

## **Article @ Virology**

### **Development and Evaluation of Colloidal Gold Immunochromatographic Strip for Rapid Detection of Canine Parainfluenza Virus Type 5 Strain**

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#### **ABSTRACT**

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A colloidal gold immunochromatographic test strip was firstly developed to detect Canine Parainfluenza Virus type 5 based on a sandwich format. The sensitivity of strip was tested for 10<sup>3.8</sup> TCID<sub>50</sub> mL<sup>-1</sup> to CPIV 5. No cross-reactivity with five common viruses from dogs were observed. Thirty-nine nasopharyngeal swabs samples were used to compare the consistency between RT-PCR and the strip, and the total coincidence rate was 87.18%. The colloidal gold immunochromatographic test strip thus provides a rapid and convenient method for on-site detection of CPIV 5 without any ancillary equipment.

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**Key Words:** Canine Parainfluenza Virus type 5; Monoclonal Antibody; Colloidal Gold; Strip.

**Abbreviations:** CPIV, Canine Parainfluenza Virus; BSA, Bovine Serum Albumin  
RT-PCR, Reverse Transcriptase Polymerase Chain Reaction;  
MAb, Monoclonal Antibody; CIV, Canine Influenza Virus

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## Introduction

Canine parainfluenza virus type 5 (CPIV5) is a negative-stranded non-segmented RNA virus of the family *Paramyxoviridae*<sup>[1,2]</sup>. Canine parainfluenza virus type 5 is the main infectious cofactor of canine respiratory disease complex or CITB (commonly known as kennel cough). The main clinical symptoms of this disease includes running nose, fever and cough<sup>[3, 4]</sup>. A number of trials have been done to diagnose the infection of CPIV, such as virus isolation and identification assay<sup>[4, 5]</sup>, polymerase chain reaction<sup>[6, 7]</sup>, immunohistochemistry<sup>[8, 9]</sup>, and immunofluorescence assay<sup>[10]</sup>. These methods were very useful in the accurate diagnosis of CPIV. However, these methods also have several limitation including professional techniques and operational skills, expensive equipment, and long processing times. Hence, a rapid and simple diagnostic assay would be extremely valuable for on site detection of CPIV.

Colloidal gold immunochromatographic assays has provided the latest advancements in rapid detection over past decade. In the colloidal gold immunochromatographic assay, colloidal gold labelled antibody reacts with antigen via an immunochromatographic procedure to obtain the detection result. Colloidal gold immunochromatographic assay are rapid, taking approximately 15 min to flow, also convenient, only need small volume (<20µl) of the tested

Sample<sup>[11, 12]</sup>. These characteristics are suited for on-site testing by non-professional workers. Moreover, colloidal gold immunochromatographic assays still have high sensitivity and selectivity. In this study, a colloidal gold immunochromatographic test strip was firstly developed to detect CPIV5.

## Materials and methods

### 1. Preparation of chemical and supply

Complete Freund's adjuvant, incomplete Freund's adjuvant, HAuCl<sub>4</sub> · 3H<sub>2</sub>O, trisodium citrate, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The sample pad, conjugated pad, nitrocellulose membrane, and absorption pad were obtained from Shanghai Liangxin Technology Co., Ltd. (Shanghai, china). A XYZ Platform combing motion control with BioJet Quanti3000k dispenser and AirJet Quanti3000k dispenser for solution dispensing were purchased from BioDot (Irvine, CA, USA).

### 2. Preparation of animals and biological material

BALB/c mice were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd.. Thirty nine nasopharyngeal swabs were collected from experimental dogs infected by CPIV5 in our laboratory. Monoclonal antibody (MAb) 4H1 and MAb 2B7 were

prepared in our laboratory as previously described<sup>[13]</sup>. Goat anti-mouse IgG was purchased from Beijing Wanyumeilan Technology Co., Ltd. (Beijing, china). Canine parainfluenza virus type 5 strain S0427 and canine influenza virus (CIV) strain GD03 were isolated, identified and stored in our laboratory. Canine adenovirus type 1 (CAV-1) strain Utrecht and canine adenovirus type 2 (CAV-2) strain Toronto A26/61 were purchased from ATCC. Canine distemper virus (CDV) strain Snyder Hill, and canine parvovirus (CPV) strain C154 were derived from vaccine virus.

