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Isolation and Identification of a Subgroup A Avian Leukosis Virus from Imported Meat-type Grand-parent Chickens*

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Abstract: An exogenous avian leukosis virus (ALV) strain SDAU09C1 was isolated in DF-1 cells from one of 240 imported 1-day-old white meat-type grand parent breeder chicks. Inoculation of SDAU09C1 in ALV-free chickens induced antibody reactions specific to subgroup A or B. But gp85 amino acid sequence comparisons indicated that SDAU09C1 fell into subgroup A; it had homology of 88.8% - 90.3% to 6 reference strains of subgroup A, much higher compared to other subgroups including subgroup B. This is the first report for ALV of subgroup A isolated from imported breeders.

Key words: Avian leukosis virus(ALV); Subgroup A; Envelope gp85; Imported Chicken Breeders

Avian leukosis viruses (ALV) is a member of the *α-retrovirus* genus of *retroviridae*. Based on its infectivity to different species of birds, interference between different ALV strains in cell cultures, and different antigenicity in cross viral neutralization, ALV are divided into 10 subgroups from A to J. Among them, only subgroups A, B, C, D, E and J can infect chickens. The subgroup E ALVs belongs to endogenous virus and usually it has very low pathogenicity or no pathogenicity to chickens, but other subgroups are exogenous ALV pathogenic to chickens. The chicken lymphoid leukosis and sarcoma are mainly induced by subgroup A or B ALV, which are the most common exogenous ALV in the field. Subgroup J ALV (ALV-J) was first recognized and isolated from meat-type chickens in 1989 and mainly induces myeloid tumors [5]. During the last 20 years, ALV-J was introduced into chickens in China from chickens imported for breeding.

By very strict eradication programs, exogenous ALVs of subgroup A, B, C, D (ALV-A, B, C, D) were almost totally eradicated from the chicken breeder flocks of most international breeding companies by the end of 1980's. Although ALV-J started to emerge and had caused big economic losses to the meat-type chicken industry of the whole world from 1989, it is also almost eradicated now in all big international breeding companies through strict eradication programs implemented during the last 20 years. However, ALV-J is still spreading very widely in

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chicken farms in China since it was introduced.

Since there is no strict nationwide eradication program for ALV infection on chicken farms in China, ALV infections in chickens have become more and more serious. In the last 10 years, there have been many reports on myeloid tumor cases induced by ALV-J. It was first found and confirmed in white meat-type breeders [2, 3, 11]. Later, more and more ALV-J infections and their economic loses were detected and reported in layers [8, 9], Chinese native breeds [1] and local meat- type "yellow" chickens in South China [7].

To determine whether exogenous ALV has been completely eradicated in chickens from international chicken breeding companies and to collect experiment data from tests for exogenous ALV infection in imported breeders, we attempted to isolate exogenous ALV from directly imported grand-parent baby chicks. And then its subgroup was identified by gp85 gene sequence comparisons.

MATERIALS AND METHODS

Origin of samples

In March, 2009, 240 chicks were randomly selected from a batch of ~40 000 1-day-old white meat-type grand-parent chicks imported from abroad. They were transported in boxes provided by the breeder company to the SPF facility in our laboratory from the airport just after they arrived in China. All chicks were kept in 4 isolators with filtered positive air (less than 100 particles per cubic liter) in an SPF room (with filtered positive air, 10 000 particles per cubic liter). During the period of the experiments, feeds and water were autoclaved before use.

Isolation and titration of virus

DF-1 cells (a cell line from embryo fibroblast cells of C/E type chickens resistant to subgroup E endogenous ALV) were used to isolate exogenous ALV. Plasma samples were collected from each chickens at the age of 7d and inoculated into DF-1 monolayers in 12-well culture plates respectively. Inoculated DF-1 cultures were incubated at 37℃ with 5% CO_2 for 9 days, then the supernatant of each well was tested for ALV group-specific p27 antigen with an ALV p27 antigen detection kit (Beijing IDEXX Yuanheng Laboratories, Co., Ltd) according to the instructions of the manufacturer. Supernatant samples in wells which were positive to p27 were inoculated into another fresh DF-1 cell culture at large scale to amplify virus stocks. Positive supernatant samples were kept at -80℃.

