

# Development and application of a colloidal gold test strip for the rapid detection of the infectious laryngotracheitis virus

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**ABSTRACT** Infectious laryngotracheitis disease is an acute, highly contagious viral disease seriously affecting poultry industry worldwide. In this study, a rapid and simple immune colloidal gold test strip for detecting infectious laryngotracheitis virus (ILTV) was developed based on membrane chromatography with monoclonal antibodies (mAbs) against gJ protein of ILTV and systematically evaluated for the detection of ILTV from clinical samples. mAb 2D4 1D7 was conjugated with colloidal gold as the detector antibody on the test strip. Another mAb, 1D8 1G3, was used as the capture complex at the test line (T-line), and goat antimouse IgG antibody was used as the capture antibody at the control line (C-line). The colloidal gold test strip showed high specificity in the detection of ILTV, with no

cross-reaction with other avian pathogens, including infectious bronchitis virus, infectious bursal disease virus, avian influenza virus, Newcastle disease virus, fowl adenoviruses, and Marek's disease virus. Besides, the detection limit of this method was as low as 60 ELD<sub>50</sub>/mL for the ILTV Wanggang strain. Furthermore, we evaluated its application in 260 clinical samples suspected of infection with ILTV. Results from the strip test were nearly identical with those from real-time PCR (coincidence rate 99.6%) and showed higher sensitivity than conventional PCR. All the results obtained in this study indicated that the colloidal gold test strip can be applied as a simple, rapid, sensitive, and specific diagnostic tool for the detection of ILTV, especially in resource-limited areas.

**Key words:** infectious laryngotracheitis virus, gJ protein, monoclonal antibody, colloidal gold test strip, diagnostic method

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## INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is a double-stranded DNA virus that is classified within the genus *Iltovirus*, subfamily *Alphaherpesvirinae*, and family *Herpesviridae* (Fuchs et al., 2007). ILTV induces an acute, highly contagious infectious disease in chickens and causes enormous economic losses in the poultry industry (Bagust et al., 2000; Crespo et al., 2007). The clinical features of ILTV infection are conjunctivitis, coughing, nasal discharge, reduced weight gain, expectoration of bloody mucus, decreased egg production, and increased susceptibility to other

respiratory pathogens (Gowthaman et al., 2016; Craig et al., 2017). Infectious laryngotracheitis has become an economically important disease worldwide because it was first reported in the Americas in 1925 (May HG, 1925).

The genome of ILTV is approximately 150 kbp in length and encodes at least 79 predicted proteins (Leib et al., 1987). The genome is divided into 4 distinct regions: the unique long region, the unique short region, the internal repeat region, and the terminal repeat region (Leib et al., 1987; Bagust et al., 2000; Fuchs et al., 2007). The US5 gene encodes glycoprotein J (gJ), which is considered to be responsible for stimulating humoral and cell-mediated immune responses (Thureen and Keeler, 2006). gJ is one of the most important antigen proteins of ILTV, and its function is related to cell autophagy and viral egress; however, the mechanisms underlying these functions are not fully known (Fuchs et al., 2005).

In recent years, ILTV has caused sporadic outbreaks in China and led to huge economic losses in the poultry

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industry (Zhao et al., 2015; Yan et al., 2016). Depending on the field ILTV strains, mortality among infected chickens varies from 10 to 70% (Schadler et al., 2019). Moreover, the clinical symptoms and pathological changes observed in the disease are easily confused with other avian pathogens, such as infectious bronchitis virus (IBV) and Newcastle disease virus (NDV). Therefore, the development of a simple and specific measure to diagnose ILTV infections in chicken breeding farms is urgently needed to reduce large annual economic losses.

Clinical diagnosis has played an important role in controlling infectious disease. Virus isolation and serological testing were the traditional methods for detecting ILTV (Inoue and Nishibe, 1973; Ide 1978; El-Zein et al., 1979). Currently, different types of PCR-based techniques have been developed for ILTV detection, such as conventional PCR (Abbas et al., 1996), nested PCR (Humberd et al., 2002), real-time quantitative PCR (qPCR) (Creelan et al., 2006; Mahmoudian et al., 2011; Zhao et al., 2013), and real-time Taqman PCR (Callison et al., 2007). The virus isolation and serological tests are laborious, time-consuming, and exhibit low sensitivity. In addition, although the PCR-based diagnostic techniques were widely applied in clinical detection because of their simplicity and high sensitivity, these assays require expensive instruments and relatively sophisticated laboratories, making them unsuitable for point-of-care and visible detections, especially in some remote developing world locations. In contrast, the colloidal gold test strip method provides rapid and reliable results and involves little dependence on equipment (Oliver, 2010; Ovais et al., 2017). The immune colloidal gold technique is an immunochromatographic method in which a cellulose membrane is used as the carrier and a colloidal gold-labeled antigen or antibody is used as the tracer, thus making the results readable using the naked eye (Oliver, 2010). It develops quickly and has been applied in the detection of many avian pathogens, such as avian influenza virus (AIV) (Cui and Tong, 2008), NDV (Li et al., 2019), avian leukosis virus (Yu et al., 2019), and infectious bursal disease virus (IBDV) (Nurulfiza et al., 2011). However, the colloidal gold test strip method has not been developed for ILTV.

