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Short communication

A recombination efficiently increases the pathogenesis of the novel K subgroup of avian leukosis virus

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ABSTRACT

In this study, a recombinant ALV with ALV-K env and ALV-J backbone was generated (designated ALV-K-*env*-J) and tested in *vitro* and in *vivo*. The growth curve in DF1 cells showed that the recombinant virus replicated more efficiently in comparison with the ALV-J and ALV-K. Although all the infected chickens showed growth retardation compared with the non-infected chickens, the viral and serological detection showed that the positive rate and virus load detected in blood and cloaca, and the positive rate and titer of antibody against p27 from the chickens infected with ALV-K-*env*-J were higher than those from the chickens infected with the ALV-K, but less than those from the chickens infected with the ALV-J. All these data clearly demonstrated that the recombination event in this study increased the pathogenesis of ALV-K, and the potential recombination between different ALV subgroups should be worried when the clinical co-infections occur.

1. Introduction, methods and results

Based on the host range and its envelope protein, avian leukosis viruses (ALVs) are clustered into eleven subgroups (A–K) so far. Different from other subgroups, ALV-J is highly pathogenic and its infection mainly induces hematopoietic malignancy (Rajabzadeh et al., 2010; Payne et al., 1991; Silva et al., 2007). Since its first report in 1988 from UK, ALV-J has spread globally and caused great economic losses in poultry industry (Payne et al., 1991). ALV-K, as a novel avian leukosis virus, was recently identified by Cui et al (Cui et al., 2014). Although ALV-K is thought to be a low pathogenic ALV, ALV-K has been endemic in domestic chickens in China (Dong et al., 2015). The frequent emergence of the co-infection of ALV-K with multiple ALV subgroups not only challenges the ALV eradication program in China, but also poses the risk for the generation of the novel and pathogenic recombinant ALV (Su et al., 2018). The natural recombination events between ALV-J and ALV-A or ALV-B had occured in the field and could alter the fitness of the wild type viruses (Gingerich et al., 2002; Lupiani et al., 2003). However, the virulence evolution of ALV-K recombining with other subgroups of ALV is unknown.

To evaluate such potential, a recombinant ALV virus with ALV-K env and ALV-*J* backbone was rescued and tested in *vitro* and in *vivo*. For rescuing such virus, the *env* gene of ALV-K was first amplified from the proviral DNA extracted from DF-1 cells infected with ALV-K strain GD by PCR using the following primers (Forward primer: 5′-*GCCCTCTCTTTGCAG*GCATTTCTGACTGGATATCCTG-3′ and reverse primer: 5′-*CGCGTTCGGAACCTA*CACTGTTCCATTTTCGGGCTG-3′). The linearized ALV-*J* infectious clone p-ALV-J1 without ALV-K *env* gene (ALV-*J* infectious clone p-ALV-J1 was a kindly gift from Dr. Yixin Wang, Shangdong agriculture university) was amplified by PCR using the following primers (Forward primer:5′-*TAGGTTCCGAACGCG*ATGTAACGGGGCAAG-3′

and reverse primer: 5′-*CTGCAAAGAGAGGGCT*CGCCTCATCCTTC-3′). The italicized portion of the primer is a homologous complementary sequence and the underlined portion of the primer is the sequence of the genome itself or its reverse complement. Then, the amplified *env* gene and the linearized p-ALV-J1 without ALV-K *env* gene were ligated and recombinated by the commercial recombinant enzyme ExnaseTM II as

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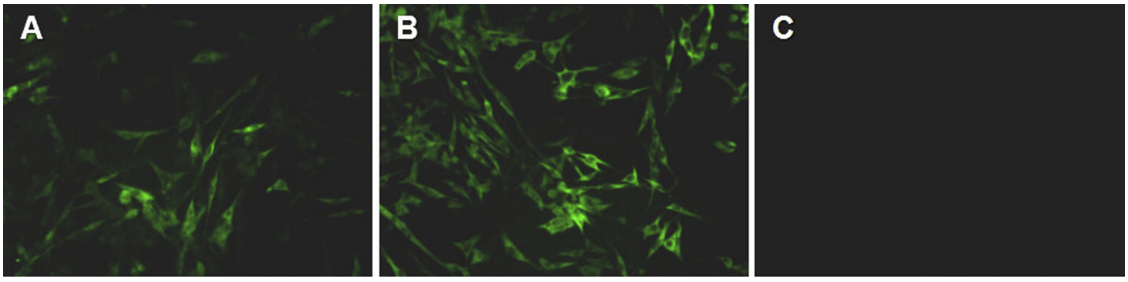