### 3. Preparation of MAbs against CPIV5

CPIV5 MAbs were produced as described previously with some modification<sup>[13]</sup>. Briefly, a group of six-week-old female BALB/c mice (n=6) were injected with 100 µl CPIV5 strain S0427 emulsified in 100 µl complete Freund's adjuvant via hypodermic injection at four different sites on the back. The mice were immunized subcutaneously by using 100µl CPIV5 strain S0427 emulsified in incomplete Freund's adjuvant in a 1:1 ration at two times every two weeks.

After multiple immunizations, the serum titer of mice was detected using CPIV5 strain S0427 by indirect immune fluorescence (IFA) assay, as described previously<sup>[14]</sup>. The 100µl CPIV5 strain S0427 without adjuvant was injected subcutaneously into mice for the final boost. The spleen cells of immunized mice with the highest mouse serum titer were collected

and fused with SP2/0 cells to obtain hybridoma cells. Positive hybridoma cells 4H1 and 2B7 were acquired by subcloning three times from a single cell.

The acquired hybridoma cells 4H1 and 2B7 were injected into liquid paraffin-pretreated BALB/c mice. Ascites 4H1 and 2B7 were harvested after one week. The ascites 4H1 and 2B7 were purified by affinity binding with protein G resin to acquire MAbs<sup>[13]</sup>. MAbs 4H1 and 2B7 titer were evaluated using CPIV5 strain S0427 by IFA assay. The isotypes were tested by the Pierce Rapid ELISA Mouse MAb Isotyping Kit (Thermo Scientific) according to the manufacturer's instructions.

### 4. Development of Colloidal Gold Strip for Detection of CPIV5

#### 4.1 Preparation of colloidal gold

Colloidal gold solution was prepared as described previously with some modification<sup>[12]</sup>. Briefly, 100 mL of 0.01% (w/v) gold chloride trihydrate solution in ultrapure water was heated to boiling, and 1.8 mL of 1% (w/v) trisodium citrate was added to the solution under constant stirring. After 15min, the color of solution were stable and the colloidal gold solution was obtained. The colloidal gold was cooling and stored at 4°C until further use.

#### 4.2 Preparation of colloidal gold labeled MAbs

The 10ml colloidal gold solution was prepared and the pH of colloidal gold solution were adjusted to 8.2 with 0.1 M  $K_2CO_3$ . With 350rpm stirring, 0.3 mg anti-CPIV5 MAb 4H1 solution was added drop-wise to label the colloidal gold solution. After 30min, the colloidal gold-MAb conjugates were then blocked with 1 mL blocking solution 10% (w/v) BSA solution. The acquired mixture was then centrifuged at 12000 rpm for 30 min to wash unconjugated MAb 4H1 and BSA. The colloidal gold-MAb was resuspended with dilution buffer to a volume of 1 mL and stored at 4 °C for further use.

#### 4.3 Test strips preparation

The NC membrane was made by spotting the capture MAb 2B7 (2.0 mg/mL) and goat anti-mouse IgG (2.0 mg/mL) onto the test line and control line at the dispensed volume of 1.0  $\mu$ l/cm. The NC membrane was then dried for 60 min at 20-25 °C and the relative humidity below 20%. The colloidal gold-MAb solution obtained was spotted onto conjugated pad. The conjugated pad was then dried in a vacuum oven at 37 °C for 60 min.

The sample pad, conjugated pad, NC membrane, and absorption pad were assembled sequentially on the PVC backing pad, and then cut into 4 mm wide and 60 mm long. All strips were stored in a desiccator until use.