To titer the virus stock, the supernatant was successively diluted at 1:10 with cell culture medium and inoculated into fresh DF-1 cells in 96-well plates; 8 wells were inoculated for each dilution. After incubation at 37℃ with 5% CO_2 for 9 d, p27 was tested as above and virus titers were determined as $TCID_{50}$.

Testing antibody responses to ALV-AB or ALV-J

All chickens were kept in the isolators for 50 d. During the period, cloaca swabs were collected 4 times from each bird and tested for p27 with an avian leukosis virus antigen detection kit (IDEXX laboratory). At 50 d, serum samples were collected from each bird for testing antibody responses to ALV-AB and ALV-J with an avian leukosis virus antibody test kit for subgroup AB or J (IDEXX laboratory). Eight birds which were negative to p27 antigen and ALV-AB or ALV-J antibodies were selected and moved into another isolator for intravenous inoculation with isolated viruses of $6\times10^5 TCID_{50}$ in 2 mL. Serum samples were collected for testing antibody reactions to ALV-AB or ALV-J 1, 2, 3, 4, 5 weeks after inoculation.

PCR with subgroup specific primers to identify subgroups of isolated viruses

DNA was extracted from DF-1 cells in wells which were p27 antigen positive by ELISA and used as templates in PCR with subgroup A, B, C, D, J specific primers CapA-F/CapA-R, CapB-F/CapB-R, CapC-F/CapC-R, CapD-F/CapD-R, H5/H7 (for J) according to Fadly *et al*[4]. The total 25 μL reaction solution, contained 10 mmol/L Tris-HCl, 1.0 mmol/L $MgCl_2$, 200 mmol/L dNTPs, 20 pmol primers, 0.5 U Taq enzyme, 100 ng template DNA. PCR procedure was: 95℃ 2 min ,95 ℃ 30 s, 57 ℃ 30 s, 72 ℃ 1 min , 30 cycles, then 72℃ 10min.

Amplification, cloning and sequencing of envelope gene (*env*)

To amplify *env* genes, the up- and down-stream sequences, which are very conservative to all ALV subgroups, were synthesized as primers. The forward primer was ALV(all)-F:5'-GGATGAGGTGACTAAGAAAG-3', the reverse primer was ALV(all)-R:5'-ACACTACATTTCCCCCTCCCTAT-3', which correspond to bp 5026-5045# and bp 7024-7047# of the subgroup. A reference strain RSA genome (GenBank accession number M37980), or to bp 5053- 5072# and bp 9118-9140# of subgroup B reference strain S-R B genome (GenBank accession number AF052428). PCR was conducted under the same conditions as listed above. The purified PCR products were cloned into pMD18-T vector for sequencing by commercial companies.

Amplification, cloning and sequencing of intact LTR

DNA fragments at both ends of LTR were amplified from proviral DNA in circular form as the template with the following primers: Forward primer F5: 5'-TGAAAGGACTGCTTTTGGGGCTTGTAGT-3', Reverse primer R-cDNA: 5'-TTTTCCCGCAATAGGTTTTACAC-3'. corresponding to bp 6697-6724# and bp 404-426# of ALV-A reference strain RSA (GenBank accession M37980) respectively.

Homologous comparison of gp85 amino acid sequences

Because the subgroups of ALV were determined only by the gp85 envelope protein, homologous comparisons of isolated virus to reference strains of subgroups were made only to the gp85 amino acid sequences. 6 strains of Subgroup A: A46 (DQ412726), B53 (DQ412727), RSA (M37980), MQNCSU (DQ365814), RAV-1 (M19113), 48 (AY131210); 2 strains of subgroup B: S-R B (AF052428), RAV-2 (M14902); 1 strain of subgroup C: Prague C (J02342); 1 strain of subgroup D: S-R D (D10652); 3 strains of subgroup E: RAV-0 (M12172), ev-1(AY013303), SD0501(EF467236); 3 strains of subgroup J: HPRS103(Z46390), NX0101 (DQ115805), ADOL-7501(AY027920). The GenBank accession numbers for sequences of each reference strains were listed in the paragraphs. The DNAstar Lasergen7.0 was used as the software in the comparisons.

