Development and application of a SYBR green real-time PCR for detection of the emerging avian leukosis virus subgroup K

Jian Chen,[*] Zijun Zhao,[*] Yangyijun Chen,[*] Jie Zhang,[*] Lifu Yan,[*] Xiaocui Zheng,[*] Ming Liao,[*,†,‡,§,#,1] and Weisheng Cao[*,†,‡,§,#,1]

[*]*College of Veterinary Medicine, South China Agricultural University, Guangzhou 510642, People's Republic of China;* [†]*Key Laboratory of Veterinary Vaccine Innovation of the Ministry of Agriculture;* [‡]*South China Collaborative Innovation Center for Prevention and Control of Poultry Infectious Diseases and Safety of Poultry Products, Guangzhou, People's Republic of China;* [§]*National and Regional Joint Engineering Laboratory for Medicament of Zoonosis Prevention and Control, People's Republic of China; and* [#]*Key Laboratory of Zoonosis Prevention and Control of Guangdong Province, People's Republic of China*

ABSTRACT Avian leukosis virus subgroup K (ALV-K) is an emerging ALV tumor virus of chickens. We developed a SYBR green-based real-time polymerase chain reaction (PCR) assay for the rapid and economical detection of ALV-K in chicken flocks. The assay was specific for ALV-K and did not cross-react with other ALV subgroup or avian influenza virus, Newcastle disease virus, or Marek's Disease virus. The method was 100 times more sensitive than conventional PCR and 10 times more sensitive than the enzyme-linked immunosorbent assay (ELISA) for the P27 antigen. The assay was also more sensitive than conventional PCR in tests of 86 clinical plasma samples. DF-1 tissue culture cells infected with 1 $TCID_{50}$ ALV-K particle were identified as negative using ELISA but tested positive with the real-time PCR method. The viral loads in organs and tissues in infected chickens were highest in kidney, lungs, and glandular stomach, and these results matched ELISA findings.

Key words: Avian leukosis virus subgroup K (ALV-K), ELISA, real-time PCR

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INTRODUCTION

Avian leukosis viruses (**ALV**) are a common source of tumor diseases in poultry and include 7 subgroups, A, B, C, D, E, J, and the emerging subgroup K in chickens (Wang et al., 2012; Cui et al., 2014; Li et al., 2016; Shao et al., 2017). ALV infections account for huge global economic losses in the poultry industry. The ALV-K subgroup possesses a long terminal repeat (**LTR**) similar to ALV-E that leads to a weaker replication capacity in DF-1 cells and less pathogenicity in SPF chickens (Li et al., 2016). Recently, an ALV-K resistant cell line was constructed for use as a diagnostic tool for the identification of ALV-K in clinical plasma samples (Mingzhang et al., 2017).

There are no vaccines or drugs available to control ALV infections, so the most effective control measures are eliminating ALV-positive breeder chickens to block vertical and horizontal transmission. An enzyme-linked immunosorbent assay (**ELISA**) targeting the ALV common P27 antigen is frequently used method to identify ALV. In addition, conventional and real-time polymerase chain reaction (**PCR**), immunofluorescence assays, and loop-mediated isothermal amplification are other methods that have been successfully applied to ALV detection (Spencer and Gilka, 1982; Zhang et al., 2010; Qiu et al., 2011; Qian et al., 2015). The real-time PCR methods included the use of the DNA intercalating dye SYBR green and Taqman probes. These assays are sensitive, rapid and accurate and can to quantitate virus copy numbers in tissues and organs (Luan et al., 2016; Marino-Merlo et al., 2017; Tien et al., 2017). Multiplexing these methods can provide an additional level of speed and efficiency. However, a multiplexed PCR assay is problematic due to difficulties with primer design (Gao et al., 2014; Kim et al., 2015; Wang et al., 2017b).

ALV-A/B and ALV-J PCR assays are currently in use, but primers for the emerging subgroup K have not been reported (Dong et al., 2012; Dai et al., 2015). In this study, we developed a SYBR green real-time PCR

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[1]Corresponding authors: mliao@scau.edu.cn (LM); caoweish@scau.edu.cn (CW)

method for ALV-K and compared our results with the current ELISA and PCR methods to compare sensitivity. We quantitated viral load in different tissues of infected chickens to evaluate the tissue affinity of ALV-K.

