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ESTABLISHMENT OF AN INDIRECT ELISA METHOD FOR DETECTING CANINE DISTEMPER VIRUS ANTIBODIES

Summary

Canine distemper virus (CDV) can cause acute infectious diseases in various carnivorous animals. This study aims to develop an indirect ELISA method for CDV antibody detecting.

Using the purified recombinant CDV antigens as coating agents, and after optimizing the conditions, a preliminary ELISA detection method for CDV specific antibodies was established. The optimal coating concentration of recombinant CDV antigen at 2,0 μg/mL 100 μL/well, overnight coating at 4 °C, blocking at 4 °C for 4 h, serum dilution at 1: 200 and incubation at 37 °C for 1,5 h or 2 h, or 4 °C overnight. No cross-reactivity was observed with CPIV, CAV-1, and CCV antibody-positive serum, indicating good specificity. Both intra-batch and inter-batch CVs were below 5 %, and the detection sensitivity reached 1:500.

Keywords: *indirect ELISA, canine distemper virus, antibody, method, animal infectious diseases.*

Резюме

Вирус чумы собак (CDV) может вызывать острые инфекционные заболевания у различных плотоядных животных. Целью данного исследования является разработка непрямого метода ИФА для обнаружения антител к CDV.

Используя очищенные рекомбинантные антигены CDV в качестве покрывающих агентов и после оптимизации условий, был разработан предварительный метод ELISA-детектирования антител, специфичных к CDV. Оптимальная концентрация рекомбинантного антигена CDV в покрытии при 2,0 мкг/мл, 100 мкл/лунка, нанесение покрытия на ночь при 4 °C, блокирование при температуре 4 °C в течение 4 часов, разведение сыворотки 1:200 и инкубация при температуре 37 °C в течение 1,5 часов или 2 часов либо 4 °C в течение ночи. Перекрестной реактивности с сывороткой, положительной по антителам к CPIV, CAV-1 и CCV, не наблюдалось, что указывает на хорошую специфичность. Коэффициент вариации как внутри, так и между партиями был ниже 5 %, а чувствительность обнаружения достигала 1:500.

Ключевые слова: *непрямой ИФА, вирус чумы собак, антитело, метод, инфекционные болезни животных.*

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INTRODUCTION

Canine distemper virus (CDV) is an acute, highly contagious, and febrile infectious disease, has emerged as a significant disease of wildlife and dogs [1]. Initially, CDV was described as an infectious disease of domestic dogs [2]. Further researches found that CDV can also naturally infects a variety of animals such as primates, macaques, and seals. So that, CDV is now recognized as a global multi-host pathogen, infecting and causing mass mortalities in a wide range of carnivore species [3]. In the past decade, a large number of CDV outbreaks have occurred in a variety of wild animal populations, causing serious damage and losses [4–6]. Therefore, surveillance and vaccination among all susceptible animals are warranted to protect animals. For us, laboratory analyses must be completed by multiple methods to determine etiology of possible interspecific viral infection, eg. RT-qPCR assay, immunohistochemistry, immunological methods etc. More, simpler, and cheaper test reagents will be more suitable for promotion worldwide.

ELISA is one of the most specific and straightforward assays for detecting biomolecules in research and clinics. Besides routine

laboratory usage, ELISA has been utilized in food industry as quality control tools [7]. With advances in analytical methods, ELISA assay has been constantly optimized to improve its specificity and sensitivity. At present, ELISA is the most commonly used immunological testing method. In this study, we aims to establish a convenient ELISA method for CDV antibody detection. In the early stage, our team has prepared the recombinant CDV antigen (a recombinant protein linked by the H and F protein sequences of CDV through a flexible linker peptide $(GGGGS)_n$ and expressed in prokaryotic cells), and verified its detection ability [8]. Using these self-made recombinant CDV antigen, we established a more convenient ELISA method, and demonstrated its high repeatability, specificity and sensitivity.

MATERIALS AND METHODS

Antigen, serum, reagents and samples. Recombinant CDV antigen (a recombinant protein linked by the H and F protein sequences of CDV through a flexible linker peptide $(GGGGS)_n$ and expressed in prokaryotic cells). carbonate buffer (pH 9,6), 0,01 mol/L phosphate buffer solution (PBS), washing buffer (PBS+0,05 % tween 20, PBST) and TMB coloring solution are self prepared in the laboratory. 96-well microtiter plate purchased from Costar. iMark microplate reader purchased from BIO-RAD. 10 positive, 36 negative serum samples of CDV, the positive serum samples of canine parainfluenza virus (CPIV), canine adenovirus type 1 (CAV-1), and canine coronavirus (CCV) were all provided by Guangzhou fumao animal hospital.