LTR sequences of ALV reference strains of different subgroups

To make homologous comparisons of LTR, the following LTR sequences of different subgroups were downloaded from GenBank: 2 strains of ALV-A, RSA (M37980) and MQNCSU (DQ365814); 1 strain ALV-B, S-R B (AF052428); 1 strain ALV-C, Prague C(J02342); 1 strain ALV-D, S-R D (D10652); 2 strain ALV-E, ev-1(AY013303) and SD0501 (EF467236)); 3 strains ALV-J, HPRS103 (Z46390), NX0101 (DQ115805), ADOL-7501(AY027920).

RESULTS

Isolation, identification and titration of exogenous ALV

DF-1 cell cultures were kept in incubators at 37℃

for 9 d and the supernatant samples were tested for ALV p27 antigen. Among wells inoculated with plasma samples of 240 chickens, only one chicken sample was positive to p27, indicating that it had viremia of an exogenous ALV. The isolate was designated as SDAU09C1 and inoculated into larger plates with fresh DF-1 cell cultures. Then the supernatant was harvested and kept at -80℃ as stock. Its titer was determined to be as high as $10^{5.5}TCID_{50}$/ mL.

Determination of subgroup specificity of antibodies

Among the 8 chickens inoculated with SDAU09C1, one was antibody positive to ALV subgroup AB 4 weeks later, and one more bird appeared positive to ALV-AB at 5 weeks after inoculation, but all sera were negative to ALV-J. The results suggest that the ALV isolate SDAU09C1 belongs to subgroups A or B.

Subgroup verification of SDAU09C1 by PCR

When DNA extracted from DF-1 cells infected with SDAU09C1 was used as template for PCR, the expected DNA band was only detected in reactions with subgroup A-specific primers CapA-F/CapA-R (Fig. 1), all other pairs of primers gave no band. The result suggested that SDAU09C1 most likely belongs to subgroup A.

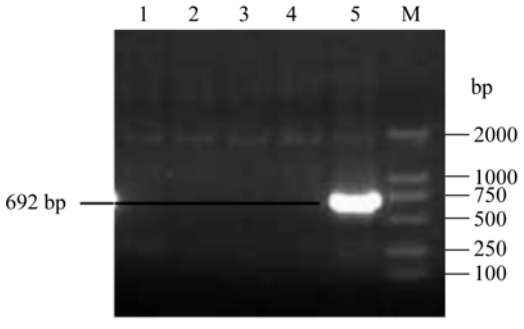

Fig.1. PCR amplification of proviral DNA extracted from SDAU09C1-infected DF-1 cell. Lane 1, subgroup B–specific primers; lane 2, subgroup C–specific primers; lane 3, subgroup D–specific primers; lane 4, subgroup J–specific primers; lane 5, subgroup A–specific primers; M, DNA marker DL2000.

Homologous comparison of SDAU09C1 gp85 amino acid sequences to reference strains

By using SDAU09C1-infected DF-1 cell genomic DNA as template, a DNA fragment of 2.2 kb was amplified with primers ALV(all)-F and ALV(all)-R. It contained 1215 bp of gp85 ORF, 612 bp of gp37 ORF and a part of the 3'terminal sequence. When its gp85 amino acid sequence was compared to other reference strains, it had 88.8% - 90.3% of identity to 6 reference strains of subgroup A. The homology to subgroups B, C, D, E was in the range of 78% - 83.6%, the homology between SDAU09C1 and 3 reference strains of subgroup J was as low as 30.9% - 35.1% (Fig. 2). The phylogenic tree indicates that the isolate SDAU09C1 definitely fell into subgroup A (Fig. 3).