In the present study, 2 anti-ILTV-gJ monoclonal antibodies (mAbs) were prepared, and an immune colloidal gold test strip for detecting ILTV was developed. The test's specificity, sensitivity, stability, and reproducibility were systematically evaluated. Furthermore, conventional PCR and real-time PCR were compared with the colloidal gold test strip for detecting ILTV in clinical samples. This test strip, which uses a chromatographic technique and colloidal gold, proved to be a useful diagnostic method for ILTV infection, and it is highly suitable for most chicken breeding environments especially in less developed countries or regions.

## MATERIALS AND METHODS

### **Virus Antigens, Cells, and Experimental Animals**

ILTV (Wanggang and Hungarian strains) was purchased from the Institute of Veterinary Drugs Control (Beijing, China). NDV (Lasota strain), IBV (H52 and H120 strains), AIV (H5 and H9 subtype), IBDV (B87 strain), and Marek's disease virus (MDV, CVI988 strain) were obtained from commercial vaccines (HILE, China). The ILTV Wanggang strain was propagated in 9- to 11-day-old specific pathogen-free (SPF) chicken embryos (MERIAL, Beijing, China), and the virus titer was determined as  $10^{4.5}$  ELD<sub>50</sub>/mL. Fowl adenoviruses (FAvV, SC-Neijiang strain) were obtained from Professor Hongning Wang (Sichuan University) and propagated in a Leghorn male hepatoma cell line as previously described (Zhai et al., 2019). Myeloma cell line SP2/0 was purchased from the ATCC (Rockville, MD). Bagg albino C mice (8 wk old), which were used for immunization and ascites preparation, were provided by the experimental animal center. Animal experiments were approved by Animal Ethics Committee of Sichuan Animal Science Academy and performed in accordance with animal ethics guidelines and approved protocols (licence: SCXK(e)2015-0018).

### **Preparation of Monoclonal Antibodies Against ILTV gJ Protein**

ILTV Wanggang strain was propagated in specific pathogen-free chicken embryos, and the virus particles were further purified using a sucrose density gradient. Viral DNA was extracted using the DNA Mini Kit (QIAGEN). The high antigenic region of the gJ gene of an ILTV Wanggang strain was amplified using the primer pair gJ-F/gJ-R (Table 1), ligated into the prokaryotic expression vector pET-sumo, and the recombinant His-gJ fusion proteins were expressed in Rosetta (DE3) *Escherichia coli* Strain and purified. The purified viral antigen of ILTV Wanggang strain was emulsified with an equal volume of Freund's Complete Adjuvant and then used to immunize specific pathogen-free 6-week-old female Bagg albino C (BALB/c) mice with approximately 100 µg per mouse. At 2-wk intervals, 2 boosters of approximately 80 µg of purified viral antigen mixed with Freund's Incomplete Adjuvant were administered. One week later, the mice received a final booster immunization with about 80 µg of purified viral antigen mixed with incomplete Freund's adjuvant. Spleen cells were harvested from immunized BALB/c mice and fused with SP2/0 cells during the logarithmic phase in the presence of PEG1450 (MERCK). The hybridomas secreting mAbs were selected by recombinant gJ-based indirect ELISA. The hybridoma cells that tested positive in indirect ELISA were used for further subcloning. Large quantities of mAbs were prepared

