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Development of Immunochromatography Strips for Detection of Canine Adenovirus Type 1 and Type 2

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ABSTRACT

Canine Adenovirus Type 1 (CAV-1) and type 2 (CAV-2) are prevalent in dogs. To develop Immunochromatographic (IC) test strips for onsite rapid detection of CAV-1 and CAV-2 simultaneously or separately, mice were injected with inactivated CAV-1 or CAV-2. The spleen cells of the immunized mice were fused with SP2/0 cells and the supernatant of hybridoma cells were screened by Hemagglutination Inhibition (HI) test. Six monoclonal antibodies (mAbs), named 1A1 and 5G4 (anti-CAV-1&CAV-2), 4F3 and 2F9 (anti-CAV-1),1C5 and 4H12 (anti-CAV-2), were chosen for the development of three types of IC test strips including CAV-1&CAV-2 strip, CAV-1 strip and CAV-2 strip. It showed that the limit of detection (LOD) of CAV-1 strip was 104.9TCID50/ml; the CAV-2 strip was 103.2TCID50/ml. The LOD of CAV-1 strip and CAV-2 strip was both similar to that of CAV-1&CAV-2 strip. There are no cross-reactivity of three kinds of strips with CDV, CPV, CPIV and CIV. Eighty seven samples were tested both by PCR and IC. Compared with PCR, the sensitivity of three types of strips was 83% (CAV-1&CAV-2 IC strip), 82% (CAV-1 IC strip) and 85% (CAV-2 IC strip), the specificity of all the three strips were 100%.The corresponding coincidence rates were 91%, 90% and 93% with PCR, respectively. The above results showed that the developed strips in this study had high sensitivity, specificity and reproducibility, and offered a rapid and convenient method for onsite detection by owners and veterinarians within 10 minutes.

INTRODUCTION

Canine Adenovirus (CAV) belongs to the genus Mastadenovirus, family Adenoviridae. At present, two types of canine adenovirus, CAV-1 and CAV-2 have been found in dogs [1,2]. CAV-1 replicated in vascular endothelial cells and causes a generalized infectious characterized by hepatitis, spans from lethargy, weight loss and inappetence to severe enteritis, hepatitis, even sudden death [3]. CAV-2 replicates mainly in the respiratory epithelium cells, therefore inducing mild and self-limiting upper respiratory disease [1,4]. At present, the diagnosis and identification of CAV are based on virus isolation, electron-microscopic observation, serological tests and Polymerase Chain Reaction (PCR) [5-11]. However, these techniques are usually laborious and time-consuming. Establishing simple, rapid, sensitive and reliable

diagnostic tests, which would help the owners or veterinarians to diagnose and prevent CAV-1 or CAV-2. The objective of the present study is to establish one-step colloidal gold-based Immunochromatographic (IC) strips for CAV-1 and CAV-2 diagnosis simultaneously or separately.

MATERIALS AND METHODS

Materials

Complete and incomplete Freund's adjuvant, Bovine Serum Albumin (BSA), HAuCl4·3H2O, and trisodium citrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). BALB/c mice were from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). The sample pad, conjugated pad, nitrocellulose membrane and absorption pad were obtained from Shanghai Liangxin Technology Co., Ltd. (China). Goat anti-mouse IgG was bought from Beijing Wanyumeilan Technology Co., Ltd. (China). A BioDot XYZ Platform combing motion control with BioJet Quanti3000k dispenser and AirJet Quanti3000k dispenser for solution dispensing were purchased from BioDot (Irvine, CA, USA). Pierce Rapid ELISA Mouse mAb Isotyping Kit was purchased from Thermo Scientific. CAV-1 strain Utrecht and CAV-2 strain Toronto A26/61 were purchased from ATCC. CAV-1 strain HN03, CAV-2 strain HN04, Canine Distemper Virus (CDV) strain HL001 [12], Canine Parvovirus (CPV) strain S2 [13], Canine Parainfluenza Virus (CPIV) strain HeN0718 [14], and Canine Influenza Virus (CIV) strain GD03 were isolated, identified and stored in our laboratory.