Homologous comparison of SDAU09C1 to different subgroup reference strains for their LTRs

A DNA fragment of 1178 bp was amplified by primers of F5/R-cDNA from SDAU09C1 infected cell genomic DNA. Sequence analysis indicated that there was an intact LTR fragment of 314 bp with U3, R, and U5 when compared to the published LTRs. As Table 1

Table 1. LTR nucleotide percentage of identity between the SDAU09C1 isolates and reference ALV sequence

Virus subgroups	Virus strains	Different part of LTR(%)		
		Intact LTR	U3	U5
ALV-A	RSA	88.8	88.8	84.4
	MQNCSU	87.3	88.3	84.4
ALV-B	S-R B	88.5	88.8	83.1
ALV-C	Prague C	90.1	88.8	92.2
ALV-D	S-R D	89.8	88.8	88.3
ALV-E	ev-1	71.9	47.3	89.6
	SD0501	71.4	47.7	88.3
ALV-J	HPRS103	86.9	89.8	80.5
	NX0101	89.2	92.6	81.8
	ADOL-7501	89.2	92.1	83.1

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1		90.3	90.3	88.8	89.1	89.7	89.4	78.0	78.0	83.3	83.6	83.3	82.1	83.3	32.5	35.1	30.9	1
2			89.7	88.8	88.2	88.8	88.8	75.1	75.7	83.0	80.9	82.9	82.0	82.4	34.4	32.8	33.2	2
3				98.8	90.9	90.3	99.1	77.3	77.9	82.9	82.9	83.5	82.3	83.5	32.5	31.8	31.3	3
4					91.7	90.9	99.4	76.5	77.1	82.4	81.8	82.4	81.8	82.4	33.1	32.1	32.9	4
5						97.9	91.4	77.6	78.2	82.3	80.5	82.0	81.4	82.0	33.1	32.1	32.9	5
6							90.9	78.2	78.8	82.4	80.6	81.5	80.9	81.5	33.1	32.1	32.9	6
7								77.0	77.6	82.0	82.0	82.6	81.4	82.6	32.5	31.8	31.3	7
8									98.8	79.2	87.0	80.6	80.0	80.1	35.4	36.4	34.2	8
9										79.8	87.6	81.2	80.6	80.6	34.7	36.0	33.9	9
10											83.9	85.0	84.2	84.2	35.4	33.4	36.2	10
11												84.7	84.1	84.4	36.7	36.4	36.5	11
12													98.6	98.6	37.0	34.4	32.2	12
13														98.3	35.4	35.1	32.6	13
14															37.0	36.4	32.2	14
15																90.3	83.7	15
16																	80.8	16
17																		17
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	

SDAU09C1, 48, A46, B53, MQNCSU, RAV-1, RSA — A
RAV-2, S-R B — B
Prague C — C
S-R D — D
ev-1, RAV-0, SD0501 — E
HPRS-103, NX0101, ADOL-7501 — J

Fig.2. Homologous comparisons of the gp85 amino acid sequence of SDAU09C1 to other reference chicken ALV strain of different subgroups. All reference sequences were obtained from GenBank with accession numbers as follows. 1) ALV-A: A46 (DQ412726), B53 (DQ412727), RSA (M37980), MQNCSU (DQ365814), RAV-1 (M19113), 48 (AY131210). 2) ALV-B: S-R B (AF052428), RAV-2 (M14902). 3) ALV-C: Prague C (J02342). 4) ALV-D: S-R D (D10652). 5)ALV-E: RAV-0 (M12172), ev-1 (AY013303), SD0501 (EF467236). 6) ALV-J: HPRS103 (Z46390), NX0101 (DQ115805), ADOL-7501 (AY027920).