**Table 1.** Sequences of primers used in the present study.

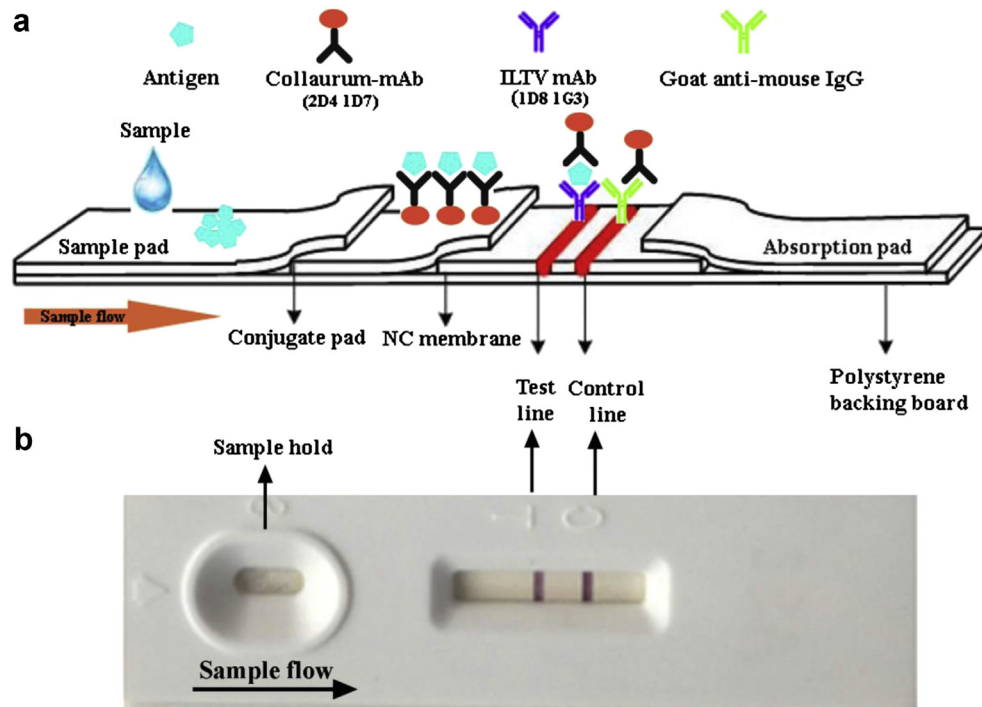
Primers	Sequences (5'-3')	Size (bp)	Reference
gJ-F	GGATCCGAATTCCGGACTAT	639	This study
gJ-R	GGAATTCATGAGATGT		
PCR-F	GTGGTGCTCGAGTGCCGCC	647	Magouz et al., 2018
PCR-R	TTATGAATACAGCTGCGCC		
qPCR-F	ACGATGACTCCGACTTTC	148	Zhao et al., 2013
qPCR-R	CGTTGGAGGTAGGTGGTA		
probe	CAGTATCTGGCATCGCCTCAT		
	CCTGGGAACAGAACCTGAACT		
	FAM-CTAACCCGTTTCG		
	CCGCACTCG-BHQ-1		

by the mouse ascites method, and the purified ascitic fluid containing the mAbs was purified using a protein G perfusion affinity chromatographic column. The purified mAbs were dialyzed in PBS (10 mmol/L) and determined using an ultraviolet spectrophotometer at 280 nm.

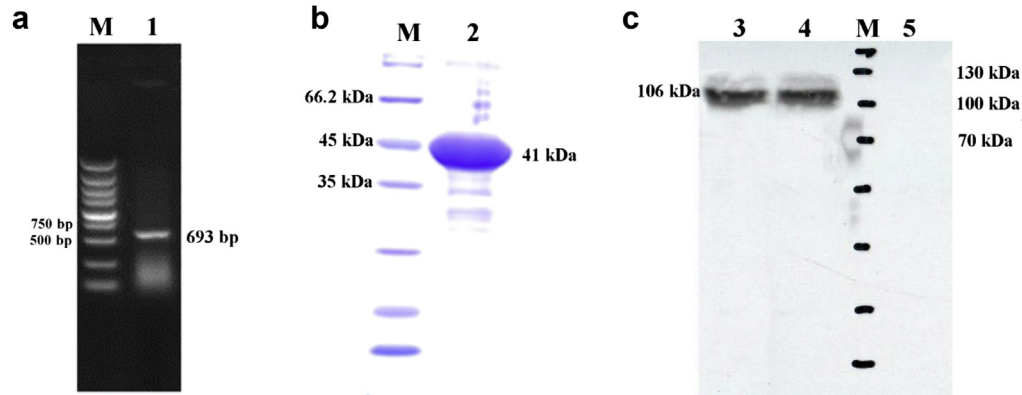
### Characterization of mAbs Against Recombinant ILTV gJ Protein

The titer of the MAbs was determined by gJ-based indirect ELISA. The supernatant culture of hybridomas and ascitic fluid were diluted in a gradient from 1:1,000 to 1:128,000. The same diluted supernatant of whole viral antigen of ILTV Wanggang strain was used as the positive control, and SP2/0 myeloma cell culture and uninfected

ascitic fluid were used as the negative control. For Western blot analysis of purified anti-gJ mAbs, the purified whole viral antigen was subjected to 12% SDS-PAGE followed by transfer to a polyvinylidene difluoride membrane. The membranes were blocked with 5% skimmed milk in Tris-buffer saline containing 0.05% Tween-20 at 37°C for 2 h. After washing 3 times with 1 × Tris-buffer saline containing 0.05% Tween-20, the membranes were incubated with the purified anti-gJ mAbs at 37°C for 1 h. The membranes were then washed 3 times as mentioned previously and incubated with 1:5,000 diluted goat anti-mouse IgG (H + L); horseradish peroxidase conjugated (1:5,000 dilution) for a further 1 h at 37 C, followed by development of the color reaction by diaminobenzidine (HRP-DAB chromogenic substrate Kit, Bio Basic Inc, Markham, Ontario, Canada).



**Figure 1.** The schematic diagram of the colloidal gold test strip (A) and the final end product of colloidal gold test strip package (B). The test strip included 3 pads (sample pad, conjugate pad, and absorption pad), an nitrocellulose membrane, and a polystyrene backing board. The conjugate pad contained the gold-labeled monoclonal antibody 2D4 1D7, which provided an easily visible red color. There were 2 lines on the nitrocellulose membrane: the test line (T-line) and control line (C-line). The T-line contained monoclonal antibody 1D8 1G3, and the C-line contained the goat antimouse IgG antibody.