Preparation of MAbs against CAV-1 and CAV-2

MAbs 1A1 and 5G4 (simultaneously against CAV-1 and CAV-2), mAbs 4F3 and 2F9 (only against CAV-1), and 1C5 and 4H12 (only against CAV-2) were produced as described previously with some modification [15]. Briefly, 3 groups of six-week-old female BALB/c mice (n=4 in each group) were used for immunization. The immunogen of group 1 and 2 was CAV-1 strain HN03, and that of group 3 was CAV-2 strain HN04. The virus was emulsified with an equal volume of complete Freund's adjuvant. The mice were injected with the immunogen (200μl/per mouse) via hypodermic injection at different sites on the back. Four booster immunizations with the same antigen emulsified with incomplete Freund's adjuvant were conducted at 2 weeks intervals. The Haemagglutination Inhibition (HI) titer against CAV in the mice serum were detected using CAV-1 strain Utrecht and CAV-2 strain Toronto A26/61, respectively14. After two weeks, the mouse in each group with the highest titer was injected with the equivalent antigen without adjuvant through intraperitoneal injection for the boost, and then their splenic cells were separately fused with SP2/0 myeloma cells with conventional hybridoma techniques. By limiting dilution, hybridomas that produced specific mAbs were selected and subcloned three times from single cells in 96-well culture plates. Murine ascites fluid was generated in paraffin-pretreated BALB/c mice, and IgG was purified by affinity binding with protein G resin. HI test was used to detect the titer of mAbs with human O-type red blood cell. Immunoperoxidase Monolayer Assay (IPMA) was performed to confirm the specificity of six mAbs with CAV-1 or/and CAV-2 as described previously with some modifications [16]. The isotyping of mAbs were identified by using Pierce Rapid ELISA Mouse MAb Isotyping Kit (Thermo Scientific) according to the manufacturer's instructions.

Virus proteins recognized by each MAbs

Construction of recombinant plasmid and transient transfection: According to the manufacturer's directions, total DNA were extracted from CAV-1 and CAV-2 using Viral Nucleic Acid Extraction Kit II (Geneaid, Taiwan, China). The complete Fiber and Penton protein coding region of CAV-1 and CAV-2 were amplified by PCR, and primer pairs with restriction sites were shown in Table 1. The amplified gene fragments of CAV-1 and CAV-2 were respectively digested with XhoI and EcoRI, and then inserted into pCAGGS mammalian expression vector.

Vero cells were seeded into 12-well plates for 24 hours, then transfected with the recombinant plasmid using Lipofection 2000 (Invitrogen) according to the manufacturer's introductions. After 48 hours, Vero cells were washed once in PBS and fixed with acetone for 30 min at 4°C. The results were observed by Immunofluorescence Assay (IFA) as described below.

IFA test: The fixed cells were incubated with 6MAbs (1A1, 5G4, 4F3, 2F9, 1C5 and 4H12) at 37oC for 1 hour. After washing three times with PBS, the cells were incubated with FITC-conjugated rabbit anti-mouse IgG (Sigma) for 1 hour at 37oC. After washed three times with PBS, cell staining was examined with a fluorescent microscope (Olympus).

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Table 1: Primers used for PCR amplification

Name	Sequence(5`-3`)
CAV-1-Fiber-*Eco*RI-F	5'-CCGGAATTCATGAAGCGGACACGAAGTGCT-3'
CAV-1-Fiber-*Xho*I-R	5'-CCGCTCGAGTCATTGATTTTCCCCCACAT-3'
CAV-1-Penton-*Eco*RI-F	5'-CCGGAATTCATGGAGTTTCCGTCGTCT-3'
CAV-1-Penton-*Xho*I-R	5'-CCGCTCGAGCTAGAATGTTTTACTGGACAGC-3'
CAV-2-Fiber-*Eco*RI-F	5'-CCGGAATTCATGAAGCGGACACGAAGA-3'
CAV-2-Fiber-*Xho*I-R	5'-CCGCTCGAGTTATTGATTTTCGCCTACAT-3'
CAV-2-Penton-*Eco*RI-F	5'-CCGGAATTCATGGAGTTTTCGTCGTCTC-3'
CAV-2-Penton-*Xho*I-R	5'-CCGCTCGAGCTAGAAGGTTTTACTGGACAG-3'

Note: The underlined letters are corresponding restriction enzyme sites.