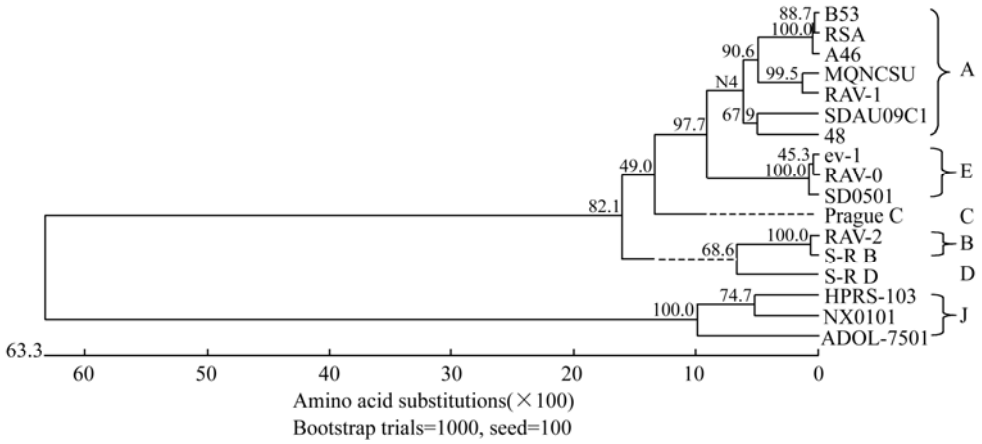

Fig.3. Phylogenetic analysis for gp85 amino acid sequences of SDAU09C1 and other ALV reference strains of different subgroups. All reference sequences were obtained from GenBank with accession numbers as follows. 1) ALV-A: A46 (DQ412726), B53 (DQ412727), RSA (M37980), MQNCSU (DQ365814), RAV-1 (M19113), 48 (AY131210). 2) ALV-B: S-R B (AF052428), RAV-2 (M14902). 3) ALV-C: Prague C (J02342). 4) ALV-D: S-R D (D10652). 5) ALV-E: RAV-0 (M12172), ev-1 (AY013303), SD0501 (EF467236). 6) ALV-J: HPRS103 (Z46390), NX0101 (DQ115805), ADOL-7501 (AY027920).Numbers at the branch points in the tree are bootstrap values.

indicates, SDAU09C1 had only 71.4%-71.9% homology to the total LTR with 2 endogenous ALV-E strains. This was because the U3 homology between SDAU09C1 and two ALV-E strains was as low as 47.3%-47.7%, but SDAU09C1 had as high as 88.3% -92.6% of homology in U3 with other exogenous viruses of subgroups A, B, C, D, and J. This lead to SDAU09C1 having a higher homology of 86.9%-90.1% in total LTR to all other exogenous ALV subgroups.

DISCUSSIONS

Since the mid 1980's, almost every big international chicken breeding companies declared that exogenous

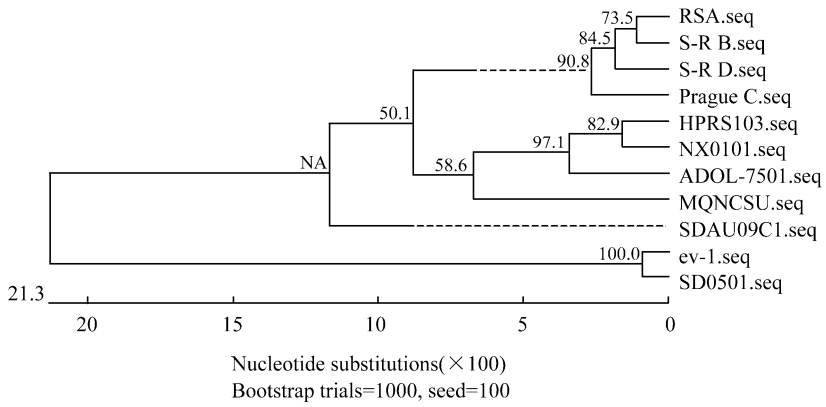

Fig. 4. Phylogenetic analysis for LTR sequences of SDAU09C1 and other ALV reference strains of different subgroups. Representatives of prototype viruses of various subgroups were obtained from GenBank with accession numbers as follows. 1) ALV-A: RSA (M37980), MQNCSU (DQ365814). 2) ALV-B: S-R B (AF052428). 3) ALV-C: Prague C (J02342). 4) ALV-D: S-R D (D10652). 5) ALV-E: ev-1 (AY013303), SD0501 (EF467236). 6) ALV-J: HPRS103 (Z46390), NX0101 (DQ115805), ADOL-7501 (AY027920). Numbers at the branch points in the tree are bootstrap values.