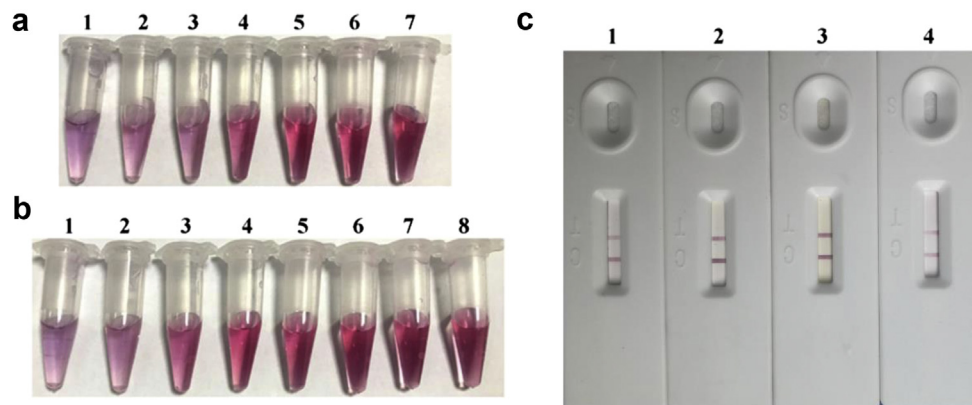
**Figure 2.** PCR amplification of the gJ gene from infectious laryngotracheitis virus (A), SDS-PAGE analysis of purified gJ protein (B), and western blot analysis of monoclonal antibodies 2D4 1D7 and 1D8 1G3 (C). Lane 1: the PCR product of gJ gene is 693 bp, lane 2: the purified gJ protein was about 41 kDa, lane 3 and 4: recognition of the whole viral antigen of infectious laryngotracheitis virus by monoclonal antibody 2D4 1D7 and 1D8 1G3 in Western blot, respectively. Lane 5: negative control.

### Optimization of the Colloidal Gold Test Strip

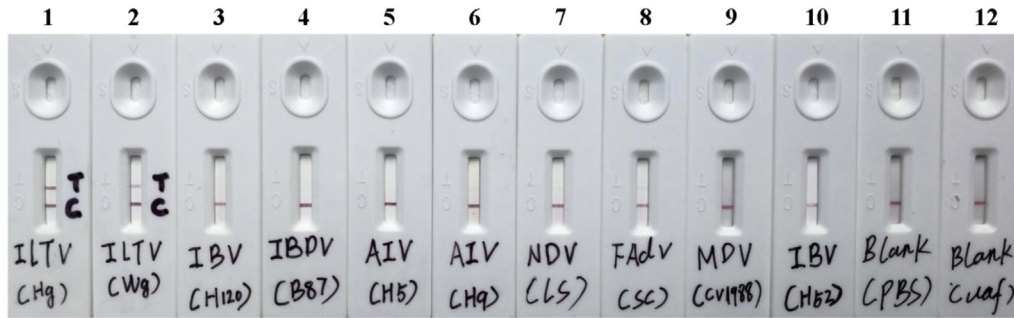
The colloidal gold (diameter of 20 nm) was prepared by a trisodium citrate reduction method as previously described (Ovais et al., 2017). The pH of the colloidal gold fluid was evaluated with 0.2 mol/L of  $K_2CO_3$  in the pH range of 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. The aforementioned colloidal gold fluid was mixed with 50  $\mu$ L of mAb 2D4 1D7 (1 mg/mL), respectively. After incubating at room temperature for 20 min, 100  $\mu$ L of 10% NaCl was added to the solutions, followed by incubating at room temperature for an additional 60 min. The optimal pH was determined by selecting the red color with the lowest value. Various amounts of ILTV mAb 2D4 1D7 (0, 5, 10, 20, 30, 40, 60, and 80  $\mu$ g) were added to 1 mL of colloidal gold fluid, followed by 2 mL of 10% bovine serum albumin to provide a final concentration of 0.4%. The optimal amount of mAb used for conjugation was 120~130% of the minimum amount.

After adding 0.2 mL 10% polyethylene glycol (MW20000), the supernatant was discarded after centrifugation at 11,000 rpm/min for 60 min. The precipitate was resuspended with 2 mL of diluent, containing trehalose (1%, *w/v*), bovine serum albumin (0.5%, *w/v*), and Tween-20 (0.1%, *w/v*), and then filtrated with a 0.22- $\mu$ m filter. The preparation was stored at 4°C until use.