Fig. 1. IFA for the identification of the rescued viruses. A. DF-1 infected with ALV-J-J1; B. DF-1 infected with ALV-K-*env*-J1; C. Uninfected DF-1 cells served as a negative control.

previously described (Li et al., 2018). After the identification by PCR and sequencing, the positive clone for the recombinant plasmid was named as p-ALV-K-*env*-J. Two viruses were then rescued by transfecting the DF-1 with p-ALV-J1 and p-ALV-K-*env*-J respectively as previously described (Shao et al., 2015). Briefly, 4 μg of the plasmid mixed evenly with 200 μL Opti-MEM, then 8 μL of the transfection reagent (MIRUS) was added and mixed well. After incubating at RT(Room-temperature) for 45 min, the transfection mixture was added into DF1 cells in 6-wells plate. After 6 h, the culture medium with transfection mixture was replaced with fresh DMEM with 1% FBS. The supernatant was blindly passed into fresh DF1 cells for 7 days. After three blind passages in DF-1 cells, the two rescued viruses were identified by immune fluorescent assay (IFA) using specific antibodies against ALV p27 and named as ALV-J-J1 and ALV-K-*env*-J respectively. As described in Fig1, antibodies against ALV p27 could efficiently react with DF-1 cells infected with ALV-J-J1 and ALV-K-env-J. The rescued ALV-J-J1 and ALV-K-*env*-J were also confirmed by sequencing the whole genome.

To investigate whether the recombinant virus ALV-K-*env*-J with *env* gene from ALV-K and backbone from ALV-J could confer the replication advantage over the ALV-K virus in *vitro*, DF-1 cells were infected with ALV-J-J1, ALV-K-*env*-J and ALV-K isolate GD respectively at MOI of 0.1, and the viral growth kinetics in the culture supernatants from the infected DF-1 cells were titrated as previously described. As shown in Fig.2, ALV-K-*env*-J replicated more efficiently and yield higher viral titer in comparison with ALV-J1 and ALV-K-GD viruses. The viral titer of ALV-K-*env*-J could reach to 5.449 log10 $TCID_{50}$/ml and 7.699log10 $TCID_{50}$/ml at day 2 and 7 post infection whereas that from ALV-J-J1 or ALV-K-GD was less than 3.949 log10 $TCID_{50}$/ml and 5.616log10 $TCID_{50}$/ml. The peak titer of ALV-K-*env*-J in DF-1 cells was more than 100 times of that from ALV-J-J1 and ALV-K-GD. Therefore, the viral growth curve in DF-1 cells clearly demonstrated that the *env* gene from ALV-K or the recombination between ALV-J backbone and ALV-K env significantly increased the viral replication ability compared with the wild type ALV-K-GD and ALV-J-J1 viruses.

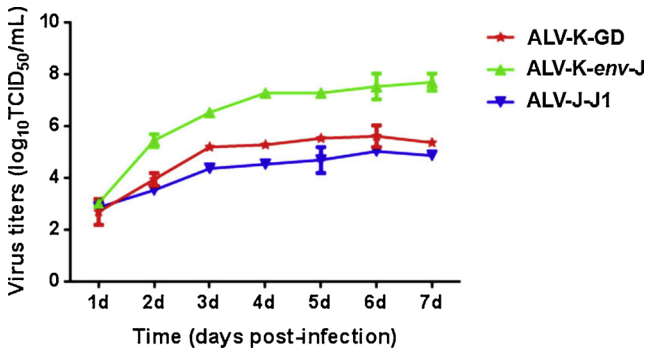

Fig. 2. Growth curve analysis for the rescued viruses. DF1 cells were infected with ALV-J-J1, ALV-K-*env*-J and ALV-K-GD at MOI 0.1 respectively, and the supernatant of the infected cells were collected at the indicated time points and titrated by TCID50. The blue, red, and green curves represent the growth behavior of ALV-J-J1, ALV-K-*env*-J and ALV-K-GD, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

