Rapid detection of avian leukosis virus using a fluorescent microsphere immunochromatographic test strip assay

Huanan Wang,[*,1] Jianchi Guan,[†,1] Xiangnan Liu,[†,‡,1] Yue Shi,[#] Qiwen Wu,[‡] Mengzhen Luo,[‡] Yujun Zhu,[§] Zizengchen Wang,[*] Lefeng Wang,[*] and Yu Pan[‡,2]

[*]*Department of Veterinary Medicine, Zhejiang Provincial Key Laboratory of Preventive Veterinary Medicine and College of Animal Sciences, Zhejiang University, Hangzhou 310058, China;* [†]*College of Animal Science and Technology, Guangdong Polytechnic of Science and Trade, Guangzhou 510640, China;* [‡]*Guangzhou Veterinary Biotechnology Co.Ltd, Guangzhou 510000, China;* [§]*Guangzhou Bozhi Biotechnology Co.Ltd, Guangzhou 510000, China; and* [#]*Beijing Senkang Biotech Development Co., Ltd, Beijing 101400, China*

ABSTRACT We developed a rapid fluorescent microsphere immunochromatographic test strip (FM-ICTS) assay for the quantitative detection of avian leukosis virus (ALV). A monoclonal antibody specific for the ALV major capsid protein encoded by the *gag* gene was coupled to label fluorescent microspheres. ALV antibodies were coated on a nitrocellulose membrane to prepare a test line for sample detection. The fluorescence signals of the test and control lines can be read either visually by exposure to UV light or using a fluorescence analyzer. ALV could be detected quantitatively using the ratio of fluorescence signals of the test and control lines (T/C). The assay threshold was determined as a T/C value of 0.0606. The fitting curve equation was established between 1 and 2,048 ng/mL P27 protein with an r^2 value of 0.9998. The assay showed no cross reactivity with Newcastle disease virus, infectious laryngotracheitis virus, infectious bronchitis virus, Marek's disease virus, infectious bursal disease, Reoviridae virus, or avian influenza virus. The repeatability was satisfactory with an overall average CV of 8.65%. The Kappa coefficient between a commercial ELISA kit was 0.7031 using clinical chicken meconium samples. Thus, a simple, rapid, sensitive, and specific fluorescent microsphere immunochromatographic test strip was developed based on specific anti-capsid protein p27 monoclonal antibodies.

Key words: avian leukosis virus, fluorescent microsphere immunochromatographic test strip, T/C value, fitting curve equation

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INTRODUCTION

Avian leukosis is an infection caused by the oncogenic retrovirus avian leukosis virus (**ALV**) that causes enormous economic losses to the poultry industry (Barbour et al., 1997; Payne et al., 1991). There are currently no vaccines or drugs available to protect chickens from ALV (Payne and Nair, 2012). ALV infections are controlled in developed countries by eliminating ALV-positive breeder chickens to avoid vertical and horizontal transmission (Feng and Zhang, 2016). The success of this control program relies on rapid, sensitive, and inexpensive virus detection procedures and many assays have been developed (Zhang et al., 2010; Wang et al., 2011; Chang et al., 2013; Yun et al., 2013; Gao et al., 2014; Chen et al., 2018). The problem with the current set of assays is that they are labor intensive and require special instrumentation that limits their field application. Thus, there is a high demand for a simple and rapid molecular assay to supplement existing methods.

Fluorescent immunochromatography is a rapid analytical method that combines fluorescent microsphere labeling with immunochromatography. The combination of fluorescence immunochromatographic test strips and a fluorescence analyzer makes it possible to quantitatively read and analyze fluorescence signals and avoids errors caused by visual observation. An analyte concentration can be calculated by establishing a fitting curve (Jiang et al., 2018; Xie et al., 2014).

The ALV genome is a 7.2 kb sequence and encodes the typical combination of retroviral structural proteins and enzymes including gag, pol, and env. ALV p27 is a major group-specific capsid antigen encoded by the *gag* gene and accounts for more than 30% of the total viral protein mass (Weiss 2006). In this study, a simple and rapid fluorescent microsphere immunochromatographic test strip was developed using anti-p27 monoclonal antibodies (mAb).