MATERIALS AND METHODS

Virus

The ALV-K strain GD1601 was isolated from clinical plasma samples of chickens infected naturally in Guangdong Province, China. ALV-A strain GD13–1, ALV-B strain CD08, ALV-E strain HN1301, ALV-J strain CHN06, avian influenza virus (**AIV**) strain H9N2 and Newcastle disease virus (**NDV**) strain GM were maintained in our laboratory. DNA of Marek's disease virus strain CVI988 that was used to check the specificity of the real-time PCR assay was extracted from a commercial vaccine. The ALV-sensitive cell line DF-1 was infected with strain GD1601 using standard procedures (Feng et al., 2014).

PCR Assay Design and Testing

ALV-K specific PCR primers were designed using commercial software based on published ALV subgroup sequences (GeneBank ID: KP686143, KP686142, KP686144, KU605774, and KF746200). (Thornton and Basu, 2011). DNAStar (Madison, WI) was used to identify genomic regions unique to ALV-K. Oligo 7 was used to design primers K1/K2 and P1/P2, which both used to distinguish ALV-K from other chicken ALV subgroups (GeneBank ID: M19113, HM452341, HM452342, HM452339, HM452340, HM446005, M12172, M12172, EF467236, DQ115805, HQ900844). The primer pair K1/K2 (5′ to 3′: CAGACAGGTTCTCGCTTCCG, CCATATACCTCCTGTGCGTGT) targets the *gp85* gene and was used for real-time PCR (amplicon = 84 bp). Primers P1/P2 (5′ to 3′: TCCAGGCCG-CAACTCAC, CATACCACCACCCA CGTACT targeted the *env* gene and was used for routine PCR (amplicon = 1214 bp). All primers were synthesized by Invitrogen (Shanghai, China).

Plasmid Standard Preparation

Total DNA was extracted from DF-1 cells infected with strain GD1601 according to the manufacturer's specifications (OMEGA, Norcross, GA). Routine PCR amplicons were identified using 1% agarose gel electrophoresis and 84 bp and 1214 bp products were recovered and purified using the E.Z.N.A. Gel Extraction Kit (OMEGA, Norcross, GA). Purified DNA was cloned into the PMD-18T Vector (Takara, Dalian, China) and then used to transform *Escherichia coli* competent DH5α cells to ampicillin resistance by plating on Luria-Bertani agar. Bacterial colonies were picked and plasmids were extracted using a Plasmid Mini Kit (OMEGA, Norcross, GA). Recombinant plasmid inserts were sequenced by Tsingke Biotechnology (Guangzhou, China). Plasmid concentration were measured by UV spectroscopy and the recombinant plasmid copy number was calculated according to the formula: number of copies = (concentration in ng $\times$ 6.02 $\times$ 10^{23})/(genome length $\times$ 10^9 $\times$ 660) (Parida et al., 2011; Wang et al., 2017a).

Plasmids (Concentration = 76.0 ng/μL) were diluted in double-distilled water (**dd$\mathbf{H_2O}$**) to obtain a standard solution containing 2.5 $\times$ 10^{10} copies. The stock solution was then diluted in 10-fold increments to achieve solutions for the standard curve containing 10^8 to 10^1 copies. Real-time PCR was carried out in a 20 μL reaction volume containing 10 μL 2 $\times$ iTaq Universal SYBRGreen Supermix (BioRad, Hercules, CA), 0.5 μL each primers K1 and K2 (20 pmol/μL),1 μL cDNA or plasmid and 8 μL ddH_2O. An ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) was used for amplification with 1 cycle of 95°C 3 min; 40 cycles of 95°C for 15 s and 60°C for 34 s. Fluorescence signals were collected after each amplification step.

Specificity of Real-Time PCR

The assay specificity was assessed using ALV-A, ALV-B, ALV-E, ALV-J, MDV, AIV, and NDV as templates in the standard reaction procedure. Routine PCR was used to distinguish ALV-K from other ALV subgroups.