Preliminary establishment of indirect ELISA. Diluted the recombinant CDV antigen with the carbonate buffer (pH 9,6), and then transferred into 96-well microtiter plate with 100 μL/well, 100 ng/well. After overnight coating at 4 ℃, discarded the coating liquid and took excess residual liquid on absorbent paper, and added 150 μL 5 % skimmed milk to each well, blocked at 37 ℃ for 2 h. Then, discarded skim milk and added 150 μL PBST to wash for 2 min, washing repeat 3 times; After the microtiter plate was wrapped, it can be directly used or packaged in a self sealing bag, and then inverted in a –20 ℃ refrigerator for backup.

Test procedure: Diluted CDV positive serum with PBS at a ratio of 1:100 and add it into the coated well, 100 μL/well, and then incubated 2 h. After that, washed 3 times with PBST, added commercial secondary antibody (1:5000 in PBS) into the well and incubated for 1 h, 100 μL/well. Then, washed 3 times with PBST, added 100 μL TMB to each well and incubated for 10 min. Finally, 50 μL 2 mol/L H_2SO_4 to terminate the reaction, and immediately detected the OD_{450} value in a microplate reader. The entire ELISA process was conducted at room temperature.

Optimization of indirect ELISA

1. Optimization of coating concentration. Diluted the recombinant CDV antigen to 0,25, 0,50, 1,0, 1,5, 2,0, 5,0 μg/mL, added (100 μL/well) and coated the 96-well microtiter plate, respectively. Performed the indirect ELISA according to the above «Test procedure» and obtained OD_{450}, then calculated P/N, and determined the coating concentration corresponding to the maximum P/N.

2. Optimization of coating temperature and time. Coated the 96-well microtiter plate with the optimal concentration of coated protein, incubated at 4 ℃ for 2 h, 4 ℃ for 4 h, 37 ℃ for 1 h, 37 ℃ for 2 h and 4 ℃ overnight, respectively. Performed the indirect ELISA according to the above «Test procedure» and obtained OD_{450}, then calculated P/N, and determined the coating temperature and time corresponding to the maximum P/N.

3. Optimization of blocking conditions. Blocked the 96-well microtiter plate at 4 ℃ for 2 h, 4 ℃ for 4 h, 4 ℃ overnight, 37 ℃ for 1 h, 37 ℃ for 2 h, and 37 ℃ 3 h, respectively. Performed the indirect ELISA according to the above «Test procedure» and obtained OD_{450}, then calculated P/N, and determined the blocking conditions corresponding to the maximum P/N.

4. Optimization of serum incubation conditions. Incubated the positive serum at room temperature for 0,5 h, 1,0 h, 1,5 h, 2,0 h and 4 ℃ overnight, respectively. Performed the indirect ELISA according to the above «Test procedure» for other steps and obtained OD_{450}, then calculated P/N, and determined the serum incubation conditions corresponding to the maximum P/N.

5. Optimization of serum dilution. Diluted the positive serum with PBS at 1:100, 1:200, 1:400, 1:800, 1:1000, and 1:2000. Performed the indirect ELISA according to the above optimal conditions, calculated P/N ra-

tion, and determined the optimized serum dilution corresponding to the maximum P/N.

Determination of the negative and positive critical values for indirect ELISA

According to the optimized detection steps, ELISA was performed on 36 negative serum samples, and the critical value of ELISA negative/positive was obtained by calculating the mean and standard deviation (SD).

Repeatability of indirect ELISA

To verify the repeatability of the indirect ELISA, 96-well microtiter plate coated in one batch and three different batches were randomly selected for repeatability valuation. 5 CDV positive serum samples were simultaneously detected using the same batch and different batches microtiter plate.

Specificity of indirect ELISA

To explore the specificity of the indirect ELISA, the positive (10 cases)/negative (36 cases) serum samples of CDV, the positive serum sample of CPIV, CAV-1, and CCV were selected for this indirect ELISA.

Sensitivity of indirect ELISA

Diluted 10 CDV positive serum samples at 1:100, 1:200, 1:300, 1:400, 1:500, and 1:600, as the samples, performed the ELISA to evaluate its sensitivity.

RESULTS AND DISCUSSION

Optimization results of the indirect ELISA

1. Optimization results of coating concentration. The ELISA results showed that when the protein concentration of the recombinant CDV antigen 2,0 μg/mL, the positive serum OD_{450} value was 0,82, the negative serum OD_{450} value was 0,098, and the corresponding P/N value was maximum (8,37, table 1). Therefore, the optimal coating concentration for the recombinant CDV antigen was determined to be 2,0 μg/mL.