Development of IC test strips for detecting CAV-1 and CAV-2

Conjugation of MAbs with colloidal gold: Colloidal gold solution was synthesized as previously mentioned with minor modifications [17].The optimal pH value and concentration of the antibody (5G4, 4H12, 2F9) for preparing the colloidal gold-mAb conjugation were determined separately. Briefly, a serial of colloidal gold with pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 were prepared, and then 0.025mg/ml mAb were added and mixed thoroughly. After 15 minutes, 10% NaCl was added, followed by stirring for 30 minutes. The mixture was centrifuged at 2000rpm for 10 minutes, the supernatant was collected and analyzed on an ultraviolet spectrophotometer. The sample exhibiting the highest absorbance was considered the optimal reaction. Similarly, the optimal concentration of antibody was determined by varying the amounts of mAb in the reaction system. The 1.2-fold minimum protein concentration was chosen for labeling. The optimal pH value of the colloidal gold solution was pH7.5 (mAb 5G4 and 4H12), pH8.0 (mAb 2F9). The optimal concentration of the mAb was 0.024mg/ml (mAb 5G4), 0.030mg/ml (mAb 4H12) and 0.030mg/ml (mAb 2F9).

The colloidal gold-mAb 5G4, 4H12 and 2F9 was prepared under the optimal reaction condition, respectively. Each gold-labeled IgG was incubated at room temperature for 30 minutes, and then the non-specific binding sites were blocked with 1% BSA solution for 30 minutes. The mixtures was centrifuged at 12000 rpm for 20 minutes at 4°C, and the sediment was resuspended in 1ml dilution buffer, and store at 4°C until use.

Preparationof IC test strips: Each gold-labeled conjugation was sprayed onto glass fiber membranes as conjugated pad, and dried at 37°C for 2 hours. The capture mAb (anti-CAV-1&CAV-2 mAb 1A1, anti-CAV-1 mAb 4F3 or anti-CAV-2 mAb 1C5) and goat anti-mouse IgG were placed onto the test line (T line) and control line(C line) on the nitrocellulose membrane, respectively. There was 5 mm distance between the T line and C line. The nitrocellulose membrane was then dried for 2 hours at room temperature. The sample pad, gold conjugate pad, nitrocellulose membrane and absorbent pad were assembled on a PVC backing pad in the suitable order [18]. The test strip was sheared into 4 mm wide using a cutting machine and stored in a desiccator until use. The rapid IC test strip was the final assembled product with the plastic cover. Three kinds of IC test strips, namely, CAV-1&CAV-2 IC test strip, CAV-1 IC test strip and CAV-2 IC test strip were prepared.

Based on sandwich principle, when test sample dissolved by the sample solution is applied to sample pad, it migrates by capillary action through gold conjugate pad, mobilizes gold-labeled conjugation coated on the gold conjugate pad and reacts with the conjugation for forming early complex. The

early complex continues to move along the nitrocellulose membrane, and interacts with the capture mAb immobilized onto the T line area. If the CAV content of the measured sample is present at levels of the detection limit or above, the antigen-antibody complex will be formed and then appeared a colored band in the T line region (judged by naked eye within 10 min as positive). If not, the T area will remain colorless. The immune complex subsequently moves to the C line region and forms a pink or purple color, showing the detection is working and the result is valid, otherwise it is invalid.

Characteristics and application of IC test strips

Sensitivity of the IC test strip: At first, CAV-1 strain Utrecht (107.2TCID50/ml) and CAV-2 strain Toronto A26/61 (105.5TCID50/ml) were gradually diluted by 10, 50, 100, 200, 400 and 800 folds with sample dilution solution. All of dilutions of virus were added to sample pad, each with 100μl, and detected triplicate by CAV-1, CAV-2, and CAV-1&CAV-2 strips, respectively.

Specificity of the test strip: Apart from sample solution (negative control), CDV, CPV, CPIV and CIV were tested to estimate the specificity of strips in triplicate.

Application of the test strip in detecting clinical samples: A total of 87 clinical samples, consisting of 60 eye and nose swabs, 12 anal swabs and 15 faeces from dogs were collected from pet hospitals and freshly dissolved in sample solution. 25 eye and nose swabs, 12 anal swabs and 15 faeces were CAV-1, and 35 eye and nose swabs were CAV-2. All of the samples were identified by PCR to amplify the CAV E3 genes (508bp for CAV-1; 1030bp for CAV-2). The PCR was performed according the reported method using the forward primer: CGCGCTGAACATTACTACCTTGTC; the reverse primer: CCTAGAGCACTTCGTGTCCGCTT [7].