Sensitivity Analysis of Real-Time PCR, Routine PCR and ELISA

The assay sensitivity was evaluated using 10^8 to 10^1 standard curve dilutions as templates for routine PCR that were analyzed by 1% agarose gel electrophoresis. Titers of GD1601 virus stocks used for testing were in the range of 10^3 to 1 $TCID_{50}$/0.2 mL and were used to inoculate DF-1 cells (Dai et al., 2015). The DF-1 cell supernatants were collected for ELISA detection of the ALV P27 antigen after infection for 7 d. Total RNA was extracted from cell pellets and used for real-time PCR analysis.

Reproducibility of Real-Time PCR

RNA extracted from 3 ALV-K-positive liver tissue samples was used to evaluate the reproducibility of the real-time PCR assay. The coefficient of variation (**CV**) values of intra-assay and inter-assay were calculated.

Animal Experiment, Tissue Processing and Clinical Plasma Samples

We used 20 specific-pathogen-free (**SPF**) chickens (White Leghorn) obtained from Jinan SAIS Poultry

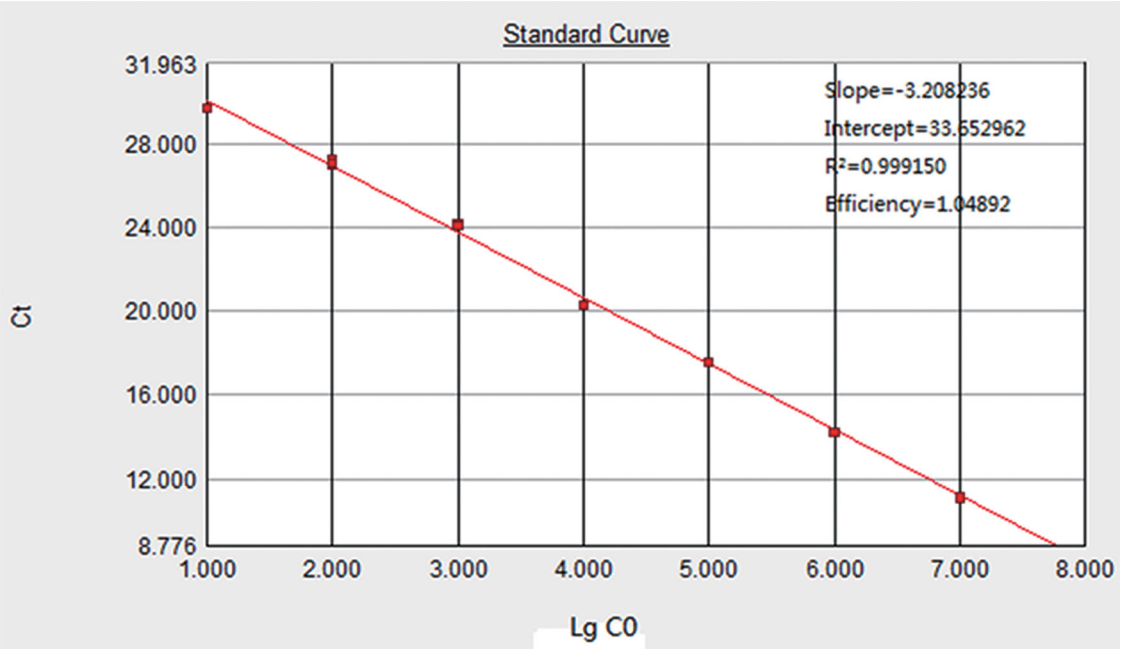

Figure 1. The standard curve of real-time PCR detecting ALV-K. The plasmids (10^8–10^1 copies) was used to establish the standard curve and the ABI7500 SDS software 1.4 was used to analyze the relationship of Ct values and plasmid copy numbers.

Table 1. Real time PCR assay for ALV-K.

