Re-emergence of Avian leukosis virus subgroup J in the rooster of Hy-line brown and its transmission pattern in flocks

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Abstract

Avian leukosis virus subgroup J (ALV-J) is the most prevalent subgroup in chickens and exhibits increasing pathogenicity and stronger horizontal and vertical transmission ability in different kinds of chicken. Although vertical transmission of ALV-J from hens infected through artificial insemination with ALV-J infected semen was reported before by the detection of swabs and serum, there was no further research on the trasmission pattern of ALVs in the roosters. In the present study, the introduction of Hy-line brown roosters infected with ALV-J significantly increased the p27 positive rate of ALV in a indigenous flock detected by virus isolation. Sequence analysis and IFA showed that it is classified into ALV-J subgroup, locating in a new branch compared with the domestic and foreign referential sequences. Meanwhile, the gp85 gene of the ALV-J isolated in the hens and its albumens had a homology of 94.1-99.7% with that in the roosters, which means that the strain is quite likely transmitted to the hens and their offspring through insemination of the roosters. Moreover, Semens are directly detected by ELISA method is not completely accurate. There are four ALV-J infection status in plasma and semen of rooster (V+S+, V-S+, V+S-, V-S-), so the eradication of ALV in rooster requires simultaneous virus isolation of semen and plasma. Therefore, we speculate that the reason why there are still some sporadic findings of ALV-J in laying hens is probably due to the incomplete eradication process of roosters.

1 —Introduction

Avian leukosis (AL), inducing malignant or benign tumorigenic diseases by avian leukosis virus (ALV), has caused immense economic losses all over the world in the poultry industry since its emergence(Payne & Nair, 2012; Weiss & Vogt, 2011). ALVs were divided into 11 different subgroups (designated A to K) based on cross neutralization and gp85 sequences(Dong et al., 2015). Among those, avian leukosis virus subgroup J (ALV-J) is the most prevalent subgroup in chickens and exhibits increasing pathogenicity and stronger vertical transmission ability in recent years (Li et al., 2016; Payne, Howes, Gillespie, & Smith, 1992; Venugopal, Howes, Flannery, & Payne, 2000). ALV-J infection used to be a common problem in poultry industry during 2003–2010, when its widespread causing the myelocytoma of broilers and haemangioma of layers(Cui, Du, Zhang, & Silva, 2003; Lai et al., 2011; Li et al., 2018; Li et al., 2013; Pan et al., 2012) . Because of the widespread distribution of the ALV-J strains, together with the less organized nature of the poultry industry especially among the local breeds of chickens, control and eradication of ALV in China

remain a major challenge. Since 2018, a more tumorigenic ALV-J strain has appeared in the imported broiler breeders in China, inducing severe osteomas in keel and ribs, causing a massive pandemic, and hence becoming a major avian health concern. Therefore, many domestic farms have started self-checking for ALV infection.

Rubin and his colleagues defined four serological classes of susceptible birds: viraemia, no antibody (V+A-); no viraemia, with antibody (V-A+); viraemia, with antibody (V+A+); and no viraemia, no antibody (V-A-)(Payne & Nair, 2012; Rubin, Fanshier, Cornelius, & Hughes, 1962). Yet the researchers did not elaborate on the ALV infection status of male semen or the role of males in the transmission of ALV. During another study, Li found that roosters could spread ALV-J to hens by insemination and cause vertical transmission(Li et al., 2017). Therefore, roosters may be selected for freedom from Avian leukosis virus by ELISA tests of cloacal swabs or semen sampling, as well as virus isolation. However, the accuracy of these detection methods remains to be defined.

In this study, it was discovered ALV-J infection re-emerged in Hy-line brown roosters in recent years. Moreover, the hens were infectecd by insemination, and virus can be transmitted vertically to the breeding eggs. Several infection states of ALV-J in roosters were clarified and the reliability of different methods for detecting samples of males were compared. All those data may provide more detailed basis for the eradication of ALV.