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[1]These authors contributed equally to this work.
[2]Corresponding author: pyn2019@163.com

MATERIALS AND METHODS

Materials

ALV p27 protein was expressed and purified in our laboratory followed the protocol as previously described (Yun, 2013). Anti-p27 capsid mAbs were also produced and purified from 2 hybridoma clones (3F6 and 5B7) at our laboratory. Clinical chicken fecal samples were collected from 3 chicken farms in Heshan, Qingyuan and Zhaoqing, China. Newcastle disease virus (**NDV**) strain F48E9, infectious laryngotracheitis virus (**ILTV**) strain N-71,851 (ATCC VR-783), infectious bronchitis virus (**IBV**) Massachusetts, Marek's disease virus (**MDV**) 814, infectious bursal disease virus (**IBDV**) B87, and avian influenza virus (**AIV**) H7N2 were maintained in our laboratory. Avian leukosis virus A, B, J, and Reoviridae virus (**REOV**) were provided by Guangdong Laboratory Animals Monitoring Institute**.** Albumen samples purified from specific pathogen free (**SPF**) chickens were purchased from Guangdong Wens Dahuanong Biotechnology (Guangdong, China). A commercial ALV ELISA kit (Product No. GM420) was obtained from Idexx Laboratories (Westbrook, ME). Fluorescent microspheres (Product number MD021, 210 nm) were purchased from Nanjing Microdetection Bio-Tech (Nanjing, China). Goat anti-mouse IgG was bought from Beijing Biosynthesis Biotechnology (Beijing, China). Lateral chromatographic test strip materials including nitrocellulose membranes, glass cellulose membranes (Product number CN95) and a base plate were purchased from Shanghai Jieyi Biotechnology (Shanghai, China).

Antibody Coupling to Fluorescent Microspheres

Antibody was coupled to fluorescent microspheres by carboxyl activation coupling (Zhang et al., 2016). In brief, 10 μL of the commercial fluorescent microsphere solution was diluted with 50 mM MES (pH 6.5) to a total volume of 400 μL and 10 μL of 50 mM N-hydroxysuccinimide (NHS) and 10 μL 50 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide were added and the mixture was incubated for 20 min while shaking at room temperature. The reaction was stopped by centrifugation and the beads were suspended in 400 μL 50 mM MES after the supernatant was removed. The mAb 5B7 was added along with 50 μL 10% BSA and the mixture was incubated for 30 min at room temperature. The beads were recovered after centrifugation and suspended in 400 μL of 0.05 mol/L PBS (pH 7.4) containing 1% BSA, 2% PEG 20000, and 1% Dextran 4000 and stored at 4°C.

Preparation of the Fluorescent Microsphere Test Strip

Fluorescent microspheres labeled with ALV 5B7 antibody were sprayed onto a glass cellulose membrane to prepare a conjugate pad. ALV 3F6 antibodies were coated on a nitrocellulose membrane to prepare a T-line for sample detection. Goat anti-mouse IgG antibodies were coated on a nitrocellulose membrane to prepare a C line for quality control. The above materials were assembled with a base plate, a sample pad, and absorbent paper and cut into test strips using a strip cutting machine.

The test strip was used to detect negative and positive samples. The test strips were exposed to UV illumination and the fluorescence signal was detected using a fluorescent analyzer (FD-100, Nanjing Microdetection Bio-Tech Co., Ltd, Nangjing, China). SPF albumen samples were used as negative controls and the T/C values were recorded statistically. The threshold value of the test strip was calculated as threshold value = mean $\pm$ 3 $\times$ standard deviation.

Assay sensitivity was tested by using ALV P27 protein samples that were serially diluted in 2-fold increments using 0.01 M PBS (pH 7.4) from 1,024 to 0.125 ng/mL, and also using ALV B virus samples that were serially diluted in 10-fold increments from TCID50 10^3/ml to TCID50 10^0/mL. The T/C value was compared with the threshold value to define the minimum detection levels for the test strip. Fitting curve equation was constructed using ALV P27 protein samples that were serially diluted as per above from 16,384 to 1 ng/mL. The T/C value was recorded and the relationship between the T/C value and the concentrations were analyzed by fitting curve equation. The specificity was determined using different types of ALV including ALVA, B, J, and a series of avian viruses that included NDV, ILTV, IBV, MDV, IBDV, REOV, and AIV.