#### 4.4 Optimization of test strip parameters

The concentration of labeled MAb/colloidal gold (i.e., 20, 30, 40 $\mu$ g/ml), the spotted volume of colloidal gold probe per cm conjugated pad (i.e., 1.5, 2.0, 2.5  $\mu$ l), and concentration of test line MAb (i.e., 0.5, 1.0, 2.0mg/ml) was optimized in the test strip. All experiments were performed in triplicate.

#### 5. Validation of the test strip

##### 5.1 Sensitivity of the test strip

These different concentrations of CPIV5 samples were prepared by diluting CPIV5 at concentrations of  $10^{5.8}$  TCID<sub>50</sub>/mL at a percentage of 1:4, 1:20, 1:100, 1:500. Exactly 100 $\mu$ l of these test samples were added into sample well of the test strip. After 10min, the detection result was been observed, and the sensitivity of the test strip was evaluated according to the detection result. All experiments were performed in triplicate.

##### 5.2 Specificity of the test strip

The specific sample were prepared including CPIV5 strain S0427, canine distemper virus strain Snyder Hill, canine parvovirus strain C154, canine adenovirus type 1 strain Utrecht, canine adenovirus type 2 strain Toronto A26/61, and canine influenza virus strain GD03.

All of these samples were mixed with the sample solution in a 1:1 dilution respectively. The prepared samples and 0.01 M PBS (negative control) were added into sample well of the test strip.

After 10min, the specificity of the test strip was evaluated according to the detection result.

### 5.3 Detection of clinical samples

Nasopharyngeal swabs samples were produced from experimental dogs infected by CPIV5, and 39 nasopharyngeal were collected for the experiment. The collected nasopharyngeal swabs were immediately immersed in sample collection tubes. The processed samples were respectively tested by the prepared test strips and the corresponding commercial RT-PCR Kit from Beijing ANHEAL Laboratories Co., Ltd. to evaluate the applicability of test strip.

## Results

### 1. Determination of test results

In the CPIV5 colloidal gold immuno-chromatographic strip, colloidal gold was labeled by detection MAb 4H1. The test line of strip was coated by the capture MAb 2B7, and the control line of strip was coated by goat anti-mouse IgG. The sample was added into the sample well, and the solution was migrated towards the absorbent pad by capillary action. If the concentration of CPIV5 antigen in the sample was at the limit of detection or higher, the colloidal gold-MAb 4H1 conjugate would react with CPIV5 antigen. Colloidal gold-MAb 4H1-CPIV5 conjugate migrated to the test line. The conjugate would react with the capture MAb 2B7 on test line to form

colloidal gold-MAb 4H-CPIV5-MAb 2B7 conjugate, and a red line will be appeared on the test line which indicates a positive result.

The colloidal gold-MAb 4H1 conjugate must bind to goat anti-mouse IgG on control line to form a red line as a procedural control. No red band was appeared on control line indicated that the result was invalid, and the result should be retested using a new strip.

### 2. Production of MAbs against CPIV5

The serum titer of third immunized mice to CPIV5 was detected by IFA assay. The anti-CPIV5 serum titer of third immunized mice was 1:1600-1:6400 by IFA tests. The spleen cells of mice with highest antibody titer were collected and fused with SP2/0 cells to obtain the hybridoma cells. The obtained hybridoma cells were subcloned three times to obtain positive hybridoma cells. Two strains positive hybridoma cells were used to immunize mice to produce MAbs 4H1 and 2B7. The combination status and titers of MAbs 4H1 and 2B7 against CPIV5 were evaluated by IFA assay.

As showed in Figure 1, MAbs 4H1 and 2B7 both reacted with CPIV5 antigens fixed in the well of 96-well cell plates to produce strong fluorescent signals, which indicated the specific binding of 4H1 and 2B7 MAbs with CPIV5 antigen. MAbs 4H1 and 2B7 titer were both no less than 1:3200 by IFA assay detection.

The isotypes of MAb 4H1 and 2B7 were tested to be IgG1/Kappa and IgG1/Kappa.