2 — Materials and methods

2.1 — Sample origin

The positive rates of virus isolation and p27 antigen in breeding eggs of an indigenous chicken were both within 1% for two consecutive generations. In order to introduce new genes, the introduction of Hy-line brown fast feathering roosters and artificial insemination were performed. Ever since, about 7.61% (7 from 92 samples) were found to be p27 positive in the albumen samples. Therefore, semen and plasma samples from the same males (46 samples), plasmas from hens (92 samples) and albumens from the five ALV positive hens (10 samples) were collected for virus isolation of for ALV, respectively.

2.2 — virus isolation and identification

Blood samples were collected aseptically from the Hy-Line brown layer chickens, and semens were collected as sterile as possible. The semen samples were diluted 5 times with PBS containing 2% penicillin and streptomycin. After mixing upside down gently, the supernatant was centrifuged to precipitate cells at 4000 r/min for 2 min. Albumens were collected aseptically, and diluted 4 times with DMEM in 5ml syringes. Virus isolation of plasmas, semen and albumens were performed by inoculation of DF1 cells as previously described (Meng et al., 2018). The ALV group-specific antigen p27 in the culture supernatant was detected by an enzyme-linked immunosorbent assay.

The cell wells were washed with PBS and fixed with cold acetone–alcohol mixture (3:2) for 5 min. Then, the cells were incubated with mouse anti-ALV-J monoclonal antibody JE9 (Qin et al., 2001) at 37°C for 60 min, following by incubation with goat anti-mouse IgG antibody conjugated with fluorescein isothiocyanate (Sigma, California, USA) at 37°C for another 60 min. Finally, the cells were observed under fluorescence microscope.

2.3 — Primers and PCR Amplification

A pair of universal primers (ALV-F/R) , targeting env and LTR, was adopted to detect exogenous ALVs as previously reported (ALV-F:GATGAGGCGAGCCCTCTCTTTG/ALV-R: TGTGGTGGGAGGTAAAATGGCGT). DNA was extracted from the DF-1 cells. The conditions for PCR with primers ALV-F/R were as follows: 95degC for 5 min; followed by 31 cycles of 95degC for 50 s, 55degC for 40 s, and 72degC for 140 s; with a final elongation step of 10 min at 72degC. The PCR product was analyzed by electrophoresis in 0.8% agarose in Tris-acetate-EDTA buffer. Molecular cloning of positive amplicons was performed and positive clones were confirmed and subjected to Sanger sequencing.

2.4 — Sequence Analysis

Sequence alignments were assembled with other ALV-J referential sequences retrieved from the National Center for Biotechnology Information database (Table 1). Phylogenetic analysis was based on the neighbor-joining method with 1000 bootstrap replicates by MEGA ver. 5.1 (Tamura et al., 2007). The GenBank accession numbers of the strains used in this study are listed in Table 1.

3 — Results

3.1 — The introduction of Hy-line brown roosters with ALV-J significantly increased the positive rate of ALV in the flock

Before the introduction of Hy-line brown roosters, the positive rate of virus isolation and p27 in the albumens in the flock was within 1% for two consecutive generations, and the peak egg production was about 70%. However, after the introduction of the roosters, the positive rate of virus isolation and p27 in the albumens increased to 5.4% (5/92) and 7.6% (7/92), respectively (Table 2). The peak egg production dropped to about 65% from 75%. Two breeding eggs from each positive hen were chosen for virus isolation with albumen, and only one albumen showed positive result (1/10).

3.2 — The infection status of Avian leukosis virus in semen and plasma of a rooster

Virus isolation was performed on the semen and plasmas of 46 roosters simultaneously. In total, 14 roosters were tested to be positive for ALV in plasma or semen (30.43%). Among those, 12 were positive in plasma (26.09%) and 8 were positive in semen (17.39%). According to the results of virus isolation from semen and plasma of the same rooster, there are 4 types of infection status of Avian leukosis virus in the 46 roosters (Table 2). 6 chicken showed viraemia, with semen positive (V+S+, 13.04%); 6 chickens showed viraemia, with semen negative (V+S-, 13.04%)); 2 chickens showed no viraemia, with semen positive (V-S+, 4.35%), while the remaining 32 were double-negative (V-S-, 69.56%).