Table 1 – Optimization results of coating concentration

Serum	Recombinant CDV antigen concentration (μg/mL)					
	0,25	0,5	1,0	1,5	**2,0**	5,0
Positive(P)	0,32	0,45	0,58	0,78	**0,82**	0,85
Negative(N)	0,078	0,086	0,092	0,096	**0,098**	0,11
P/N	4,10	5,23	6,30	8,13	**8,37**	7,73

2. Optimization results of coating temperature and time. The ELISA results showed that when coating condition of the recombinant CDV antigen was 4 ℃ overnight, the positive serum OD_{450} value was 0,83, and the negative serum OD_{450} value was 0,092, and the corresponding P/N value maximum (9,02, table 2). Therefore, the optimal coating condition was determined to be 4 ℃ overnight.

Table 2 – Optimization results of coating temperature and time

Serum	Antigen coating condition				
	4 ℃ 2 h	4 ℃ 4 h	37 ℃ 1 h	37 ℃ 2 h	**4 ℃ overnight**
Positive(P)	0,32	0,45	0,52	0,79	**0,83**
Negative(N)	0,059	0,072	0,078	0,094	**0,092**
P/N	5,42	6,25	6,67	8,40	**9,02**

3. Optimization results of blocking conditions. The ELISA results showed that when blocking condition was 4 ℃ 4 h, the positive serum OD_{450} value was 0,81, and the negative serum OD_{450} value was 0,095 and the corresponding P/N value maximum (8,53, table 3). Therefore, the optimal blocking condition was determined to be 4 ℃ 4 h.

Table 3 – Optimization results of blocking conditions

Serum	Blocking condition					
	4 ℃ 2 h	**4 ℃ 4 h**	4 ℃ overnight	37 ℃ 1 h	37 ℃ 2 h	37 ℃ 3 h
Positive(P)	0,65	**0,81**	0,76	0,69	0,84	0,72
Negative(N)	0,25	**0,095**	0,096	0,21	0,11	0,097
P/N	2,6	**8,53**	7,92	3,29	7,64	7,42

4. Optimization results of serum incubation conditions. The ELISA results showed that when serum incubation conditions were room temperature 1,5 h and 2,0 h, 4 ℃ overnight, their corresponding P/N value was 9,14, 9,13 and 9,13, respectively, which are very close (table 4). Therefore, the optimal serum incubation conditions was determined to be room temperature 1,5 h or 2,0 h, or 4 ℃ overnight.

Table 4 – Optimization results of serum incubation conditions

Serum	Serum incubation condition				
	0,5 h	1,0 h	**1,5 h**	**2,h**	**4 ℃ overnight**
Positive(P)	0,52	0,74	**0,85**	**0,84**	**0,84**
Negative(N)	0,091	0,092	**0,093**	**0,092**	**0,092**
P/N	5,71	8,04	**9,14**	**9,13**	**9,13**

5. Optimization results of serum dilution. The ELISA results showed that when the serum dilution was 1:200, the positive serum OD_{450} value was 1,85, the negative serum OD_{450} value was 0,12, and the corresponding P/N value maximum (15,42, table 5). Therefore, the optimal serum dilution was determined to be 1:200.

Table 5 – Optimization results of serum dilution

Serum	Serum dilution					
	1:100	**1:200**	1:400	1:800	1:1000	1:2000
Positive(P)	2,13	**1,85**	0,92	0,33	0,25	0,11
Negative(N)	0,31	**0,12**	0,098	0,088	0,087	0,09
P/N	6,87	**15,42**	9,39	3,75	2,87	1,22

Determination results of the negative and positive critical values for indirect ELISA

36 negative serum samples were tested according to the optimal conditions, the mean OD450 value was 0.11, with the standard deviation was 0,018. The critical values of the negative and positive are as follows: when the OD_{450}value > mean + 3×SD = 0,17, it is considered positive; when the $OD4_{50}$ value < mean + 2×SD = 0,15, it is considered negative, and between the two values, it is considered suspicious samples.

Repeatability results of indirect ELISA

5 positive serum samples were tested using 96-well microtiter plate prepared from the same batch, repeated 5 times in each sample. The results showed that the coefficient of variation (CV) ranged from 1,70 % to 3,56 %, all less than 5 %, indicating the repeatability of the intra-batch was good; 5 positive serum samples were tested using 96-well microtiter plate prepared from the three different batches, repeated 5 times in each sample. The results showed that CV ranged from 2,57 % to 4,55 %, all less than 5 %, indicating the repeatability of the inter-batch was good. The results were shown in table 6.

