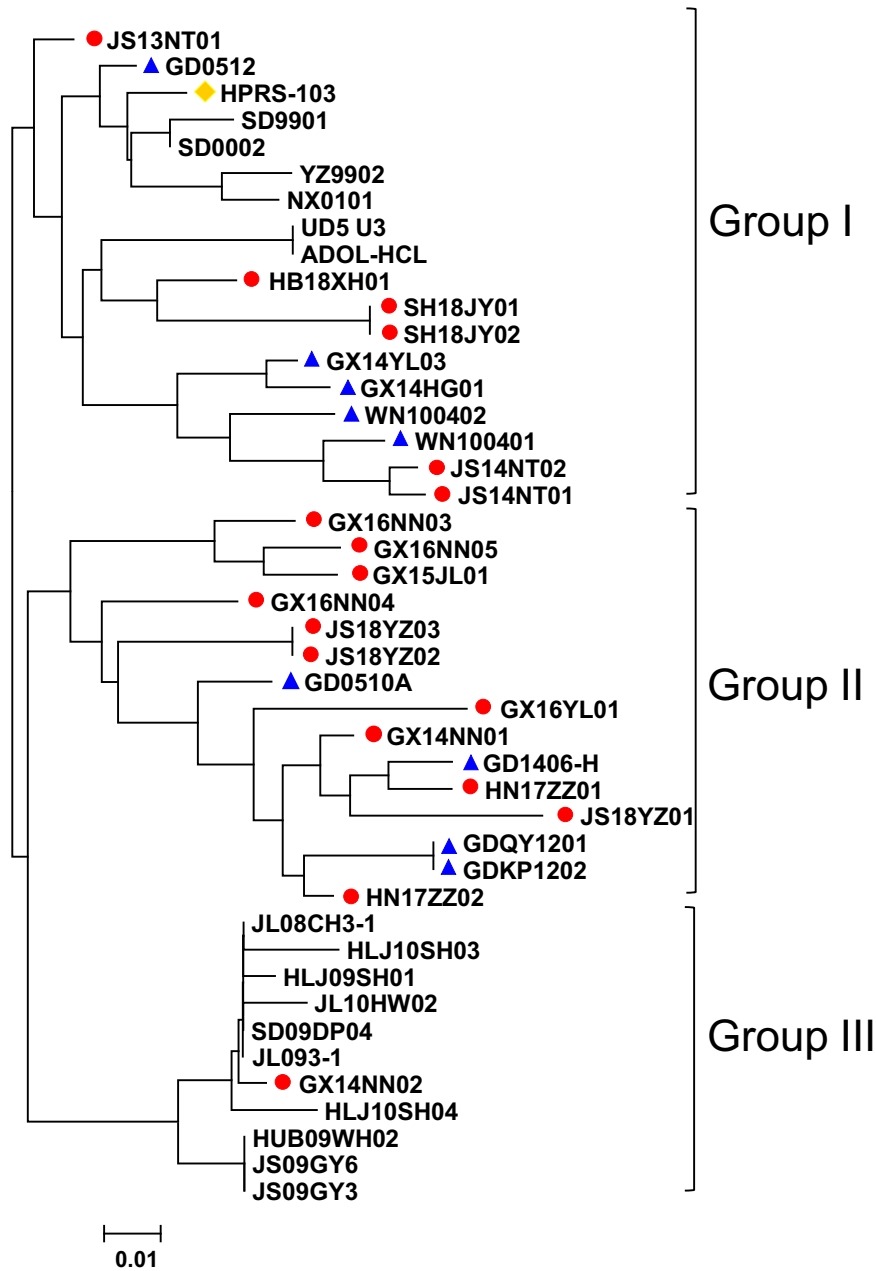


Figure 5. Phylogenetic analysis of the U3 region of ALV-J local chicken isolates. The 3 groups are marked. The red circles represent the 18 isolates in this study. The blue triangles represent ALV-J early-isolated local strains. The yellow diamond represents the ALV-J prototype strain HPRS-103. Abbreviation: ALV-J, Avian leukosis virus subgroup J.

addition, our previous studies have shown that most ALV-J *gp85* sequence variations occurred primarily near the hr1 and hr2 regions (Gao et al., 2012). Therefore, it was concluded that the division of *gp85* gene-variation regions in the other subgroups (ALV-A to ALV-E) may not be conducive to ALV-J and that the host range region and variant regions of the ALV-J *gp85* gene should be redefined.

The 3'UTR contains effective regulatory sequences that influence the expression of the chromosome and viral genes. It is a vital region that controls viral replication and pathogenesis (Zavala et al., 2007). A 205-bp deletion in the rTM and DR-1 region of the 3'UTR was first observed in ALV-J layer chicken isolates in

China in 2012 (Gao et al., 2012); a 205-bp deletion in the rTM and DR-1 region was subsequently observed in ALV-J broiler isolates (Ma et al., 2018). In the present study, all the 18 isolates displayed a 195–210 bp deletion in the rTM and a 16–28 bp deletion in the DR-1 region. Based on the above analysis, it was hypothesized that these deletions in the rTM and DR-1 regions of the 3'UTR may constitute unique molecular characteristics of ALV-J strains in China. In addition, our previous studies have demonstrated that ALV-J strains containing a 205 bp deletion in the 3'UTR showed higher pathogenicity and oncogenicity in SPF chickens (Wang et al., 2012a). Thus, the deletions in the rTM and DR-1 regions may be the result of natural evolution of ALV-J, which