3.3 — High proportion of false positives in semens directly detected by ELISA

Semens were detected directly using ELISA, and the results showed that 10 samples were positive, but only 6 corresponding samples showed positive from virus isolation in plasmas or semen (accurucy rate 42.86% (6/14)). At least 4 samples showed negtive in the ELISA were false negtives. Therefore, the remaining 4 positive semen sample in the ELISA were false positives (mistaken rate 8.69%) (Table 2).

3.4 — Identification of Avian leukosis virus isolated from roosters, hens and albumens

The positive samples from roosters, hens, and albumens were went through virus isolation and sequence analysis, and were classified into ALV-J by the *gp85* gene and phylogenetic tree analysis (Figure 1). IFA also showed positive with the monoclonal antibody JE9 (Figure 2). However, these strains are located in a new branch of the subgroup J, and do not show obvious homology through the comparison with domestic and foreign referential sequences (Figure 1).

3.5 — The homology analysis of ALV-J strains in roosters, hens and albumens

The viruses isolated from roosters, hens, as well as albumens, show a homology between 94.1-99.7%, and are located in the same branch of the phylogenetic tree (Figure 2), which demonstrate that they are derived from the same source. The comparison of the *gp85* sequence between semen and plasma sample of roosters shows that the nucleotide homology in most roosters (5/6) is more than 98.6%, but the other one (1/6) only has a homology of 96%, and its homology with that of the other 5 chicken is as low as 94.1%.

4 — Discussion

Since the first strain of ALV-J, HPRS-103, 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., 1992; Zhang et al., 2020). Being the most pathogenic subgroup of ALV, ALV-J could induce malignant or benign tumorigenic diseases and immunosuppression in chicken, including hemangiomas, myelomas and

fibrosarcomas(Payne & Nair, 2012; Sun et al., 2016; Wang et al., 2016a, 2016b). The outbreak of hemangioma associated with ALV-J was reported between 2006 and 2010 in China in commercial layer chickens(Cheng, Liu, Cui, & Zhang, 2010; Lai et al., 2011). However, with the implementation of the ALV purification project by a number of major breeder companies, successful eradication has been achieved at pedigree and multiplier levels. Therefore, there is few reports of ALV-J infection in layers in recent years. Zhao discovered that co-infection with avian hepatitis E virus and avian leukosis virus subgroup J is the cause of an outbreak of hepatitis and liver hemorrhagic syndromes in a Hy-line brown layer chicken flock in China recently(Sun et al., 2020). This study also found subclinical infection of ALV-J in the roosters of Hy-line layers, which reminded us that the purification of ALV can not be overlooked, and needs to be continued through monitoring by proportional sampling.

Different methods were used to detect various samples of roosters and hens in the same flock. The ELISA method is used to detect semen directly, which showed that only 50% samples are consistent with the results of virus isolation. Virus isolation is used as the gold standard for detecting ALV, so the results achieved by ELISA has false positives and false negatives, resulting in missed and false mistaken of chickens. At the same time, different infection status in semen and plasma of the same rooster were clarified, namely, viraemia, with semen positive (V+S+, 13.04%), no viraemia, but semen positive (V-S+, 4.35%), viraemia, but semen negative (V+S-, 13.04%), double-negative (V-S-, 69.56%). The positive rate of virus isolation from plasma is higher than that of semen, but the two are not one-to-one. Therefore, this suggests that we need to choose both semen and plasma for virus isolation when performing ALV purification of roosters in order to obtain a better purification effect.

Li previously found that ALV-J in semen could be transmitted to hens by insemination through animal experiments in SPF chicken(Y. Li et al., 2017), but they only discovered the antibody of ALV-J, without viraemia in the hens and their offspring. This study is based on the infected clinical flocks. The ALV-J was isolated not only in the hens, but also in its breeding eggs. Although the positive rate of virus isolation in albumens was low, one strain of ALV-J was found and sequenced. The homology analysis showed that the strain had a homology of 94.1-99.7% with that in the roosters. Up to 100% similarity means that the strain is quite likely transmitted to the hens and their offspring through insemination of the roosters. This study comprehensively described that ALV-J from roosters can be transmitted to hens and their offspring through insemination.