To further evaluate the pathogenesis of the recombinant virus ALV-K-*env*-J, a total of 120 1-day-old SPF chickens were randomly divided into four groups (Thirty chickens per group; Group I: chickens without infection; Group II: chickens infected with ALV-K-*env*-J; Group III: chickens infected with ALV-K-GD; Group IV: chickens infected with ALV-J-J1) and inoculated through intraperitoneal injection. The infection dose for chickens in Group II–IV was 10^4 $TCID_{50}$ in 0.2 mL PBS. At day 7, 14, 21, 28 and 35 post infection (dpi), the blood and the cloacal swab of the chickens were collected and tested for viral titers. In brief, the collected blood and the cloacal swab samples were first inoculated into DF-1 cells. After six days post inoculation, the supernatant from the inoculated DF-1 cells were collected and tested for p27 antigen of ALV by using a sandwich ELISA as previously described (Li et al., 2018). As shown in Fig3A, the viral positive rate in the plasma from chickens infected with ALV-J-J1 kept high level with 96%,

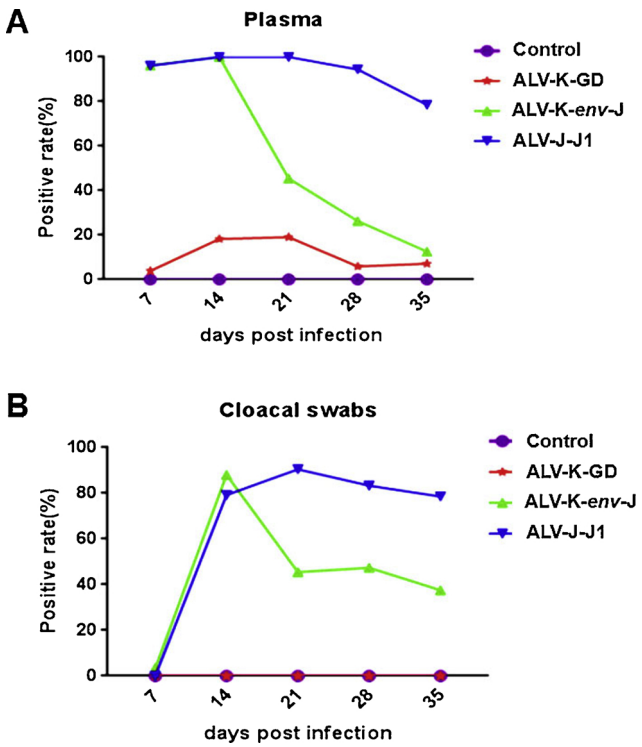

Fig. 3. Detection of viruses in the infected chickens. The blue, red, green and purple curves represent the ALV-J-J1, ALV-K-*env*-J and ALV-K-GD, respectively. At day 7, 14, 21, 28 and 35 post infection (dpi), the blood and the cloacal swab of the chickens were collected and tested for viral titers. (A) Comparison of the viral positive rate in the plasma from chickens infected with ALV-J-J1, ALV-K-*env*-J and ALV-K-GD. (B) Comparison of the viral positive rate in the cloacal swab from chickens infected with ALV-J-J1, ALV-K-*env*-J and ALV-K-GD (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