Assay repeatability was determined using ALV P27 serial dilutions as above from 2,048 to 1 ng/mL. Three repeated experiments were performed on each concentration and the coefficient of variation coefficient (CV) of the T/C value for each concentration group was calculated. Clinical samples consisting of 984 meconium samples were tested using the test strips. The Kappa coefficient of the detection results were compared with a commercial ELISA kit.

RESULTS

Positive and Negative Detection with Fluorescent Microsphere Test Strip for ALV Antigens

The test strip color intensity in the T-lines positively correlated with the ALV P27 protein concentrations at 10, 100, 1,000, and 10,000 ng/mL (Figure 1). The T/C values determined with the fluorescence analyzer were positively correlated with the sample concentrations (Table 1). PBS was used as the negative control which generated no color on its T-line.

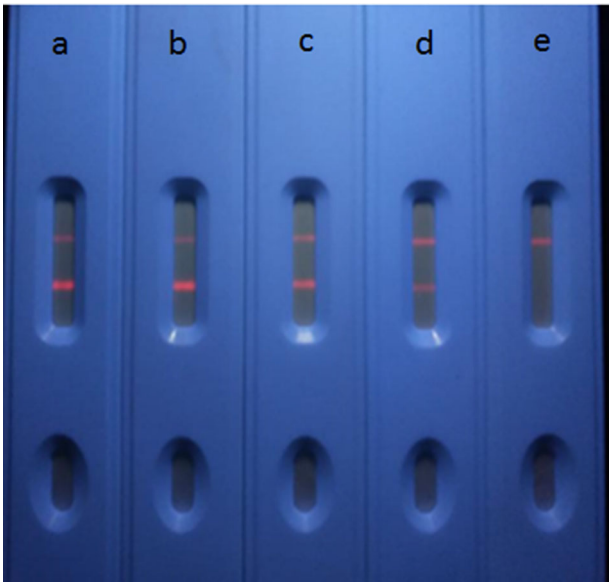

Figure 1. Positive and negative detection with fluorescent microsphere test strip for ALV antigens. ALV P27 proteins were added to the test strips at 10, 100, 1,000, and 10,000 ng/mL. (a) 10,000 ng/mL; (b) 1,000 ng/mL; (c) 100 ng/mL; (d)10 ng/mL; (e) PBS.

Table 1. Positive and negative detection with fluorescent microsphere test strip for ALV antigens.

c (ng/mL)	T value	C value	T/C
10,000	23,729	3,442	6.8940
1,000	24,152	3,624	6.6645
100	14,860	4,567	3.2538
10	7,710	8,320	0.9267
0	39	10,900	0.0036

Note: c (concentration); T value (T-line value); C value (C-line value).

Table 2. The threshold assay of FM-ICTS with 40 album samples from SPF chickens.

Samples	T/C value			
Negative albumin samples	0.0134	0.0224	0.0031	0.0018
	0.0126	0.0324	0.0909	0.0025
	0.0109	0.0236	0.0027	0.0367
	0.0306	0.0129	0.0039	0.0136
	0.0155	0.0003	0.0016	0.0258
	0.0042	0.0197	0.0025	0.0056
	0.0131	0.0021	0.0016	0.0013
	0.0017	0.0016	0.0025	0.0020
	0.0026	0.0041	0.0031	0.0012
	0.0242	0.0004	0.0008	0.0026
	Mean = 0.0114			
	Standard deviation = 0.0164			
	Threshold = 0.0606			

Note: T (T-line value); C (C-line value).

Threshold and Sensitivity Testing

To determine the threshold setting we examined 40 albumin samples from SPF chickens and found a threshold level of 0.0606. A T/C value > 0.0606 indicated a positive test result (Table 2). To determine assay sensitivity, we used dilutions of purified ALV P27 protein or ALV virus. We found that that when the concentration was ≥1 ng/mL ALV P27 Protein, or ≥TCID50 10^1/mL ALV virus, the T/C value exceeded the threshold value. Therefore, the sensitivity of our test strips was 1 ng/mL for ALV P27 protein (Table 3) or TCID50 10^1/mL for ALV virus (Table 4).