Previous studies have demonstrated that ALV-J displays a high level of genetic variation and recombination (Dong et al., 2017; Meng et al., 2016), which allows the development of new variants with changes in antigenicity, tissue tropism, host range and pathopoiesis (Lupiani, Hunt, Silva, & Fadly, 2000). Gao found that ALV-J is subject to greater selective pressure in the hen's follicles, which can promote the evolution of the virus(Gao et al., 2020). In this study, we found that the ALV-J in the semen of the same rooster had only 96% homology with that in the plasma, which indicated that semen of roosters may suffer heavy selective pressure that promoted the evolution of ALV-J;and that is why the isolated strains located in a new branch in the phylogenetic tree compared with the referential stains.

In summary, we isolated ALV-J from Hy-line brown layers, and discovered the complete chain of the transmission of ALV-J from roosters to hens and then to the offspring through insemination and vertical transmission. Semen are detected by ELISA method is not completely accurate. There are four ALV-J infection status in plasma and semen of rooster, so the purification of ALV in rooster requires simultaneous virus isolation of semen and plasma. Therefore, we speculate that the reason why there are still some sporadic findings of ALV-J in laying hens is probably due to the incomplete purification process of roosters.

Date Availability

The data used to support the findings of this study are included within the article.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1 The referential strains for the comparison of*gp85* gene

Strain	Year	Origin	Source	Genbank	Subgroup
RAV-1	1980	USA	/	MF926337	A
MAV-2	1993	CAN	/	L10922	B
RSR B	1998	USA	/	AF052428	B
Prague C	1977	USA	/	J02342	C
RSR D	1992	JPN	/	D10652	D
EV-1	2000	USA	/	AY013303	E
SD0501	2007	CHN	CEF	EF467236	E
JS11C1	2014	CHN	Gallus	KF746200	K
JS14CZ01	2017	CHN	Gallus	KY490695	K
QL1	2020	EGY	Layer	MN496121	J
HPRS-103	1989	UK	Brioler	Z46390	J
NX0101	2001	CHN	Brioler	DQ115805	J
ADOL-7501	1997	UK	CEF	AY027920	J
ADOL-HC1	1993	UK	Brioler	AF097731	J
JS09GY3	2009	CHN	Layer	GU982308	J

HuB09WH02	2009	CHN	Layer	HQ634804	J
HN1001-1	2010	CHN	Layer	HQ260974	J
GD1109	2011	CHN	Layer	JX254901	J
NG_VX29	2017	CHN	Vaccine	MH669345	J
EO59	2017	NGA	Layer	MF926336	J
SVR807	2008	RUS	Gallus	HM776937	J
10022-2	2006	USA	Gallus	GU222396	J
SDGM1801	2018	CHN	Broiler	MN413674	J
GD14J2	2014	CHN	Yellow	KU500032	J
YZ9902	2010	CHN	Broiler	HM235670	J
CLB908U	2009	RUS	Gallus	JQ935966	J
HB201101	2020	CHN	Layer-P	MW476816	J
HB201101-1	2020	CHN	Layer-E	MW476817	J
HB201102	2020	CHN	Layer-P	MW476818	J
HB201102-1	2020	CHN	Layer-S	MW476819	J
HB201103	2020	CHN	Layer-P	MW476820	J
HB201103-1	2020	CHN	Layer-S	MW476821	J
HB201104	2020	CHN	Layer-P	MW476822	J
HB201104-1	2020	CHN	Layer-S	MW476823	J
HB201105	2020	CHN	Layer-P	MW476824	J
HB201105-1	2020	CHN	Layer-S	MW476825	J
HB201106	2020	CHN	Layer-P	MW476826	J
HB201106-1	2020	CHN	Layer-S	MW476827	J
HB201107	2020	CHN	Layer-P	MW476828	J
HB201107-1	2020	CHN	Layer-S	MW476829	J