### 3. Optimization of test strip parameters

The optimal parameters of test strip were obtained by an orthogonal L9 (3)3 test design. The concentration of labeled MAb/colloidal gold, the spotted volume of colloidal gold probe per cm conjugated pad, and concentration of test line MAb were considered as the most important factors that affected the cut off limit of the strip and the color intensity of the test line<sup>[15, 16]</sup>. The cut off limit was defined as the minimum analyse concentration required for showing apparent color on test line to the naked eye<sup>[17]</sup>.

As shown in Table1, the concentration of labeled MAb/colloidal gold was a key factor to influence the sensitivity of test strip. When the concentration of labeled MAb/colloidal gold were 20 µg/ml, colloidal gold-MAb 4H1-CPIV5 could not be captured by MAb 2B7 effectively. Therefore, the color intensity on test line was weak and the sensitivity of test strip was low despite of the concentration of CPIV5 sample was at 10<sup>5.2</sup> TCID<sub>50</sub>/mL. When the concentration of labeled MAb /colloidal gold were 30µg/ml or 40 µg/ml, colloidal gold-MAb 4H1-CPIV5 could be captured by MAb 2B7 effectively.

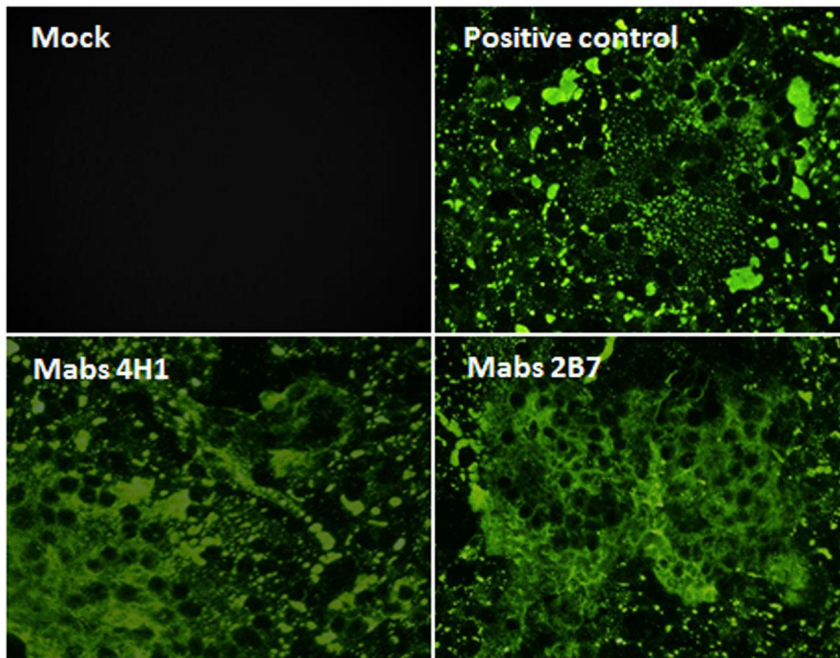


Figure 1: Results of Mabs 4H1 and Mabs 2B7 analyzed by IFA.

Positive control was the serum of immunized mice.

Mock was Vero cells. Magnification × 200

Table 1. Optimization of test strip parameter

No.	Concentration ( $\mu\text{g/ml}$ ) of labeled MAB/colloidal gold	The spotted volume ( $\mu\text{l}$ ) of colloidal gold probe per cm conjugated pad	Concentration ( $\text{mg/ml}$ ) of test line MAB	Color intensity of test line	Cut off limit of the strip ( $\text{TCID}_{50}/\text{mL}$ )
1	20	1.5	2	weak	$10^{4.6}$
2	20	2	1	weak	$10^{4.9}$
3	20	2.5	0.5	weak	$10^{4.9}$
4	30	1.5	1	weak	$10^{4.2}$
5	30	2	0.5	weak	$10^{4.5}$
6	30	2.5	2	medium	$10^{3.8}$
7	40	1.5	0.5	weak	$10^{4.0}$
8	40	2	2	strong	$10^{4.5}$
9	40	2.5	1	medium	$10^{4.2}$