RESULTS

Production of six mAbs against CAV-1/CAV-2

In order to obtain mAbs against CAV-1/CAV-2, HI test was used for evaluating the antibody titers in the sera of all the immunized mice after multiple immunizations. The highest HI titers of the immunized mice in each group could reach to 1:1280. As described above, the hybridoma cells were produced and screened by HI, 6 positive mAbs including 1A1 and 5G4 (anti-CAV-1&CAV-2), 4F3 and 2F9 (anti-CAV-1), 1C5 and 4H12 (anti-CAV-2) were chosen and the ascites were prepared for further study. The isotyping of six mAbs were listed in Table 2.

After purification, the specificity of mAbs were evaluated by HI and IPMA. As shown in Table 2, the 1A1 and 5G4 could react with both CAV-1 and CAV-2 and the HI titer could reach to 1:5120-1:40960. The 4F3 and 2F9 could react with only CAV-1 with HI titer 1:5120-1:20480. The 1C5 and 4H12 could react only CAV-2 with HI titer1:10240-1:20480.

Table 1: Primers used for PCR amplification

Antigen	mAb	Isotyping (Heavy/light chain)	HI titer	
			CAV-1	CAV-2
CAV-1&CAV-2	1A1		1:10240	1:40960
CAV-1&CAV-2	5G4	IgG1/Kappa	1:5120	1:40960
CAV-1	4F3	IgG2a/Kappa	1: 5120	<1:10, negative
CAV-1	2F9	IgG1/Kappa	1: 20480	<1:10, negative
CAV-2	1C5	IgG1/Kappa	<1:10, negative	1:10240
CAV-2	4H12	IgG1/Kappa	<1:10, negative	1:20480

Reactivity of six mAbs

Vero cells transfected with the recombinant plasmids pCAGGS-Fiber/Penton from CAV-1 and CAV-2 were applied to analyze the six mAbs reactivity by IPMA and IFA. As shown in (Figure 1&2), all the six mAbs showed good reactivity with recombinant Fiber protein, but not with the recombinant Penton protein (data not shown). The 1A1 and 5G4 mAbs reacted with recombinant Fiber protein of CAV-1 and CAV-2. The 4F3 and 2F9 mAbs only interacted with recombinant CAV-1 Fiber

protein. The 1C5 and 4H12 mAbs just reacted with recombinant CAV-2 Fiber protein. Nevertheless, no positive signals were found in the Vero cells transfected with the empty vector pCAGGS and normal Vero cells.