Table 2 The results of virus isolation and ELISA with different samples

Number	**Genbank No.**	**Status**	**Plasma VI**	**Semen VI**	**Semen VI**	**Semen ELISA**	**Sem**
9	/	P(-)S(-) (32/46) 69.56%	Neg	Neg	Neg	Pos	Pos
11	/		Neg	Neg	Neg	Pos	Pos
20	/		Neg	Neg	Neg	Pos	Pos
21	/		Neg	Neg	Neg	Pos	Pos
39	/		Neg	Neg	Neg	Pos	Pos
24	/	P(+)S(-) (6/46) 13.04%	Pos	Neg	Neg	Pos	Pos
27	/		Pos	Neg	Neg	Pos	Pos
28	/		Pos	Neg	Neg	Neg	Neg
35	/		Pos	Neg	Neg	Neg	Neg
51	/		Pos	Neg	Neg	Neg	Neg
55	/		Pos	Neg	Neg	Neg	Neg
17	HB201102/-1	P(+)S(+) (6/46) 13.04%	Pos	Pos	Pos	Pos	Pos
25	HB201103/-1		Pos	Pos	Pos	Pos	Pos
26	HB201104/-1		Pos	Pos	Pos	Pos	Pos
30	HB201105/-1		Pos	Pos	Pos	Pos	Pos
31	HB201106/-1		Pos	Pos	Pos	Neg	Neg
57	HB201107/-1		Pos	Pos	Pos	Neg	Neg
29	/	P(-)S(+) (2/46) 4.35%	Neg	Pos	Pos	Neg	Neg
34	/		Neg	Pos	Pos	Neg	Neg
Hens	Hens	Hens	Hens	Hens	Hens	Hens	Hen
E-4	/	Plasma	Pos	Pos	/	/	/
E-7	/	Plasma	Pos	Pos	/	/	/

E-9	/	Plasma	Pos	Pos	/	/	/
E-20	/	Plasma	Pos	Pos	/	/	/
E-63	HB201101	Plasma	Pos	Pos	/	/	/
E-63	HB201101-1	Albumen	Pos	Pos	/	/	/

Note: The numbers are the original number in the flock. P=Plasma, S=Semen; VI=Virus isolation; Pos=Positive, Neg=Negtive, "/"= no result.

Figure Legends

Figure 1. The phylogenetic tree of *gp85* gene in the isolates and the referential strains

The phylogenetic tree is generated using the neighbor-joining method using MEGA5.1 software (bootstrap method with 1000 replicates). ALV-A to E, K and J were divided and marked on the right. The source of ALV-J reference strains is in parentheses. Purple rhombus represent the strains were isolated in the plasmas of the rooster. Green triangles represent the strains were isolated in the semens of the rooster. The other strains in this study are also highlighted by the red figure. The sources of reference and isolated strains are marked after each strain. The following indicates the abbreviations used for country names: USA=United States, UK=United Kingdom, CHN=China, RUS=Russia, EGY=Egypt, NGA=Nigeria.

Figure 2. The results of immunofluorescence assay on the DF1 cells with JE9 monoclonal antibody. (A) Negative control, 100×; (B) DF-1 cells infected with semens, 100×.

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Figure 1 The phylogenetic tree of *gp85* gene in the isolates and the referential strains

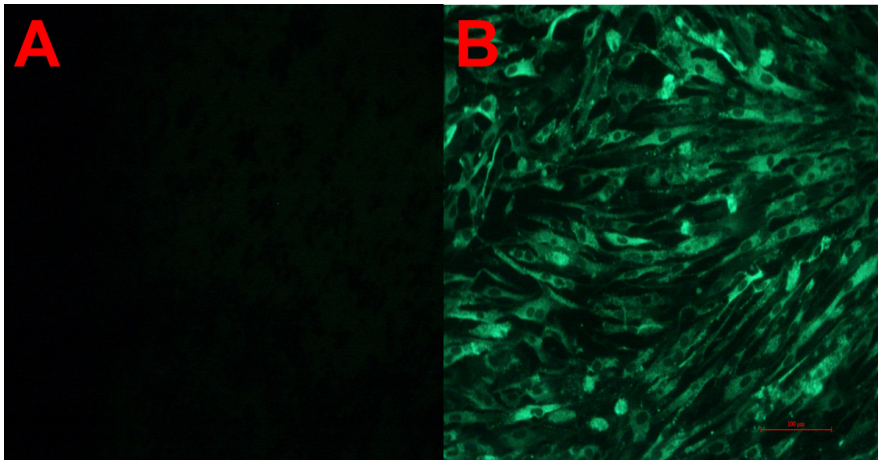

Figure 2 The results of immunofluorescence assay on the DF1 cells with JE9 monoclonal antibody.