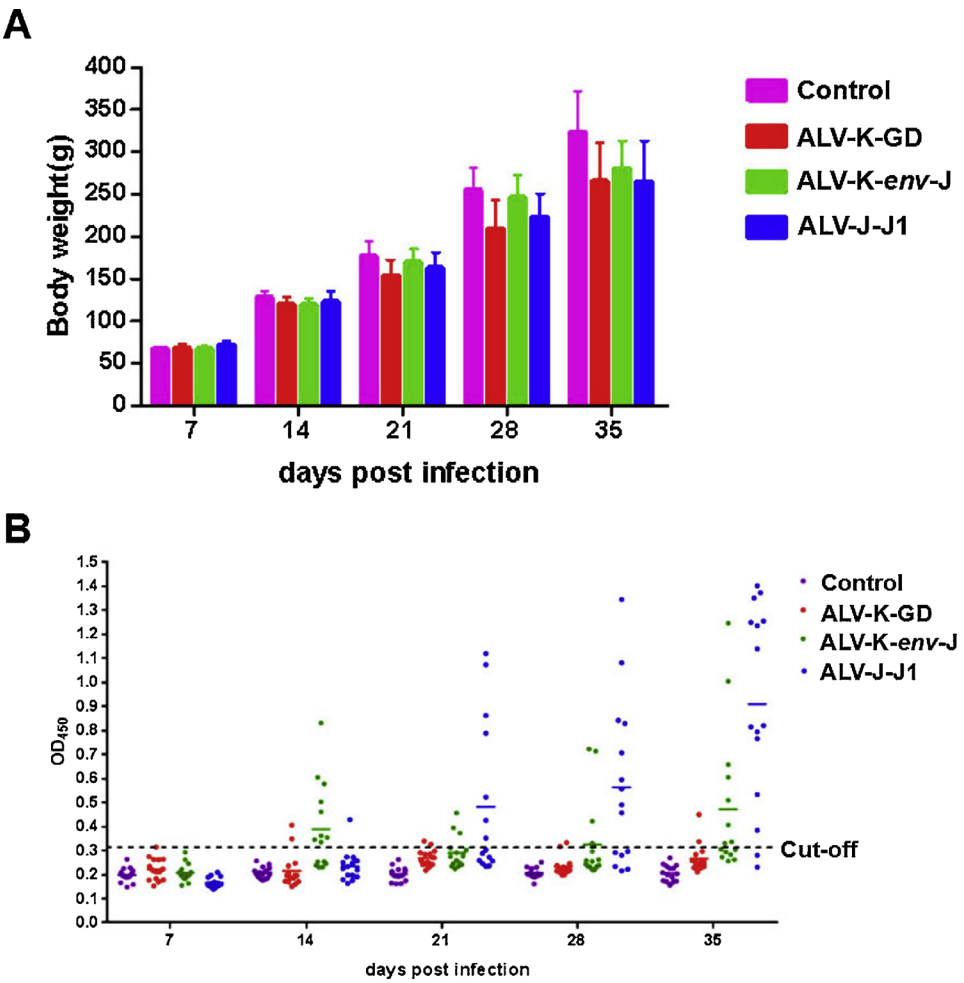

Fig. 4. Detection of bodyweight and antibody against ALV in the infected chickens. The blue, red, green and purple column strips or spots represent the ALV-J-J1, ALV-K-*env*-J and ALV-K-GD, respectively. (A) Comparison of the bodyweight measured at different times are presented. The chickens infected with ALV-J-J1 or ALV-K-*env*-J or ALV-K-GD grew more lower than the control chickens without infection. (B) The results of the antibody titer against p27 antigen measured at different times are shown (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

100%, 100%, 94% and 78% respectively at 7, 14, 21, 28 and 35 dpi. In chickens infected with recombinant virus ALV-K-*env*-J, the viral positive rate in the plasma was 96% and 100% respectively at 7 and 14 dpi whereas that was dramatically decreased to 45%, 26% and 12% at 21, 28 and 35 dpi. Notably, the viral positive rate in the plasma from chickens infected ALV-K-GD kept low level with 5%, 20%, 23%, 5% and 8% respectively at 7, 14, 21, 28 and 35 dpi. For the viral shedding in cloaca as described in Fig3B, the positive rate from chickens infected with ALV-J-J1 and ALV-K-*env*-J was very similar at 14 dpi, whereas that from chickens infected with ALV-J-J1 kept high level with 85%, 80% and 78% respectively at 21, 28 and 35 dpi and that from chickens infected with ALV-K-*env*-J was dramatically decreased to 45%, 47% and 37% respectively at 21, 28 and 35 dpi. It was noted that no virus was detected in the cloaca from the chickens infected ALV-K-GD at 7, 14, 21, 28 and 35 dpi. All these demonstrated that the chickens infected with recombinant virus ALV-K-env-J shed more viruses in cloaca and caused severe viremia compared with the wide type ALV-K.

In addition to detection of viral shedding and viremia in the infected chickens, the bodyweight and the antibody titer against p27 antigen were also measured at different time points. As described in Fig4A, although no significant difference for the bodyweight was found among different Groups, the chickens infected with ALV-J-J1 or ALV-K-*env*-J or ALV-K-GD grew more lower than the control chickens without infection. For the detection of antibody titer against p27 antigen, an indirect ELISA method was used as previously described (Qiu et al., 2011). The mean OD450 value for antibody titer against p27 from chickens infected with ALV-J-J1 was 0.36, 0.58 and 0.91 respectively at 21, 28 and 35 dpi whereas that from chickens infected with ALV-K-*env*-J was 0.28, 0.3 and 0.5 respectively. However, the mean OD450 value for antibody titer against p27 from chickens infected with ALV-K-GD or control chickens was all less than 0.3 (the cut-off value of the ELISA) at 21, 28 and 35 dpi.