The ILTV mAb 2D4 1D7 with conjugated colloidal gold (1.4 mL/cm) was evenly sprayed on a piece of glass fiber membrane and then dried at 37°C overnight. The ILTV mAb 1D8 1G3 was serially diluted across a range of 1 mg/mL, 1.5 mg/mL, 2 mg/mL, and 2.5 mg/mL and then microsprayed onto a nitrocellulose (NC) membrane (UniSart CN140) at a position that allowed to make the test line (T-line). Goat antimouse IgG antibody in PBS was diluted in a different concentration gradient (0.5 mg/mL, 1 mg/mL, 1.5 mg/mL, and 2 mg/mL) and then microsprayed onto the same NC membrane at a position that allowed to make the control line



**Figure 3.** Optimization of the colloid gold test strip assay. (A) The PH of the colloidal gold fluid (lane 1-7: 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5), (B) the amount of monoclonal antibody 2D4 1D7 required for conjugation with colloidal gold particles (lane 1-8: 0  $\mu$ g/mL, 5  $\mu$ g/mL, 10  $\mu$ g/mL, 20  $\mu$ g/mL, 30  $\mu$ g/mL, 40  $\mu$ g/mL, 60  $\mu$ g/mL, and 80  $\mu$ g/mL), and (C) the concentration of monoclonal antibody 1D8 1G3 (lane 1-4: 2.5 mg/mL, 2 mg/mL, 1.5 mg/mL, and 1 mg/mL) and goat antimouse IgG antibody (lane 1-4: 2 mg/mL, 1.5 mg/mL, 1 mg/mL, and 0.5 mg/mL) were tested to determine the optimal test strip system for detecting infectious laryngotracheitis virus.



**Figure 4.** Specificity of the colloidal gold test strip. The positive specimens suspected of being infected with infectious laryngotracheitis virus Hungarian (lane 1), infectious laryngotracheitis virus Wanggang (lane 2), infectious bronchitis virus H120 (lane 3), infectious bursal disease virus B87 (lane 4), avian influenza virus H5 (lane 5), avian influenza virus H9 (lane 6), Newcastle disease virus Lasota (lane 7), Fowl adenoviruses SC-neijiang (lane 8), Marek's disease virus CVI988 (lane 9), infectious bronchitis virus H52 (lane 10), and negative samples from PBS (lane 11) and uninfected allantoic fluid (lane 12) were simultaneously tested by this strip test.

(C-line). The test strips were dried at 37°C overnight and stored at 4°C. The optimal concentration was selected using the color developing index.

### Preparation of the Test Strip Package

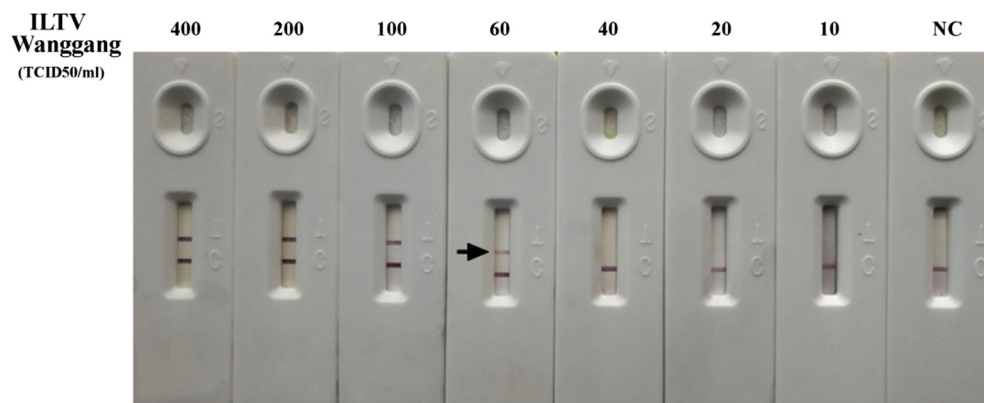
To assemble a colloidal gold test strip package, the NC membrane was arranged with ILTV mAb 2D4 1D7 conjugated with colloidal gold at one end, the T-line microsprayed with mAb 1D8 1G3 was set upstream, and the C-line was set downstream, with a distance between the T-line and the C-line of 0.5 cm. Besides, an absorbing pad was set at the opposite end. The polystyrene backing board was subsequently cut into a 2.8-mm-wide and 6-cm-long strip (Figure 1A). Finally, each strip was placed in a plastic case, which was stored individually in a desiccated plastic bag. The test results were determined by the number and position of the detection lines on the test strips. Positive samples yielded one band at the C-line and one band at the T-line, whereas negative samples yielded one band at the C-line and no band at

the T-line. The results were discarded if there was no band at the C-line (Figure 1B).

### Specificity and Sensitivity of the Colloidal Gold Test Strip

To evaluate the specificity of the colloidal gold test strip, ILTV (Wanggang and Hungarian strains) chicken embryonic culture and antigens of IBV (H52 and H120 strains), AIV (H5 and H9 subtype), NDV (Lasota strain), IBDV (B87 strain), MDV (CVI988 strain), and FAdV (SC-Neijiang strain) were used in this study. The PBS and uninfected allantoises fluid were used as the negative groups. A pipet was used to add the sample or allantoic fluid to the sample pad (3 drops, ~100 µL), which was then placed on a horizontal surface for 10 min before the test strip was examined for bands. The tests in this study were repeated at least 3 times.