The spotted volume of colloidal gold probe per cm conjugated pad, and concentration of test line MAb were two key parameters to influence the color intensity on test line and the sensitivity of test strip. The orthogonal test results indicated that the optimal conditions of test strip were that 30  $\mu\text{g/ml}$  the concentration of labeled MAb/colloidal gold, 2.5  $\mu\text{l}$  the spotted volume of colloidal gold probe per cm conjugated pad, and 2  $\text{mg/ml}$  concentration of test line MAb. At the optimal conditions, the cut off limit of the strip was lowest, and color intensity of test line was modest for naked eyes observation.

#### 4. Validation results of the test strip

##### 4.1 Sensitivity analysis of the test strip

CPIV5 solution at the concentration of  $10^{5.8}$   $\text{TCID}_{50}/\text{ml}$  was prepared. The prepared CPIV5 solution were respectively diluted at 1:4, 1:20, 1:100, 1:500 times to obtain different concentrations of test samples. As shown in Figure 2, These different concentrations of CPIV5 solution were tested by the strips. After 10 min, the detection results were observed by naked eyes. It was observed that red lines was respectively appeared on the test line and the color intensity changed from weak to strong as the concentration of

CPIV5 solution increased from  $10^{3.8}$  TCID<sub>50</sub>/ml (100 times dilution) to  $10^{5.2}$  TCID<sub>50</sub>/ml (4 times dilution). When the concentration of CPIV5 solution was  $10^{3.1}$  TCID<sub>50</sub>/ml (500 times dilution), no a red line was observed on the test line by the naked eyes.

The detection limit of strip was defined as the minimum analyze concentration required for showing no obvious visual color on the test line<sup>[17]</sup>. According to above results, the detection limit of CPIV5 test strip were  $10^{3.8}$  TCID<sub>50</sub>/ml.

#### 4.2 Cross-reactivity analysis of the test strip

Some common viruses from dogs were tested to evaluate the specificity of test strip.

As shown in Figure 3, canine parainfluenza virus type 5 (CPIV5) strain S0427 were detected for positive in test strip only, whereas canine distemper virus (CDV) strain Snyder Hill, canine parvovirus (CPV) strain C154, canine adenovirus type 1 (CAV-1) strain Utrecht, canine adenovirus type 2 (CAV-2) strain Toronto A26/61, and CIV strain GD03 were detected for negative in test strip. Therefore, the test strip had high specificity with CPIV5 and had no cross-reactivity with the above viruses from dogs.



Figure 2. The sensitivity results of test strip.

CPIV5 at concentrations of  $10^{5.8}$  TCID<sub>50</sub>/mL were diluted in 1:4, 1:20, 1:100, 1:500 with 0.01 M PBS from No. 1–4, respectively.



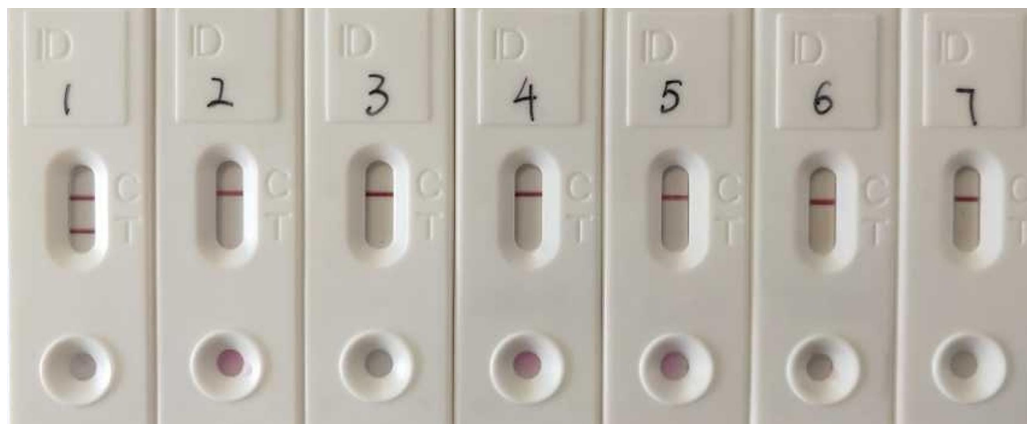


Figure 3. The specificity results of test strip.