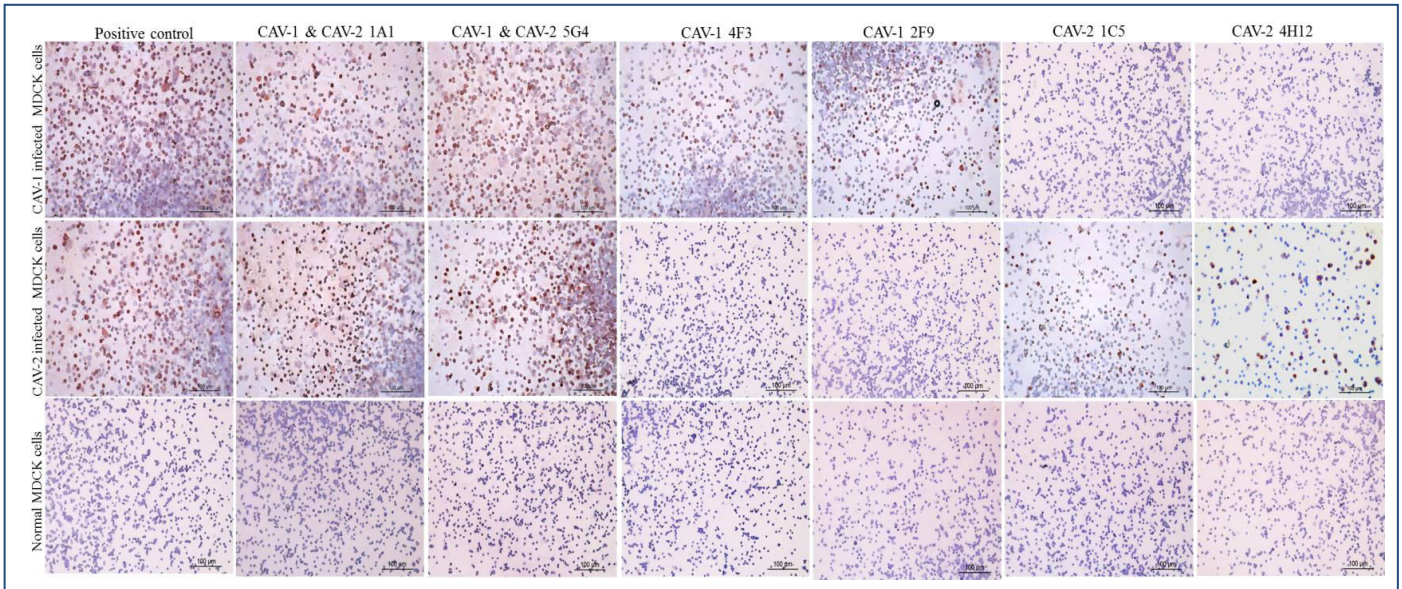


Figure 1: Specificity results of six mAbs1A1, 5G4, 4F3, 2F9, 1C5 and 4H12 analyzed by IPMA. Positive control was the serum of immunized mice.

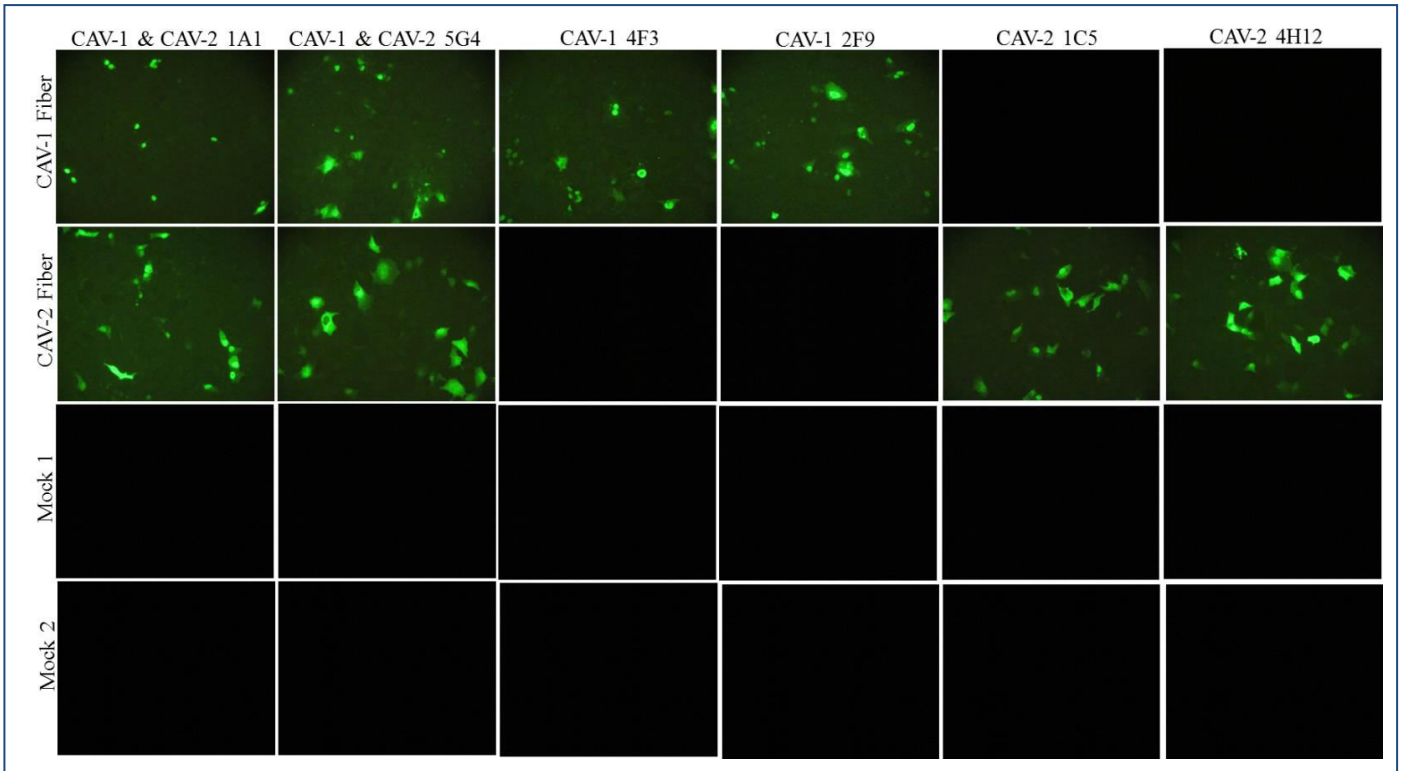


Figure 2: Reactivity of six MAbs with the Vero cells transfected with the fiber and penton protein of CAV-1 and CAV-2 by IFA. Mock 1, Vero cells transfected with the empty vector pCAGGS; Mock 2, Normal Vero cells.