To evaluate the sensitivity of the test strip, different contents of ILTV Wanggang strain (400 ELD<sub>50</sub>/mL, 200 ELD<sub>50</sub>/mL, 100 ELD<sub>50</sub>/mL, 60 ELD<sub>50</sub>/mL, 40 ELD<sub>50</sub>/mL, 20 ELD<sub>50</sub>/mL, 10 ELD<sub>50</sub>/mL, and NC) were tested with the strips. The uninfected allantoises fluid was used as the negative control (NC). The arrow indicates the lowest detection limit of the colloidal gold test strip.



**Figure 5.** Sensitivity of the colloidal gold test strip for infectious laryngotracheitis virus Wanggang strain. Different virus titers of the infectious laryngotracheitis virus Wanggang strain (400 ELD<sub>50</sub>/mL, 200 ELD<sub>50</sub>/mL, 100 ELD<sub>50</sub>/mL, 60 ELD<sub>50</sub>/mL, 40 ELD<sub>50</sub>/mL, 20 ELD<sub>50</sub>/mL, 10 ELD<sub>50</sub>/mL, and NC) were tested with the strips. The uninfected allantoises fluid was used as the negative control (NC). The arrow indicates the lowest detection limit of the colloidal gold test strip.

**Table 2.** Specificity of the colloidal gold test strips in expiration date experiments.

Detection method	Mo	ILTV	IBV	IBDV	AIV	NDV	MDV	FAdV	Blank
Test strips <sup>1</sup>	3	+	–	–	–	–	–	–	–
	6	+	–	–	–	–	–	–	–
	9	+	–	–	–	–	–	–	–
	12	+	–	–	–	–	–	–	–
	15	+	–	–	–	–	–	–	–

“+” indicates the positive results by the test strips, while “–” indicates the negative results by the test strips.

Abbreviations: AIV, avian influenza virus; FAdV, fowl adenoviruses; IBDV, infectious bursal disease virus; IBV, infectious bronchitis virus; ILTV, infectious laryngotracheitis virus; MDV, Marek’s disease virus; NDV, Newcastle disease virus.

<sup>1</sup>The test strips stored at 4°C were tested after 3, 6, 9, 12, and 15 mo in this study.

ELD<sub>50</sub>/mL, 20 ELD<sub>50</sub>/mL, and 10 ELD<sub>50</sub>/mL) were tested, along with the uninfected allantoises fluid used as the negative control.

### Stability and Repeatability of the Colloidal Gold Test Strip

To determine the stability and repeatability of the colloidal gold test strip assay, the test strips that had been stored for 1, 3, 6, 9, 12, and 15 mo at 4°C were tested with known ILTV-positive and -negative specimens. Meanwhile, the specificity and sensitivity of the test strips were also tested, with 15 test strips repeat tested for each sample.

### Clinical Evaluation of the Colloidal Gold Test Strip

To determine how the strip test performed in analyzing clinical samples, 260 clinical samples collected from 45 breeder-broiler farms in southwestern China were simultaneously subjected to conventional PCR (primers PCR-F/PCR-R, Table 1), real-time PCR (primers qPCR-F/qPCR-R, Table 1), and the colloidal gold test strip assay. Tissue samples of infected chickens, including larynx tissue, heart, liver, and lung, were brought back to laboratory on ice, and about 1.0 g of each sample was homogenized separately in cold PBS (PH 7.2) using TissueLyser (2,000 r/min, 3 cycles) for DNA extraction. The supernatants were collected by centrifugation at 4,000 × *g* for 20 min at 4°C. Viral DNA was extracted from the supernatants using the TIANGEN Genomic DNA Kit (Beijing, China) following the manufacturer’s instructions. The quality of the extracted DNA samples was measured by using Nanodrop 2,000 (Thermo Fisher, Waltham, MA). The amplified PCR products were

analyzed by electrophoresis, the detection of real-time PCR was analyzed by the presence of a Ct value, and the colloidal gold test strip reaction results were analyzed by visualizing the detection lines using the naked eye.

## RESULTS

### Expression, purification, and Western Blot Analysis of the Recombinant Protein pET-sumo-gJ

The expression fragment of about 693 bp long of the gJ gene was amplified by PCR from the DNA of ILTV and cloned into a pET-sumo vector. PCR and sequences analysis showed that prokaryotic expression plasmid pET-sumo-gJ was successfully constructed (Figure 2A). The recombinant pET-sumo-gJ protein was expressed in a form of inclusion body after induction with IPTG and then purified and identified to be approximately 41 kDa by SDS-PAGE (Figure 2B). Western blot analysis demonstrated that 2 mAbs (2D4 1D7 and 1D8 1G3) could recognize the whole viral antigen of ILTV (Figure 2C).