Copy number	CT value			Mean ± SD	CV(%)
	1	2	3		
10^8	8	8.07	7.97	8.01 ± 0.05	0.62
10^7	11.13	11.09	10.87	11.03 ± 0.14	1.26
10^6	14.24	14.20	14.24	14.23 ± 0.02	0.14
10^5	17.83	18.05	17.99	17.96 ± 0.11	0.61
10^4	20.17	20.21	20.33	20.24 ± 0.08	0.39
10^3	24.85	24.21	24.88	24.65 ± 0.38	1.54
10^2	27.22	27.30	27.11	27.21 ± 0.09	0.33
10^1	29.47	29.47	29.36	29.43 ± 0.06	0.20

Shangdong Province, China. The chickens were randomly divided into 2 groups and 10 were inoculated intraperitoneally with 0.2 mL ALV-K GD1601 (10^3TCID$_{50}$/0.2 mL) and 10 in a control group were not infected. All chickens were raised in SPF chicken isolators. Heart, liver, spleen, lung, kidney, thymus, bursa, and glandular stomach from each bird were collected at 13 wk post-infection and stored at −80°C until processed.

Tissue samples (200 mg) were homogenized in 1 mL phosphate-buffered saline (**PBS**) and centrifuged at 14,000 × *g* for 5 min at 4°C after 3 rounds of continuous freeze-thawing. The supernatant was then stored at −80°C. ELISA testing of the supernatant (100 μL) used a commercial avian leukosis virus p27 antigen test kit (IDEXX, Inc., Westbrook, ME). RNA was extracted from the remaining tissue homogenates using the RNA fast 200 kit (Fastagen, Shanghai, China) and 500 ng total RNA was reverse transcribed using the PrimeScript RT Master Mix (Perfect Real Time)(Takara, Dalian, China). The cDNA generated was used for real-time PCR amplification.

A total of 86 positive plasma samples that had been confirmed by the ELISA P27 antigen kit were used for real-time PCR and routine PCR.

RESULTS

Establishment of a Standard Curve

Plasmid DNA containing cloned gene copies of the *gp85* and the *env* gene were used to construct standard curves. Over the range of 10^8 to 10^1 copies per reaction, the threshold cycle (**CT**) and copy numbers displayed a good linear relationship with an R^2 of 0.999 and the equation y = –3.208236x + 33.652962 (Figure 1). The CV values at each point were below 1.54% (Table 1).

Sensitivity, Specificity, and Reproducibility of Real-Time PCR

We tested the specificity of the assay using DNA or cDNA samples from ALV-A/B/E/J, MDV, AIV and NDV as templates. We found no amplification signals

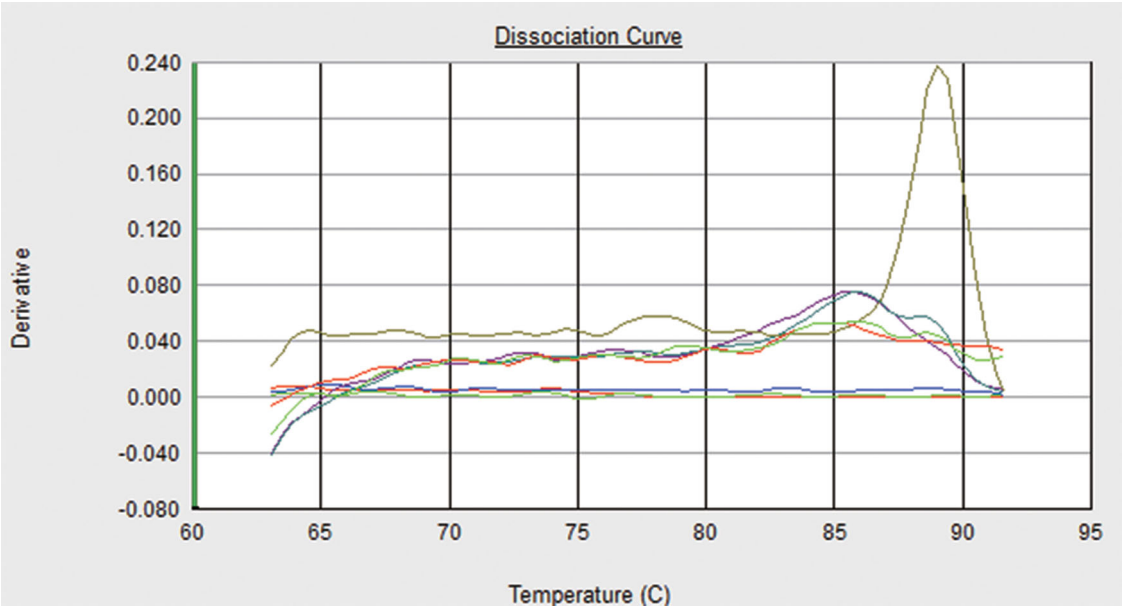

Figure 2. Specificity of real-time PCR. The dissociation curves showed specific curve at about 88.5°C only for the templates of ALV-K. The dissociation curves were analyzed by the ABI7500 SDS software 1.4.