Table 6 – Repeatability results of indirect ELISA

№	Intra-batch			Inter-batches		
	mean	SD	CV	mean	SD	CV
1	1,83	0,031	1,70	1,826	0,053	2,91
2	1,74	0,026	1,49	1,716	0,048	2,81
3	2,02	0,042	2,07	2,014	0,052	2,57
4	2,16	0,04	1,85	2,146	0,074	3,43
5	2,0988	0,075	3,56	2,046	0,093	4,55

Specificity results of indirect ELISA

Table 7 showed that OD_{450} values of CPIV, CAV-1, and CCV positive serum and 36 CDV negative serums were all lower than 0,15, while the OD_{450} value of 10 CDV positive serums were all higher than 0,17, indicating the indirect ELISA has high specificity to CDV antibody.

Table 7 – Specificity results of indirect ELISA

Serum	P CDV	N CDV	CPIV	CAV-1	CCV
OD_{450}	All>0,17	All<0,15	0,095	0,13	0,14
P or N	P	N	N	N	N

Sensitivity results of indirect ELISA

Tested 10 positive serum samples at the dilution of 1:100, 1:200, 1:300, 1:400, 1:500, and 1:600, respectively. ELISA results showed that the positive serum still was positive at 1:500 dilution (OD_{450} > 0,17, table 8), indicating the indirect ELISA has high sensitivity.

Table 8 – Sensitivity results of indirect ELISA

№	Serum dilution					
	1:100	1:200	1:300	1:400	**1:500**	1:600
1	2,34	2,12	1,85	1,24	**0,74**	0,36
2	1,82	1,6	1,27	0,68	**0,42**	0,23
3	2,51	2,46	2,07	1,64	**1,16**	0,67
4	1,78	1,28	1,03	0,66	**0,43**	0,19
5	2,14	1,79	1,46	0,86	**0,48**	0,24
6	2,25	1,89	1,62	0,94	**0,57**	0,22
7	1,48	1,01	0,75	0,52	**0,39**	0,14
8	1,82	1,45	1,17	0,84	**0,51**	0,27
9	2,23	2,07	1,67	1,20	**0,69**	0,37
10	2,11	1,90	1,47	1,06	**0,84**	0,53

CDV is a highly contagious and fatal systemic disease in free-living and captive carnivores worldwide. CDV-infected animals may simultaneously or sequentially develop respiratory, gastrointestinal, dermatologic [9]. The widespread clinical symptoms hampers early and accurate clinical diagnosis of CDV [10]. Therefore, rapid and accurate diagnosis of CDV will help to implement appropriate strategies in time to prevent the outbreak of CDV. Molecular detection techniques are cold chain dependent and require relatively expensive equipment with experienced technicians, making them difficult to implement on-site and in underdeveloped areas [11]. A simple, rapid, accurate and user-friendly platform is needed for early basic detection of CDV infection. ELISA is precisely such a method, also with more cost-effective [12, 13]. This study established a indirect ELISA, which was conducted at room temperature, with the entire detection process taking only 2 hours and requiring no professional equipment or experienced technicians. This indirect ELISA is a simple, rapid and user-friendly detection tool for early diagnosis of CDV infection.

ELISA techniques are broadly classified into direct, indirect, competitive and sandwich ELISA [14]. Indirect ELISA method is the most common method of antibody detection. The principle is to detect antibodies that have been bound to the solid phase by using antibodies labeled with enzymes. This method only requires the replacement of different solidphase antigens, the antibodies against various antigens can be detected with an enzyme labeled antibody [15]. The indirect ELISA method revealed efficient detection and promising application prospects in various virus antibody tests, eg. African swine fever virus antibodies [16], duck hepatitis A virus type 1 antibodies [17], Pseudorabies (Aujeszky disease) virus antibodies [18], which making them ideal for epidemiological surveillance. In CDV antibody detection, indirect ELISA also revealed efficient detection and promising application prospects: all CVs below 5 %, high specificity to CDV antibody, and sensitivity reached 1:500.

Overall, the detection of anti-CDV antibodies with indirect ELISA demonstrated good repeatability, specificity and sensitivity, which provide a simple, rapid and user-friendly detection tool that is suitable for CDV antibody detection and epidemiological surveillance.

CONCLUSION

The development of indirect ELISA method for CDV antibodies provided a reference for clinical diagnosis and lays a foundation for effective monitoring of CDV infection.

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