2. Discussion

In China, the pathogenic ALV-J was first identified and reported in 1999 (Du et al., 1999). ALV-J has rapidly spread and caused severe economic losses to the poultry industry in China. Although the eradication program of ALV in *China* since 2010 has significantly reduced the disease caused by ALV-J, the emerging of novel subgroup of avian leukosis virus ALV-K is challenging the ALV eradication program in China. ALV-K or ALV-K like viruses have been not only frequently isolated from the indigenous chicken flocks in mainland China, but also reported from Japan and Taiwan (Cui et al., 2014; Dong et al., 2015; Shao et al., 2017; Li et al., 2016). Several groups have characterized ALV-K as a low pathogenic ALV mainly due to its LTR derived from the endogenous ALV-E virus (Shao et al., 2017). Notably, ALV-A, ALV-B, ALV-J and ALV-K infections were prevalent in some indigenous chicken flocks in China in the past few years (Su et al., 2018). Recently, ALV-A, ALV-J and ALV-K were identified in an indigenous chicken flock by our group (Data not shown, manuscript under review). It should be noted

that the high genetic diversity of ALV among the indigenous chickens in China and the frequent emergence of the co-infection of ALV-K with multiple ALV subgroups pose a risk for the generation of the novel and pathogenic recombinant ALV. Previous study showed that the natural recombinant ALV-J with an ALV-A envelope could significantly change the viral pathogenicity (Lupiani et al., 2003). In this study, we generated a recombinant ALV virus ALV-K-*env*-J using the *env* gene of ALV-K and the backbone of ALV-J infectious clone J1 to evaluate such potential. Our data showed that ALV-K-*env*-J virus replicated more efficient and yield higher viral titer in DF-1 cells compared with the wild type ALV-K-GD and the ALV-J-J1 rescued, highlighting the *env* gene of ALV-K could significantly increase the viral replication of ALV-J-J1 in DF-1 cells. Different from other ALV-J viruses, ALV-J-J1 virus rescued did not show efficient replication in DF-1 cells. Further analysis revealed that the *env* gene from the ALV-J infectious clone J1 carried unique mutations which might response to the low replication ability of ALV-J-J1 in DF-1 cells. However, such mutation in env of ALV-J-J1 might not obviously affect on the growth of ALV-J *in vivo*. Chicken infection study further demonstrated that ALV-K-*env*-J shed more viruses in cloaca and caused more severe viremia than the wide type ALV-K, but not the ALV-J-J1. The high level expression of viral receptors of ALV-J, but not ALV-K in chickens might contribute to the more efficient replication of ALV-J-J1 than ALV-K-*env*-J *in vivo*. Notably, although wide type ALV-K-GD did not shed the detectable viruses in cloaca and caused low viremia/antibody positive rate, the chickens infected with ALV-K-GD, similar with ALV-K-*env*-J and ALV-J-J1, did grow lowly compared with the chickens in the control group. This observation indicated that although ALV-K in infected chickens was hardly detected, ALV-K infection did affect the health of the chickens. In addition, no obvious tumor was found in the chickens infected with all three viruses possibly due to the short experimental time in the study. And in the HE staining sections, the typical tumor cell was also not found (Data not shown).

In summary, it is the first demonstration of a novel recombinant virus carrying *env* gene from ALV-K and other genome from ALV-J with increased pathogenesis. The frequent co-infection of ALV-K with multiple ALV subgroups in domestic chicken flocks in mainland China poses the risk for the emergence of novel recombinant ALVs and burdens the control strategy for ALV, highlighting the significance of the epidemiological monitoring for such recombinant viruses like ALV-K-*env*-J generated in this study.

Authors' contributions

LL, JY and AQ conceived and designed the experiments. LL, MH, JD and YL performed the experiments. TL, AQ, HS and JY analysed the data. TL,QX and LL contributed reagents/materials/analysis tools. LL, TL and JY contributed to the writing of the manuscript. TL and JY prepared the figures. All authors read and approved the final manuscript.

Ethics statement

All animal experiments were performed in accordance with institutional animal care guidelines, and the protocol, #06R015, was approved by the Animal Care Committee at Yangzhou University in China.

Competing interests

The authors declare that they have no competing interests.

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References

Cui, N., Su, S., Chen, Z., Zhao, X., Cui, Z., 2014. Genomic sequence analysis and biological characteristics of a rescued clone of avian leukosis virus strain JS11C1, isolated from indigenous chickens. J. Gen. Virol. 95, 2512–2522. https://doi.org/10.1099/vir.0.067264-0.