detected except for the ALV-K template indicating the assay was specific for ALV-K (Figure 2). The assay could detect as few as 25 copies (Figure 3a). In contrast, the limit of detection using conventional PCR was 10^3 copies (Figure 3b). This indicated that our real-time PCR assay was about 100-fold more sensitive.

We next applied the assay to the detection of ALV-K in 86 P27 positive clinical plasma samples from 4 breeding poultry farms and found 10 ALV-K positive samples (11.63%). This was slightly greater than conventional PCR (9.30%). Compared with the ELISA assay, the limit of detection for our real-time PCR was 1 $TCID_{50}$ compared with 10 $TCID_{50}$ for ELISA (Figure 4). Moreover, the CV values for both intra- and inter-assay tests of ALV-K-positive liver tissue samples were less than 1.95% (Table 2), which indicated that the method had a stable repeatability.

Assessment of Virus Tissue Affinity

We also compared organ tissues samples from infected chickens and control groups by ELISA and real-time PCR. In both assays, all negative controls were negative (data not shown). The ELISA assay detected a high positive rate for the lungs, glandular stomach and the kidney (Table 3). The real-time PCR results were consistent with these findings (Figure 5). The tissues with the lowest positive rate were the hearts by ELISA. Similarly, real-time PCR determined that the lowest viral loads were in the thymus and hearts (Table 3 and Figure 5).

DISCUSSION

The identification of a virus by cell culture identification is expensive and time-consuming. One solution to the problem was ELISA, which could determine whether the sample contains the ALV P27 antigen or not, but the method cannot be used to identify ALV subgroups or differentiate the exogenous ALV from endogenous ALV. Similarly, conventional PCR is not a good choice to quantify viral gene copy numbers. The real-time PCR assay could provide the solution to both these problems.

Based on the gene sequence of gp85 of ALV-K, we designed PCR primers that possessed good specificity, sensitivity, and reproducibility. In our assays, only those samples containing ALV-K gave positive fluorescence signals. We also found significant linear relationships between gene copy numbers and CT values ($R^2 = 0.999$, Efficiency = 1.04). Our real time method had a detection limit of 25 copies that was 100-fold more sensitive than conventional PCR. Among the 86 clinical plasma samples we tested, 10 samples were ALV-K positive by real-time PCR, and conventional PCR found only 8 of these. The extra 2 samples were not the result of false positives because both the 2 samples were shown to have exogenous ALV viruses after 3 consecutive generation cell culture, furthermore the nucleotide homology sequence analysis of *gp85* showed that the ALV viruses in above 2 samples were 99.8% similar to ALV-K strain GD1601. DF-1 cells infected at levels as low as 1 $TCID_{50}$ ALV-K could be detected by real-time PCR and was 10-fold more sensitive than ELISA. These results indicated that real-time PCR is more sensitive than conventional PCR and ELISA.

The CV values that were indicators of reproducibility assay were 0.15–1.95% for our new PCR method, indicating good accuracy and reproducibility. The positive rate in hearts were 20% by ELISA among 10 chickens, and low gene copy numbers could be detected by real-time PCR in heart tissues. These results indicated