Characteristics and application of IC test strip

Sensitivity analysisin three types of IC test strips: In this study, the detection limit (LOD) of strips were defined as the minimum concentration required for revealing no obvious visual color on the T line [19]. According to above-mentioned methods, all the CAV-1 related solutions, including 1 (undiluted), 10, 50, 100, 200, 400 and 800 ratios, were separately measured by CAV-1 & CAV-2 IC test strips and CAV-1 IC test strips. The test results displayed both of them could be measured at the concentration of 400 ratio (weakest positive, corresponding concentration: 104.9TCID50/ml), as shown in (Figure 3). By contrast, all of CAV-2 related diluents, also covering 1 (undiluted), 10, 50, 100, 200, 400 and 800 ratios, were respectively tested by CAV-1 & CAV-2 IC test strips and CAV-2 IC test strips, and the consequence was the same as the dilution ratio (400-fold, weakest positive, corresponding concentration: 103.2TCID50/ml). In particular, the color intensity on the T line decreased gradually as the concentration of CAV-1 or CAV-2 reduced. But for the sake of observation, we defined the visual LOD of CAV-1&CAV-2 IC test strip as 104.9TCID50/ml (CAV-1, 200-fold) and 103.2TCID50/ml (CAV-2, 200-fold). The experimental results demonstrated that every strip had a good consistency and repeatability.