### Optimization of the Colloid Gold Test Strip Assay

To optimize the colloid gold test strip assay, the optimal PH of the colloidal gold fluid, the minimum amount of mAb 2D4 1D7 required for conjugation with colloidal gold particles, and the amounts of mAbs (1D8 1G3) and goat anti-mouse IgG were determined based on coloration. The results showed that the optimal pH of the colloidal gold fluid was 8.0 in the range of pH experimented (Figure 3A), and the minimum amount of mAb 2D4 1D7 was 10 µg/mL as 1 mL can stably capture colloidal gold particles with a minimum amount of

**Table 3.** Sensitivity of the colloidal gold test strip for infectious laryngotracheitis virus Wanggang strain in expiration date experiments.

Detection method	Mo	400 ELD <sub>50</sub> /mL	200 ELD <sub>50</sub> /mL	100 ELD <sub>50</sub> /mL	60 ELD <sub>50</sub> /mL	40 ELD <sub>50</sub> /mL	20 ELD <sub>50</sub> /mL	10 ELD <sub>50</sub> /mL	Negative control
Test strips <sup>1</sup>	3	+	+	+	+	–	–	–	–
	6	+	+	+	+	–	–	–	–
	9	+	+	+	+	–	–	–	–
	12	+	+	+	+	–	–	–	–
	15	+	+	+	+	–	–	–	–

“+” indicates the positive results by the test strips, while “–” indicates the negative results by the test strips.

<sup>1</sup>The test strips stored at 4°C were tested after 3, 6, 9, 12, and 15 mo in this study.

**Table 4.** Comparison of conventional PCR, real-time PCR, and immune colloid gold test strip for the detection of infectious laryngotracheitis virus in clinical specimens.

Detection methods	Conventional PCR			Real-time PCR		
	Positive (%)	Negative (%)	Row totals (%)	Positive (%)	Negative (%)	Row totals (%)
Test strip						
Positive	44 (16.9)	4 (1.5)	48 (18.5)	47 (18.1)	0 (0)	47 (18.1)
Negative	0 (0)	212 (81.5)	212 (81.5)	1 (0.4)	212 (81.5)	213 (81.9)
Column totals	44 (16.9)	216 (83.1)	260 (100)	48 (18.5)	212 (81.5)	260 (100)

antibody (Figure 3B). As shown in Figure 3C, when the concentrations of mAb 1D8 1G3 and goat antimouse IgG reached 1.5 mg/mL and 1 mg/mL, respectively, the colloid gold test strip presented the most evident bands.

### Specificity of the Immune Colloidal Gold Test Strip

To determine the specificity of the colloid gold test strip assay, 2 ILTV strains (the Wanggang and Hungarian strains) and other viral species (IBV, IBDV, AIV, NDV, FAdV, and MDV) were tested. The results were positive for allantoic fluid of both ILTV Wanggang and Hungarian strains. In contrast, the colloid gold test strip did not yield positive reactions when tested on with other viruses or blank groups (Figure 4). Thus, the results indicated that the test strip could be used to specifically detect ILTV.

### Sensitivity of the Colloidal Gold Test Strip

To evaluate the sensitivity of colloidal gold test strip, the detection limit of the assay was determined by testing against dilutions of the ILTV Wanggang strain. As shown in Figure 5, the colloidal gold test strip was able to detect ILTV Wanggang strain at a level of 60 ELD<sub>50</sub>/mL.

### Stability and Reproducibility of the Colloidal Gold Test Strip

The test strip packages were sealed and stored at 4°C up to 15 mo and were tested with known ILTV-positive and ILTV-negative samples at 3, 6, 9, 12, and 15 mo (15 strips were randomly selected and tested at each time point). The results showed that all the test strips stored from 3 to 12 mo were comparable and that all known ILTV-positives yielded positive results and all known ILTV-negatives yielded negative results. Meanwhile, the detection limit was still 60 ELD<sub>50</sub>/mL. However, after 15 mo of storage, 6.7% (1/15) of the known positives tested negative (Table 2), and the detection limit of 20.0% (3/15) of the strips increased to 100 ELD<sub>50</sub>/mL (Table 3). The aforementioned tests indicated that results from the immune colloid gold test strips were stable and repeatable when stored for a 12-mo period, and false-positives were never detected.

### Comparative Analysis of Field Samples

To confirm the applicability of the immune colloid gold test strip in the clinical field, 260 clinical specimens were simultaneously tested using the conventional PCR, real-time PCR assay, and the ILTV colloid gold test strip assay. The results showed that a total of 44 specimens out of the 260 clinical samples were identified as positive for ILTV by conventional PCR (16.9% positive). Meanwhile, 47 and 48 specimens tested positive using the test strip (18.1% positive) and real-time PCR (18.5% positive), respectively (Table 4). Notably, for the positive samples, 44 specimens were simultaneous detected by the 3 methods, whereas 3 specimens were detected using real-time PCR and the colloid gold test strip, and one specimen was only detected using real-time PCR. Therefore, the 4 specimens were further tested by the virus isolation method using 9- to 11-day-old chicken embryos, which is regarded as the gold standard (El-Zein et al., 1979). DNA was extracted from the specimens of infected chicken embryos, and PCR analysis demonstrated that the 4 specimens were positive. This indicated that the colloidal gold test strip assay has a higher sensitivity than conventional PCR and a slightly lower sensitivity than real-time PCR. The detection methods between conventional PCR and the test strip agreed on the diagnosis “positive” in 16.9% and “negative” in 81.5% of clinical specimens. Thus, their coincidence rate is  $p_o = 256/260 = 16.9 + 81.5\% = 98.4\%$ . Similarly, the coincidence rate of the methods between real-time PCR and the test strip is  $p_o = 259/260 = 18.1 + 81.5\% = 99.6\%$  (Table 4).