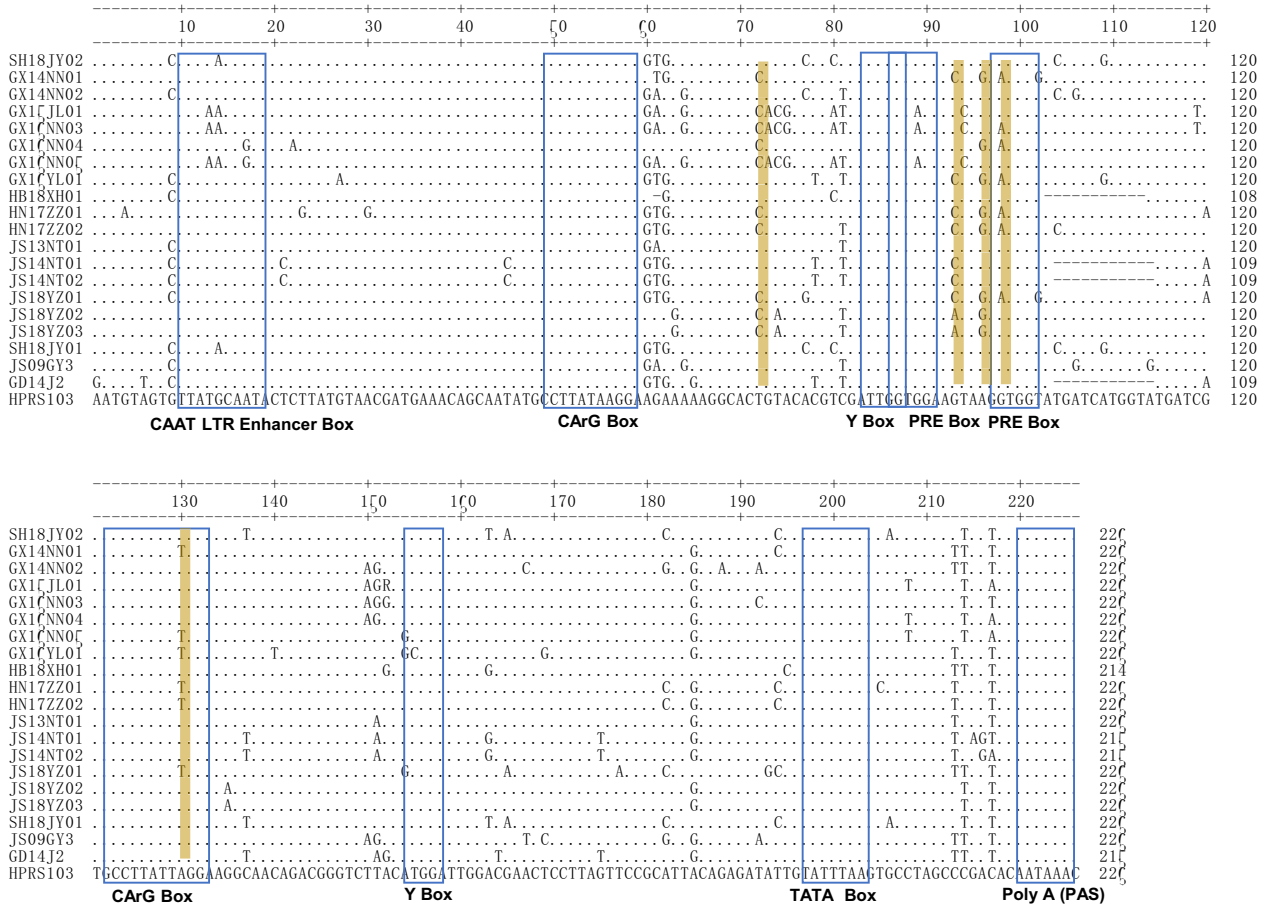


Figure 6. Analysis of the nucleotide sequences of the U3 region of ALV-J local chicken isolates. The letters indicate nucleotide substitutions; the dots indicate identical nucleotides; the dashes indicate gaps produced in the alignment. The putative transcription regulatory elements are indicated by blue boxes. Yellow shading indicates unique nucleotide substitutions in the ALV-J local chicken isolates. Abbreviation: ALV-J, Avian leukosis virus subgroup J.

was influenced by genetic selection of the breeding stock and immune pressures (Zavala et al., 2007; Gao et al., 2012).

The U3 region contains the promoter and enhancer, which are closely associated with viral replication, transcription, and translation (Zachow and Conklin, 1992). In addition, the U3 region is prone to variation in ALV-J strains. However, few studies have systematically analyzed the ALV-J U3 gene in local chicken. Our results indicated that the U3 sequences of 61.1% (11/18) of the 18 isolates shared high identity (94.6–97.3%) with ALV-J early-isolated local strains, forming their own unique genetic branch. Sequence analysis showed 5 unique nucleotide mutations in the U3 region in most ALV-J local chicken isolates. One of 5 nucleotide mutations occurred in the second CArG box, an element that is essential for cell type-specific expression of oncogenes (Cui et al., 2014). Thus, future studies should investigate the possible effects of this mutation on cell type-specific expression of oncogenes.

The ALV-J host range has gradually expanded, and its pathogenicity has become more complex in China (Xu et al., 2004). Currently, ALV-J causes relatively serious damage in Chinese local chickens (Cheng et al., 2005; Zhang et al., 2018). The present study systematically

analyzed the whole genome sequence of the 18 isolates from local chickens from 5 provinces in China from 2013 to 2018. The *gp85* gene and U3 of ALV-J local chicken isolates showed obvious variation and differed from the corresponding entities in the ALV-J broiler isolates and ALV-J layer isolates, gradually forming a distinct branch. In addition, it was determined that the gene deletions in the rTM and DR-1 regions of the 3'UTR have proved to be a unique molecular characteristic of ALV-J in China. These findings will further expand on the epidemiological data of ALV-J in Chinese local chickens and contribute to a better understanding of the pathogenic mechanism of ALV-J in Chinese local chickens.

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