Table 3. Sensitivity assay of FM-ICTS testing ALV P27 protein.

c (ng/mL)	T/C	Results
1,024	5.0122	+
512	4.0763	+
256	3.2056	+
128	2.1832	+
64	1.5959	+
32	0.9245	+
16	0.5233	+
8	0.3028	+
4	0.2303	+
2	0.1372	+
1	0.0736	+
0.5	0.0380	−
0.25	0.0090	−
0.125	0.0003	−
0	0.0015	−
Threshold	0.0606	/

Note: c (concentration); T (T-line value); C (C-line value).

Table 4. Sensitivity assay of FM-ICTS testing ALV virus.

c(TCID50/mL)	T/C	Results
1,000	1.8076	+
100	0.562	+
10	0.0841	+
1	0.0212	/
Negative	0.0118	/
Threshold	0.0606	/

Note: c (concentration); T (T-line value); C (C-line value).

Sample Concentration Fitting Curve Equation, Specificity and Repeatability

ALV P27 protein samples were serially diluted from 16,384 to 1 ng/mL and used to construct a fitting curve equation. Sample concentrations (c) between 1 and 2,048 ng/mL [Log_2 (c) = 0–11] generated T/C values that were positively correlated with the sample concentration. P27 concentrations between 4096 and 16,384 ng/mL generated T/C values that decreased. A T/C-Concentration fitting curve equation could be established according to concentrations between 1 and 2,048 ng/mL and was calculated as $y = 0.0052\ x^3 - 0.0099\ x^2 + 0.0612\ x + 0.0549$ with a correlation coefficient (r^2) = 0.9998 where x = Log_2(c) and c = 1–2,048 ng/mL, y = T/C. The recovery of the fitting curve was between 85 and 115% (Table 5).

Samples of related and unrelated viruses were also used to determine the level of cross reactivity. The NDV, ILTV, IBV, MDV, IBDV, REOV, and AIV samples all displayed T/C values less than the threshold indicating negative test results, while the ALV A, ALV B, and ALV J samples displayed T/C value higher than the threshold which declared the strip is available for different ALV types. Therefore, non-specific binding

Table 5. The relationship between concentration and T/C value.

c(ng/mL)	Log_2 (c)	T/C value	Calculated c (ng/mL)	Recovery (%)
1	0	0.0625	1.1	109.08
2	1	0.0996	1.7	86.40
4	2	0.1938	4.5	112.00
8	3	0.2717	7.3	90.91
16	4	0.4510	14.9	93.20
32	5	0.7807	33.1	103.41
64	6	1.2481	69.3	108.32
128	7	1.7716	126.7	98.98
256	8	2.5112	243.9	95.29
512	9	3.6357	524.8	102.50
1,024	10	4.8734	1022.3	99.83
2,048	11	6.4601	2054.9	100.34
4,096	12	6.6667	2230.0	/
8,192	13	5.5414	1394.7	/
16,384	14	3.8901	609.1	/

Note: c (concentration); T (T-line value); C (C-line value).

Table 6. Specificity assay of FM-ICTS for ALV.

Samples	T/C	Results
ILTV	0.0063	−
IBV	0.0009	−
MDV	0.0041	−
IBDV	0.0022	−
AIV	0.0046	−
ALV A	1.0305	+
ALV B	6.0246	+
ALV J	2.9043	+
REOV	0.0270	−
Control	0.0040	−
ALV P27 Protein	1.2481	+
Threshold	0.0606	

Note: T (T-line value); C (C-line value).

Table 7. Repeatability assay of FM-ICTS for ALV.