## DISCUSSION

ILTV is associated with respiratory diseases and decreased egg production in chickens, causing severe economic losses in the poultry industry (Bagust et al., 2000; Crespo et al., 2007). The prevention and control of ILTV requires rapid and accurate detection methods (Davidson et al., 2016; Schadler et al., 2019). Previous studies have reported that the common methods for detecting ILTV include virus isolation and identification and serological testing (Inoue and Nishibe, 1973; El-Zein et al., 1979); however, these methods are time-consuming and laborious. Although various PCR-based technologies are widely applied in ILTV detection (Vogtlin et al., 1999; Humberd et al., 2002; Creelan et al., 2006; Kirkpatrick et al., 2006;

Callison et al., 2007), these methods require relatively sophisticated biological manipulation. Thus, the development of a simple and rapid diagnostic tool to detect ILTV would be valuable for etiological surveillance and prediction of the severity of ILTV infection in chicken flocks. In the present study, a colloidal gold test strip method was developed, based on the use of mAbs against gJ of ILTV coated with gold nanoparticles, to detect ILTV from field samples in less time and make the eradication of ILTV on large breeding farms possible. Furthermore, the virus-detection capacity of the colloidal gold test strip was systematically evaluated. All the results obtained indicated that the test strip developed in this study is a simple and reliable method for the detection of ILTV and is convenient for the detection of clinical samples on chicken breeding farms.

Timely and accurate diagnostic methods are very important for the prevention and control of infectious diseases (Kirkpatrick et al., 2006; Crespo et al., 2007; Magouz et al., 2018). To obtain more accurate results using the colloidal gold test strip, the reaction conditions of the test strip were optimized, including the pH of the colloidal gold fluid, the amount of labeled mAb 2D4 1D7 used, and the concentrations of colloidal gold-1D8 1G3 conjugate and goat antimouse IgG. After optimization, the colloidal gold test strip gave an accurate and clear result, which was visualized within 10 min by the naked eye. Finally, an end-product of the test strip package was produced. We further evaluated its specificity, sensitivity, stability, reproducibility, and application in the field.

Previous studies have shown that ILTVs are often involved in coinfection with other avian viruses, such as IBV, IBDV, NDV, and MDV (Cong et al., 2018; Magouz et al., 2018). To evaluate the specificity of the colloidal gold test strip, 6 different DNA or RNA avian viruses were used in the present study. It showed that the test strips were positive only for ILTV and ILTV-gJ antigen, which indicated that the test strips can be used to differentiate ILTV from other avian viruses, including IBV, IBDV, AIV, NDV, MDV, and FAdV. Owing to a limited availability of specimens, further research is needed to verify that the ILTV test strip does not produce positive results with other avian pathogens, such as chicken anemia virus, avian leukosis virus, reticuloendotheliosis virus, avian reovirus, and so on.

Conventional PCR and real-time PCR are the most commonly used molecular detection methods in the field (Abbas et al., 1996; Vogtlin et al., 1999; Creelan et al., 2006; Zhao et al., 2013). In this study, a total of 260 clinical specimens suspected of harboring ILTV were collected from southwestern China and were tested using conventional PCR, real-time PCR, and the colloidal gold test strips. The results of the strip test were nearly identical to those of real-time PCR (coincidence rate 99.6%) and showed higher sensitivity than conventional PCR. Determining whether the strip test had a similar sensitivity to real-time PCR and a greater sensitivity than conventional PCR will require the

analysis of more clinical samples and additional tests. Overall, the data suggest that the test strip developed in this study could be a useful tool for specific diagnosis and epidemiological investigation of ILTV.

In conclusion, a rapid, sensitive, and specific colloidal gold test strip package was first developed for the detection of ILTV, based on the use of mAbs coated with gold nanoparticles. Furthermore, we evaluated the test strips with 260 clinical samples collected in the field, and the test strips' performance was excellent. The whole test process takes only about 15 min, including sample treatment time, and the detection results are directly visible to the naked eye, with no requirement for expensive instruments and sophisticated laboratory equipment. In addition, the test strips displayed good stability and repeatability of results when stored at 4°C for up to 12 mo, which further enhances the feasibility of their practical application. In summary, the colloidal gold test strip developed in this study should be useful for clinical diagnosis of ILTV and the elimination of infected chicks from flocks.

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