Dong, X., Zhao, P., Xu, B., Fan, J., Meng, F., Sun, P., Ju, S., Li, Y., Chang, S., Shi, W., Cui, Z., 2015. Avian leukosis virus in indigenous chicken breeds, China. Emerg. Microbes. Infect. 4, e76. https://doi.org/10.1038/emi.2015.76.

Du, Y., Cui, Z., Qin, A., 1999. Subgroup J of avian leukosis viruses in China. China Poult. Sci. 3, 124.

Gingerich, E., Porter, R.E., Lupiani, B., Fadly, A.M., 2002. Diagnosis of myeloid leukosis induced by a recombinant avian leukosis virus in commercial white leghorn egg laying flocks. Avian Dis. 46, 745–748. https://doi.org/10.1637/0005-2086(2002)046[0745:DOMLIB]2.0.CO;2.

Li, X., Lin, W., Chang, S., Zhao, P., Zhang, X., Liu, Y., Chen, W., Li, B., Shu, D., Zhang, H., Chen, F., Xie, Q., 2016. Isolation, identification and evolution analysis of a novel subgroup of avian leukosis virus isolated from a local Chinese yellow broiler in South China. Arch. Virol. 161, 2717–2725. https://doi.org/10.1007/s00705-016-2965-x.

Li, T., Xie, J., Lv, L., Sun, S., Dong, X., Xie, Q., Liang, G., Xia, C., Shao, H., Qin, A., Ye, J., 2018. A chicken liver cell line efficiently supports the replication of ALV-J possibly through its high level viral receptor and efficient protein expression system. Vet. Res. 49, 41. https://doi.org/10.1186/s13567-018-0537-7.

Lupiani, B., Williams, S.M., Silva, R.F., Hunt, H.D., Fadly, A.M., 2003. Pathogenicity of two recombinant avian leukosis viruses. Avian Dis. 47, 425–432. https://doi.org/10.1637/0005-2086(2002)046[0745:DOMLIB]2.0.CO;2.

Payne, L.N., Brown, S.R., Bumstead, N., Howes, K., Frazier, J.A., Thouless, M.E., 1991. A novel subgroup of exogenous avian leukosis virus in chickens. J. Gen. Virol. 72 (Pt. 4), 801–807. https://doi.org/10.1099/0022-1317-72-4-801.

Qiu, Y., Qian, K., Shen, H., Jin, W., Qin, A., 2011. Development and validation of an indirect enzyme-linked immunosorbent assay for the detection of Avian leukosis virus antibodies based on a recombinant capsid protein. J. Vet. Diagn. Invest. 23 (5), 991–993. https://doi.org/10.1177/1040638711416966.

Rajabzadeh, M., Dadras, H., Mohammadi, A., 2010. Detection of avian leukosis virus subgroups in albumen of commercial and native fowl eggs using RT-PCR in Iran. Trop. Anim. Health Prod. 42, 1829–1836. https://doi.org/10.1007/s11250-010-9645-8.

Shao, H., Fan, Z., Wan, Z., Tian, X., Chen, H., Perez, D.R., Qin, A., Ye, J., 2015. An efficient and rapid influenza gene cloning strategy for reverse genetics system. J. Virol. Methods 222, 91–94. https://doi.org/10.1016/j.jviromet.2015.06.001.

Shao, H., Wang, L., Sang, J., Li, T., Liu, Y., Wan, Z., Qian, K., Qin, A., Ye, J., 2017. Novel avian leukosis viruses from domestic chicken breeds in mainland China. Arch. Virol. 162, 2073–2076. https://doi.org/10.1007/s00705-017-3344-y.

Silva, R.F., Fadly, A.M., Taylor, S.P., 2007. Development of a polymerase chain reaction to differentiate avian leukosis virus (ALV) subgroups: detection of an ALV contaminant in commercial Marek's disease vaccines. Avian Dis. 51, 663–667. https://doi.org/10.1637/0005-2086(2007)51[663:DOAPCR]2.0.CO;2.

Su, Q., Li, Y., Li, W., Cui, S., Tian, S., Cui, Z., Zhao, P., Chang, S., 2018. Molecular characteristics of avian leukosis viruses isolated from indigenous chicken breeds in China. Poult. Sci. 97, 2917–2925. https://doi.org/10.3382/ps/pex367.