	T/C				
c ng/mL	Repeat 1	Repeat 2	Repeat 3	Mean	CV (%)
0	0.0152	0.0157	0.0152	0.0154	1.82
1	0.0598	0.0579	0.0697	0.0625	10.14
2	0.0912	0.1066	0.1010	0.0996	7.79
4	0.1879	0.1749	0.2185	0.1938	11.57
8	0.2389	0.3182	0.2580	0.2717	15.23
16	0.4308	0.4658	0.4565	0.4510	4.02
32	0.7826	0.7464	0.8131	0.7807	4.28
64	1.3010	1.3805	1.0629	1.2481	13.24
128	2.0188	1.5518	1.7443	1.7716	13.25
256	2.3415	2.7000	2.4921	2.5112	7.17
512	3.4857	3.7738	3.6477	3.6357	3.97
1,024	4.5743	4.4618	5.5842	4.8734	12.68
2,048	5.9342	6.8531	6.5929	6.4601	7.33
				Mean CV:	8.65

Note: T (T-line value); C (C-line value); variation coefficient (CV).

reactions were not a problem for the test strip assays (Table 6).

To determine the repeatability of the assay, P27 protein samples were serially diluted to generate 13 concentrations ranging from 0 to 2,048 ng/mL and performed 3 sets of repeated experiments at each concentration. It was found a maximum CV of 15.23% with an average of 8.65% for all the samples. These results indicated that the repeatability of the assay was satisfactory (Table 7).

Table 8. Clinical sample detection with FM-ICTS and ELISA.

Result	ELISA positive	ELISA negative	Total	Kappa coefficient
FM-ICTS positive	68	23	91	0.7031
FM-ICTS negative	27	866	893	
Total	95	889		

Clinical Sample Detection

To test the effectiveness of the assay using clinical samples, 984 meconium samples were obtained and subjected to the test strip assays and a commercial ELISA assay. The ELISA assay generated 95 positive and 889 negative results. The fluorescent microsphere test strip assay found 91 positive and 893 negative results. However, only 68 positive results and 886 negative results were found agreed between two methods. Therefore the Kappa coefficient between the strips and ELISA was 0.7031(Table 8).

DISCUSSION

ALV is the most common naturally occurring avian retrovirus associated with neoplastic diseases and other production problems in chickens (Fadly, 1997). Due to the increased variability of tumor types and extended host range of ALV, its eradication has become even more complex and challenging (Qian et al., 2015). There is currently still no effective drug therapy or preventive treatment for the control of ALV infections, so it is very important to achieve rapid diagnosis for eradication of the virus. The detection methods for ALV include virus isolation, ELISA, real-time PCR, immunofluorescence assays, colloidal gold test strips, and LAMP that have been successfully applied to ALV detection (Zhang et al., 2010; Yun et al., 2013; Gao et al, 2014; Chen et al., 2018; Yu et al., 2019). However, these methods require extended time and specialized laboratory instruments, which makes them often unaffordable for most chicken farms. The colloidal gold test strip and LAMP assays can be used at field sites and the results can be read directly by naked eye. However, the colloidal gold test strip method has low sensitivity and may fail to detect low titers of RV (Jiang et al., 2018). The technique that we employed in the current work uses fluorescent microspheres instead of colloidal gold particles, which leads to a higher assay sensitivity (Oliver, 2010). This method has the advantages of low cost and rapid detection, but its potential for quantitative detection has not been fully explored. Reaction bands can be recognizable by the naked eye following illumination with an ordinary UV lamp or the fluorescence signal can be analyzed using special equipment. The concentration of the sample can be calculated by establishing the fitting curve equation and this technique is sensitive and stable (Li et al., 2010; Aizik et al., 2017). At sample concentrations between 1 and 2,048 ng/mL, the recovery between 85 and 115% indicates that the fitting curve

is accurate. At sample concentrations between 4,096 and 16,384 ng/mL, the T/C values decreased as the protein level increased due to the hook effect of the lateral chromatographic test strip. Therefore, the sample concentrations should be diluted to 1–2,048 ng/mL. The sensitivity and specificity of the test strips were evaluated and our results indicated that this technique was convenient and can be an alternative tool for the ALV detection, particular at field site. Overall, the assay effectively detected ALV in the clinical samples and the sample concentration fitting curve equation were successfully established and the curve possessed an r^2 value > 0.9998, indicating the assay is satisfactory as an analytical tool.

In summary, the fluorescent microsphere immunochromatography test strips using anti-capsid protein p27 mAbs was developed for the quantitative detection of ALV. The method is simple, rapid, sensitive and specific and does not cross react with common avian viruses.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

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