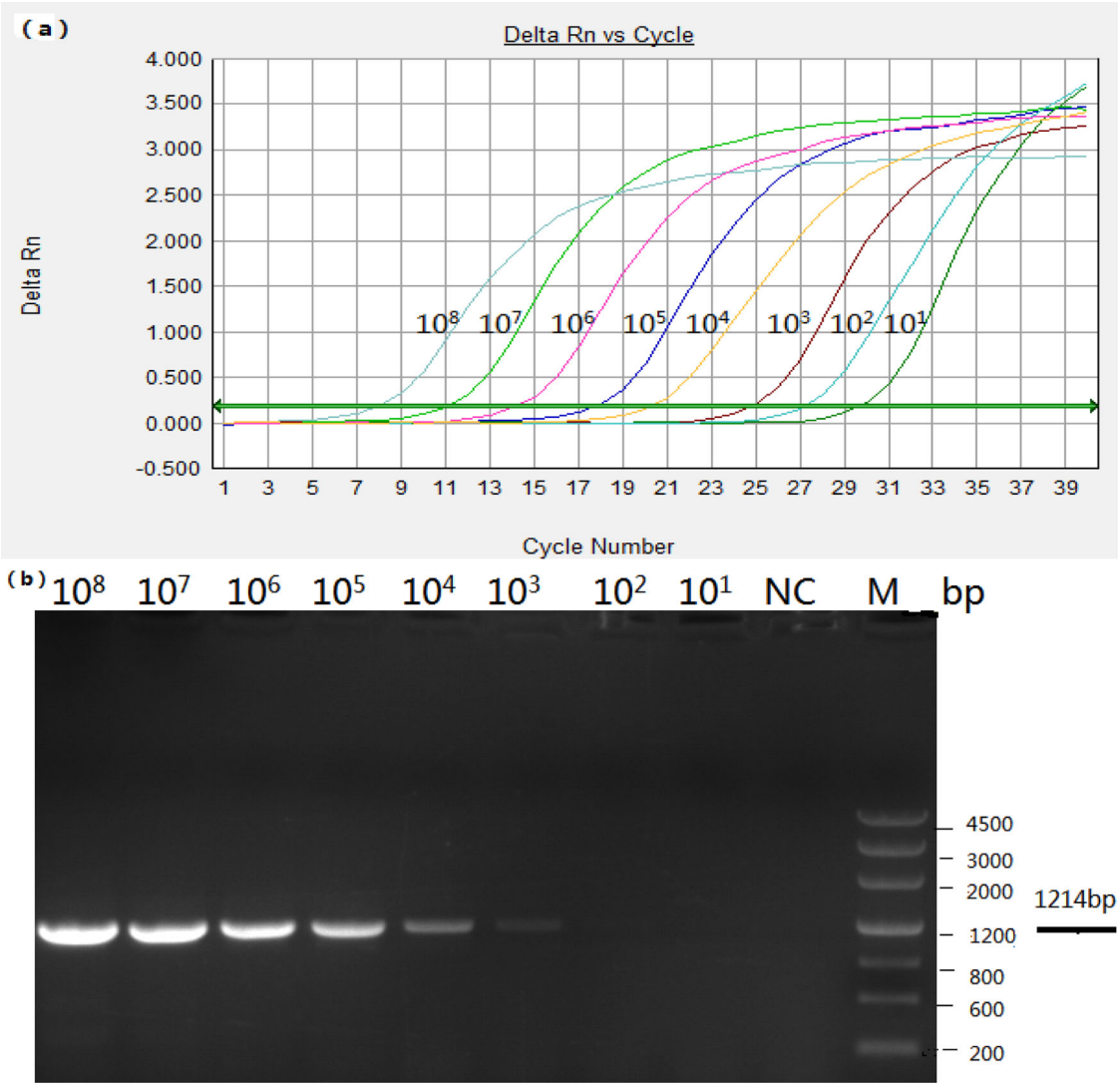

Figure 3. Sensitivity of real-time PCR and routine PCR. (a) Amplification of the 10-fold dilutions of standard plasmids (84 bp) ranging from 10^8 copies to 10^1 copies. (b) Conventional PCR products (1214 bp) were tested by 1% gel electrophoresis. M, DNA Marker 3. NC, negative control.

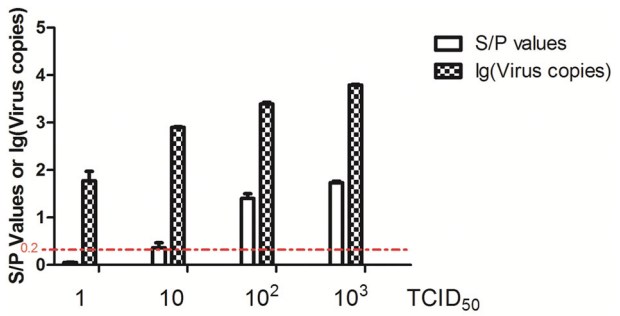

Figure 4. The minimum virus detection limits of ELISA and real-time PCR. Detection of the S/P values and virus copies according to ELISA and real-time PCR, respectively.