No. 1–7 were canine parainfluenza virus type 5 (CPIV5) strain S0427, canine distemper virus (CDV) strain Snyder Hill, canine parvovirus (CPV) strain C154, canine adenovirus type 1 (CAV-1) strain Utrecht, canine adenovirus type 2 (CAV-2) strain Toronto A26/61, and canine influenza virus (CIV) strain GD03, and PBS, respectively.

#### 4.3 Application to clinical samples

Total 39 nasopharyngeal swabs were collected from experimental dogs infected by CPIV5 to apply on test strip. The detection results were shown in Table 2. Sixteen nasopharyngeal swabs of dogs were tested positive by RT-PCR, yet only 11 samples were positive tested by strip. The results of test strip and RT-PCR were consistent in other 23 negative samples.

The total coincidence rate of the strip and RT-PCR was 87.18% (34/39), and the specific coincidence rate of the strip and RT-PCR was 100% (23/23).

#### Discussion

Serologic studies have shown that a variable but high number of dogs have been exposed to CPIV. CPIV is associated with a

highly communicable respiratory disease in the dog<sup>[18]</sup>. Several methods have been developed for the diagnosis of CPIV infection, including virus isolation and identification assay, polymerase chain reaction, immunohistochemistry, and immunofluorescence. Although all of these methods have high sensitivity and selectivity, these methods require expensive equipment, long processing times, and professional technics and operational skills. These characteristic limit the above methods to screen the samples quickly. Colloidal gold immuno-chromatographic assays have the advantage of convenient use and simple operation, short time detection and high test efficiency, which is suit for the rapid and on site screening of samples.

Table 2. The clinical results of CPIV5 nasopharyngeal swabs samples

Result by RT-PCR	CPIV nasopharyngeal swabs samples		
	Positive	Negative	Total
Result by Strip			
Positive	11	0	11
Negative	5	23	28
Total	16	23	39

In this study, MAbs 4H1 and 2B7 were respectively used as detection antibody and capture antibody, because MAbs 4H1 and 2B7 was with high titer and specific to CPIV5 detection, so MAbs 4H1 and 2B7 were crucial factors to the sensitivity and specificity of test strip. By the parameters of colloidal gold immunochromatographic strip, we found the concentration of labeled MAb/colloidal gold had a remarkable impact on to the sensitivity of test strip compared with the other parameters. Because when the concentration of labeled MAb/colloidal gold was low, weather how the other parameter was changed, the sensitivity of test strip was low. In order to evaluate the clinical effect of test strip, 39 nasopharyngeal swabs collected from experimental dogs infected by CPIV5 were

respectively detected by test strip and commercial RT-PCR kit. The results showed that the coincidence rate of the strip and RT-PCR PCR was high, but there were 5 nasopharyngeal swabs, the result of commercial RT-PCR kit was positive, however, the result of colloidal gold immunochromatographic strip was negative. These results were in accordance with previous studies in which stated PCR was more sensitive than the strip test<sup>[19]</sup>. By the detection result, it was obtained the strip had good application effect to nasopharyngeal swabs clinical sample.

A colloidal gold immunochromatographic assays with high sensitivity and good application effect was firstly developed for CPIV5 detection. The

colloidal gold immunochromatographic assays would offer a rapid and effective assay for on site screening of CPIV5.

### Authors contributions

Kegong Tian and Junhua Deng conceived the experiments.

Jingyun Wang, Liyin Hao, Fan Li, Chenyu Bai, Dingding Zheng, Yujiao Cao, Xiangdong Li, Yuxiu Liu performed the experiment.

Jingyun Wang and Xiangdong Li wrote the manuscript.

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