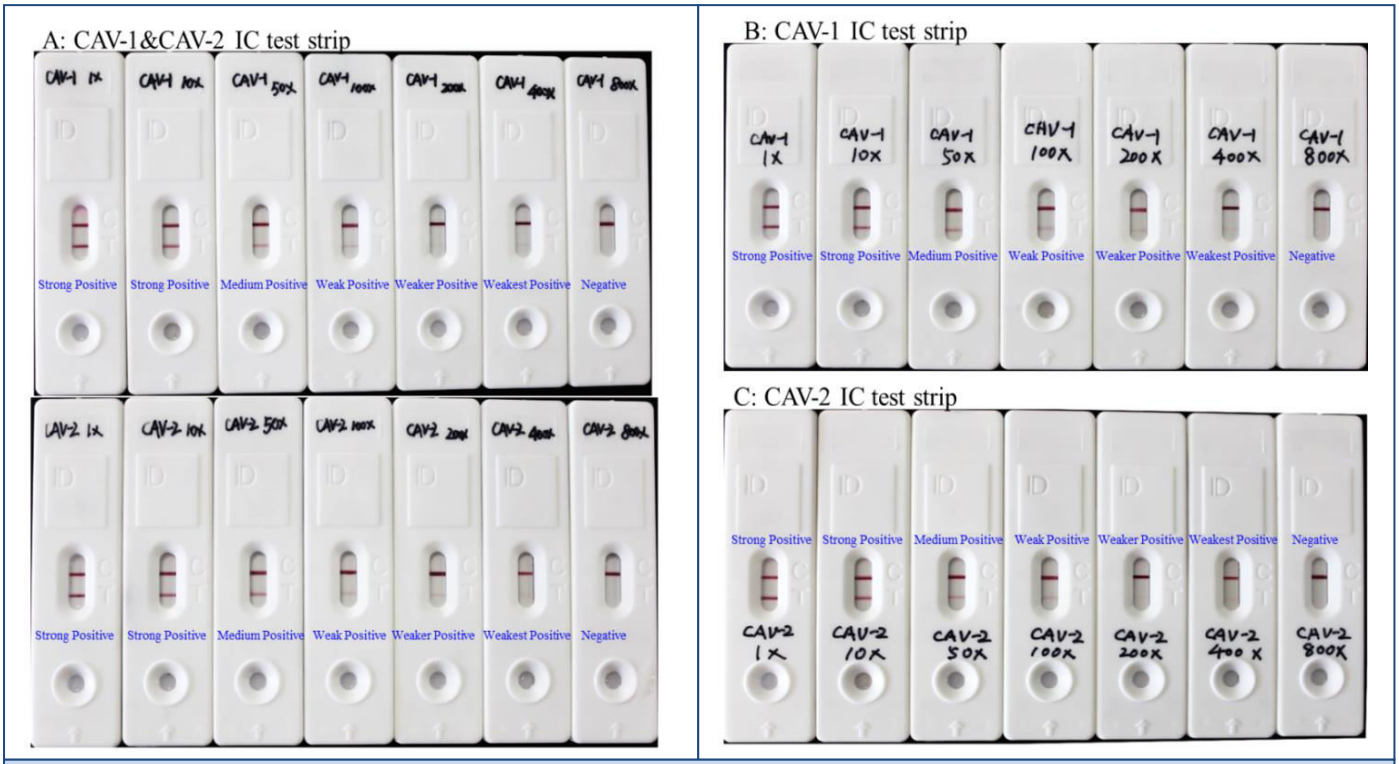


Figure 3: The sensitivity results of three types of IC test strip. Positive (including strong Positive, Medium Positive, Weak Positive, Weaker Positive, Weakest Positive) and Negative: judgment results depending on the color intensity in T line.1× (undiluted), 10×, 50×, 100×, 200×, 400×and 800×: representCAV-1 or CAV-11 (undiluted), 10, 50, 100, 200, 400 and 800 ratio diluting solutions.

Cross-reactivity analysis in three kinds of IC test strips: The sample lysate solutions and some common viruses from dogs, containing CDV, CPV, CPIV and CIV, were used in assessing the specificity of IC test strips. CAV-1 or CAV-2 was included when required. As depicted in (Figure 4), the IC test strips showed no cross-reactions with CDV, CPV, CPIV and CIV. Especially, no cross-reaction were observed in CAV-1 IC test strips with CAV-2, and CAV-2 IC test strips with CAV-1.

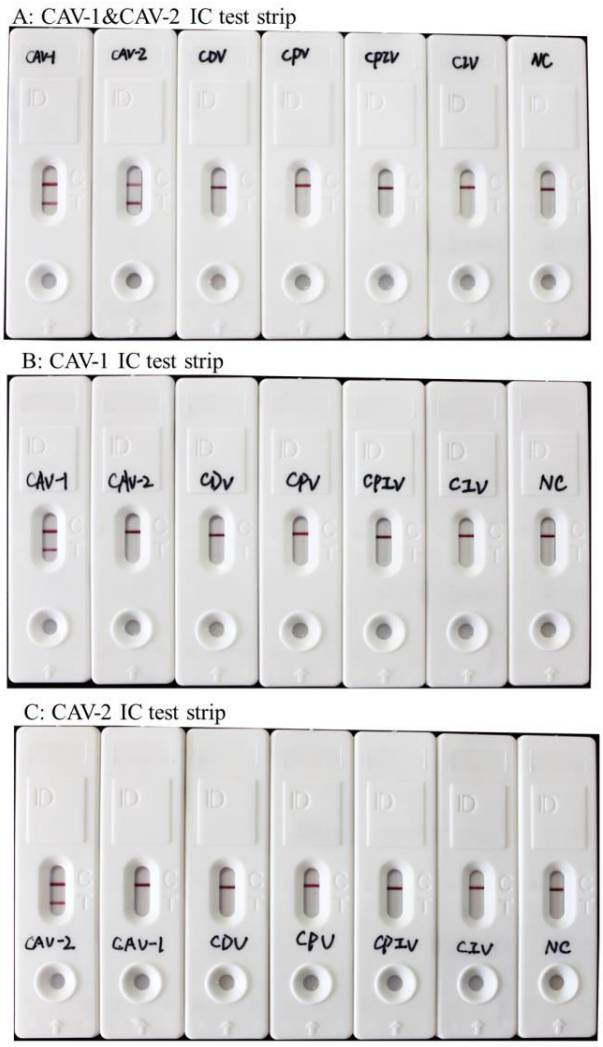


Figure 4: The specificity results of three kinds of IC test strip.