ALV of subgroups A, B, C, D has been eradicated from their breeder flocks. However, during the last 10 years, subgroup J ALV infection has become a major concern in chicken farms in China. This study indicated that an ALV-A was isolated and identified from one of 240 chickens randomly picked up from a batch of directed imported white grand parent breeder chickens. This is the first report by the valid laboratory test that exogenous ALV infection is not absolutely excluded from breeders of large international companies, although the strict eradication programs on ALV infection have been conducted for many years. However, this result is not a complete surprise since it was recently reported that there were some ALV-A contamination in the Marek's disease vaccines of several producers; such vaccines have been widely used in some farms in US [6, 10] and it is likely that some breeder farms have used such contaminated vaccines. Although these farms have initiated new eradication programs against ALV, it will be difficult to completely remove the virus from primary breeder flocks even within a few years. Although SDAU09C1 only has 88.8%-90.3% homology with strains A46 and B53 isolated from MD vaccines[6] for the gp85 protein, the gp85 gene sequences of other ALV-A isolates from MD vaccines in US [10] have not been published so far. The results in this study serve as a warning to Chinese farms that ALV-A may be introduced from breeders imported from other countries.

For classification of subgroups of ALV isolates, standard methods were based on phenotypes such as interference tests and cross viral neutralizations in cell cultures among different isolates and reference strains of different subgroups. However, these methods are too complex and need reference strains of all known subgroups[5]. Because subgroups of ALV are determined by their envelope protein gp85, some laboratories for ALV have started to use homologous comparisons of gp85 for identification and differentiation of their subgroups [2, 6, 10].

Sequence comparisons in this study indicated that the gp85 sequence of SDAU09C1 had the highest homology with known subgroup A stains (88.8% - 90.3%), compared to other exogenous ALV-B, C, D subgroups (78% - 83.6%) and endogenous ALV-E (82.1% - 83.6%); its homology to ALV-J was very low

- only 30.9% - 35.1%. The phylogenetic tree in Fig. 3 indicates that SDAU09C1 belongs to subgroup A. However, because ALV gp85 gene has high mutation rates, there is large diversity even within the same subgroup; the gp85 homology among the listed 6 reference strains of subgroup A was in the range of 88.2% to 99.4%, and the gp85 homology of SDAU09C1 to all 6 ALV-A reference strains was 88.8%-92.3% and no less than the homology of 88.2% between 2 ALV-A reference strains 48 and MQMCSU. However, LTR sequence homology comparisons suggested that LTR of SDAU09C1 was closer to subgroups C and J than A (figure 4). In particular, U3 of SDAU09C1 had highest homology (89.8% - 92.6%) to subgroup J strains whereas its U5 had highest homology (92.2%) to subgroup C. We might speculate that such phenomena could be due to recombination between the env genes and LTR between different viruses of different subgroups or else a consequence of accumulated random mutations but further studies are needed with more field strains of different subgroups to compare the relationship of LTR and subgroups.

In the past 10 years, reports or publications on ALV have been almost exclusively related to ALV-J[2, 3, 7~ 9]. So far, there have been no reports on subgroup A-induced tumors in chickens. Given the identification of subgroup A ALV in imported breeders, further studies need to be conducted on the pathogenicity and epidemiologic significance to chicken flocks, especially the relationship between the subgroup A strain SDAU09C1 and tumor or hemangioma cases in chickens in China.

Acknowledgement

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