Table 2. Reproducibility of the real-time PCR assay using liver samples as templates.

Reproducibility tests	Results (Mean ± SD)	Coefficient of variation (%)	Viral load (copies/μL)
Intra-assay reproducibility	23.89 ± 0.03	0.15	$1.12 * 10^3$
	23.42 ± 0.08	0.37	$1.58 * 10^3$
	22.02 ± 0.09	0.42	$4.32 * 10^3$
Inter-assay reproducibility	23.83 ± 0.46	1.95	$1.17 * 10^3$
	23.43 ± 0.23	0.98	$1.57 * 10^3$
	22.07 ± 0.16	0.75	$4.17 * 10^3$

that the tissue affinity of ALV-K in hearts is too weak to detect using ELISA. In addition, kidneys, lungs, and the glandular stomach tissues contained the highest viral gene copy numbers and matched the ELISA results.

In conclusion, the SYBR green I real-time PCR assay successfully quantitated viral load using gene copy numbers in organ and tissues. We determined that the lungs, glandular stomach, and the kidneys were the organs containing the highest viral titers. Compared with ALV-J, ALV-K replicates slowly and produces lower P27 titer in vitro (Li et al., 2016). In vivo, Dai et al. found that the highest viral copy numbers were present

Table 3. Detection of ALV in chickens using ELISA.

Organ tissue	1	2	3	4	5	6	7	8	9	10	Positive rate
Heart	+	−	+	−	−	−	−	−	−	−	20% (2/10)
Liver	−	−	+	+	+	+	+	+	−	−	60% (6/10)
Spleen	−	−	+	−	+	+	−	+	+	−	50% (5/10)
Lungs	+	+	+	+	+	+	+	+	+	+	100% (10/10)
Kidney	+	+	+	+	+	+	+	+	+	−	90% (9/10)
Thymus	+	+	+	+	+	+	+	−	+	−	80% (8/10)
Bursa	−	−	+	+	+	+	−	+	−	−	50% (5/10)
Glandular stomach	+	+	+	+	+	+	+	+	+	+	100% (10/10)

Note: The numbers indicate individual artificially infected SPF chickens. Each sample was tested independently 3 times. The S/P value of 0.2 is the dividing line between "+" and "−".

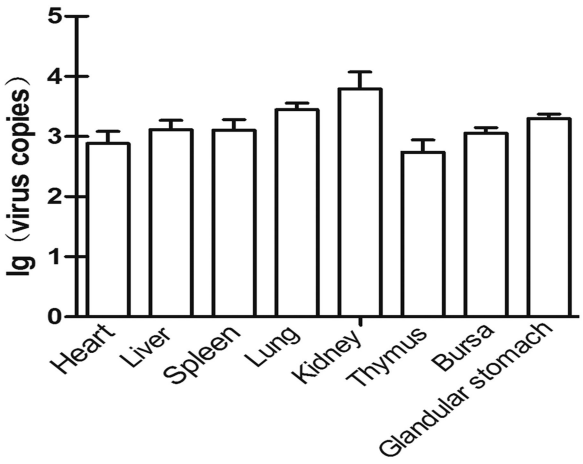

Figure 5. The virus gene copies among chicken tissues in infection group according to real-time PCR. Each sample was tested independently 3 times. Statistical analysis was made using two-way ANOVA in GraphPad Prism 5.

in hearts and kidneys of ALV-J infected SPF chicken at 30 wk post-infection (Dai et al., 2015). And Dong et al. found that the thymus and lungs infected with ALV-J possessed the highest viral copy numbers at 8 wk post-infection (Dong et al., 2012). In summary, different viral subgroups and incubation times will give different results.

ETHICS STATEMENT

The animal research obtained specific approval and guidance from South China Agriculture University's Institutional Animal Care and Use Committee. The plasma samples were from yellow chickens of Guangdong province in China during an ALV epidemiological investigation conducted by our laboratory.

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