Clinical application of three types of IC test strips: 87 clinical samples were detected by the three types of IC strips, and the results are presented in Table 3. In details, CAV-1 & CAV-2 IC test strips could obtain 40 positive and 47 negative samples. CAV-1 IC test strips could detect 23 positive and 29 negative samples, and CAV-2 IC test strips could check out 17 positive and 18 negative samples. There was no significant difference ($P > 0.05$) between the positive rate of the three CAV IC caculated by $\chi 2$ test. Compared with PCR results, the sensitivity and total coincidence rate of three strips were 83% (CAV-1&CAV-2 IC test strip), 82% (CAV-1IC test strip), 85% (CAV-2IC test strip), and 91% (CAV-1&CAV-2 IC test strip), 90% (CAV-1IC test strip), 93% (CAV-2IC test strip), respectively. There was no significant difference ($P > 0.05$) between the positive rate of the CAV IC with CAV PCR kit caculated by $\chi 2$ test. All the specificity of three strips was 100%. There was not false positive and false negative result.

In Table 3, out of 35 eye and nose swabs, 16 found positive in CAV-1 and CAV-2 IC strip where as in CAV-2 specific strip 17 found positive. The extra one sample was CAV-2 positive with PCR identified. The data showed the sensibility of CAV-2 IC strip was higher than that of CAV-1and CAV-2 IC strip for detecting CAV-2 in clinical samples. Similar type of result was obtained in case of 25 eye and nose swabs (CAV-1) between CAV-1 and CAV-2 IC strip and CAV-1 IC strip. The extra one sample was CAV-1 positive with PCR determined. The phenomenon indicated the sensibility of CAV-1and CAV-2 IC strip was higher than that of CAV-1 IC strip for detecting CAV-1 in clinical samples.

Table 3: Results of CAV-1 and CAV-2 clinical samples

Dog samples	Test method							
	CAV-1&CAV-2 strip		CAV-1 strip		CAV-2 strip		CAV PCR kit	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
35 eye and nose swabs (CAV-2)	16	19	0	0	17	18	20	15
25 eye and nose swabs (CAV-1)	11	14	10	15	0	0	13	12
12 anal swabs (CAV-1)	6	6	6	6	0	0	7	5
15 faeces (CAV-1)	7	8	7	8	0	0	8	7
Total	40	47	23	29	17	18	48	39
Sensticity	83% (40/48)		82% (23/28)		85% (17/20)			
Specificity	100% (39/39)		100% (24/24)		100% (15/15)			
Overall agreement	91% (79/87)		90% (47/52)		93% (32/35)			

There was no significant difference ($P>0.05$) between the positive rate of the three CAV IC and the CAV PCR calculated by χ^2 test.

DISCUSSION

CAV-1 and/or CAV-2 have been examined by PCR-based methods and Immunohistochemical (IHC) in some reports [7-11]. However, these approaches are time-consuming and need specific instruments and professional technicians. IC has been widely used in clinic diagnostic simply and conveniently. For example, the detection limits of commercial Canine distemper IHC could reach to 5×102 TCID50/ml, which was comprable with PCR (2×102 TCID50/ml), however, the IHC results could gained in five minutes. At present, there were no reports of CAV-associated IC. Therefore, we started to establish a simple, accurate, reproducible and suitable method for clinical samples. In the present study, mAbs, including anti-CAV-1&CAV-21A1 and 5G4, anti-CAV-1 4F3 and 2F9 and anti-CAV-2 1C5 and 4H12, were firstly produced and three types of IC test strips (namely, CAV-1&CAV-2 strip, CAV-1 strip and CAV-2 strip) were developed subsequently. CAV-1&CAV-2 strips could simultaneously detect CAV-1 and CAV-2 with high sensitivity, specificity, accuracy, and be achievable for examining various clinical specimens such as eye and nose swabs, anal swabs and faeces. Individual CAV-1 strips and CAV-2 strips were specific and sensitive detection methods for CAV-1 and CAV-2 in clinical samples with same accuracy as PCR does.

In conclusion, the IC assays could be a useful aid for early diagnosis of CAV in infected dogs. The gold strips have the advantage over other diagnostic approaches applied in clinical practice in that it is simple, sensitive, specific, convenient, and can be performed by owners and veterinarians within 10 minutes. In particular, animals from suspected infection are not generally hospitalized, and can immediately be onsite detected after conducting on dog external excretions or secretion samples. In view of these advantages, three types of IC test strips can be widely applied for onsite diagnosis. Future work should concentrate on the application of such technology to detect CAV in other wild animals such as foxes.

Conflicts of Interest

The authors declared no conflicts of interest.

Ethics approval and consent to participate

The animal trial in this study was approved by the Animal Care and Ethics Committee of China National Research Center for Veterinary Medicine and conventional animal welfare regulations and standards were taken into account.

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