

Determination of parvovirus antibodies in canine serum using magnetic bead-based chemiluminescence immunoassay

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Abstract

Canine parvovirus type 2 (CPV-2), as a highly contagious and potentially fatal disease of dogs and many other carnivores, usually causes severe gastroenteritis and myocarditis. Therefore, it is very necessary and urgent to have an accurate method to determine the CPV-2 antibodies (CPV-2-Ab) in canine samples. Here, a magnetic bead-based chemiluminescence immunoassay was established and optimized to detect the concentration of CPV-2-Ab in serum. And a commercial assay was also used to evaluate the consistency with our method. After optimization of the detection system, the CPV-2-Ab was captured by CPV-antigen-magnetic bead (8.3 $\mu\text{g/mL}$); then combined with the conjugation of anti-canine IgG antibody-acridinium ester

(0.36 $\mu\text{g/mL}$). Finally, collected the signal (read the luminosity) after 1 h reaction time. The linear correlation coefficient (R^2) is 0.9924. The limit of detection (sensitivity) is 0.36 ng/mL (the linear dynamic range: 1.32–93.75 ng/mL), and the average recovery is 100.89% without cross-reactions with other canine viral antibodies. The results' correlation between commercial assays and this method is 0.9888. This immunoassay establishes that it has high sensitivity, accuracy, and specificity in clinical analysis, indicating that this method could be suitable for quantitative detection of CPV-2-Ab and evaluation of vaccination effect. © 2019 International Union of Biochemistry and Molecular Biology, Inc. Volume 66, Number 4, Pages 586–590, 2019

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1. Introduction

Canine parvovirus type 2 (CPV-2), which belongs to genus proto parvovirus of family parvoviridae, is a highly contagious and potentially fatal disease of dogs and many other carnivores

Abbreviations: CCV-Ab, anti-canine coronavirus antibodies; CDV-Ab, anti-canine distemper virus antibodies; CIV-Ab, anti-canine influenza virus antibodies; CPV-2, Canine parvovirus type 2; CPV-2-Ab, CPV-2 antibodies; CPV-2-Ag, CPV-2 antigen; CV, coefficient of variation; EDC, 1-(3-dimethylaminyl)-3-ethylenediamine hydrochloride; ELISA, enzyme-linked immunosorbent assay; LOB, limit of blank; NHS, N-Hydroxysuccinimide; RBV-Ab, anti-rabies virus antibodies; RLU, relative light unit; SN, serum-neutralization.

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and usually causes severe gastroenteritis in juvenile dogs and myocarditis in neonatal puppies [1]. Recently, CPV appears to be endemic in almost all populations of wild and domesticated dogs [2]. Luckily, during the past decades, promptly vaccine injection for dogs has greatly reduced the incidence of canine infectious diseases [3]. Moreover, safe and effective vaccines against canine infectious virus has been considered as essential vaccines that every dog should receive, which was designated as “core vaccines” by the American Veterinary Medical Association Council on Biological and Therapeutic Agents [4, 5]. Therefore, the vaccine efficacy and duration data of immunity should be timely evaluated, which means that it is very necessary and urgent to have a quantitative and real-time method for detecting the corresponding infectious virus antibody in a sample.

Immunoassay is an easy, fast, and low-cost method for detection of antigen or its specific antibody in laboratory diagnostics. To date, these immunization techniques, colloidal gold method, enzyme-linked immunosorbent assay (ELISA), rapid fluorescent focus inhibition test, and fluorescent antibody virus neutralization test, have been most frequently proven

and widely used in clinical analysis [6, 7]. Recently, polymer magnetic bead-aided immunoassays have attracted a great deal of attention [8]. And as we all know, this technique has several advantages such as high specificity, rapidity, sensitivity, cost-effective operation, easy automatization, and so on [9]. Some researchers have reported the chemiluminescence immunoassays for various virus antibodies, such as hepatitis C virus antibodies and rubella virus antibodies [10, 11]. But to date, there is still no method for detection of CPV-ab. Hence, we have developed a highly sensitive chemiluminescence immunoassay method to determine CPV-2-Ab in canine serum.

In this study, we have established and optimized a magnetic microbead-aided indirect chemiluminescence immunoassay for CPV-2-Ab analysis. Using this assay, CPV-2-Ab in serum samples were quantitatively analyzed after vaccine immunization. Moreover, conventional ELISA was employed to evaluate and compare the effectiveness of the magnetic bead-based chemiluminescence immunoassay with the same samples.

2. Materials and Method

2.1. Conjugation of CPV-antigen to magnetic bead

The carboxyl-magnetic beads used in test are commercially available (So-Fe Biomedicine, Shanghai, China). Also, these carboxyl-magnetic beads were activated by 1-(3-dimethylaminyl)-3-ethylenediamine hydrochloride/N-Hydroxysuccinimide (EDC/NHS; Sigma, St Louis, MO, USA) method, and then coupled with the recombinant canine parvovirus antigens (YijiaBiotech, Guangzhou, China).

Briefly, the carboxyl magnetic beads were washed three times with the coating buffer (0.05 M morpholine sulfonic acid, pH 6.0) and followed by addition of equal mass of EDC and NHS and activated at room temperature for 30 Min. After the same washing, the recombinant canine parvovirus antigen (CPV-2-Ag, *E. coli* expressed) was added and reacted at room temperature for 2 H in a shaker incubator. After blocking with blocking buffer (0.05 M Tris, 2% bovine serum albumin, 0.05% Triton X-100, 0.09 NaN₃), the CPV-antigen-magnetic beads were stored at 4°C before use.

2.2. Conjugation of acridinium ester to anti-canine IgG antibodies

Rabbit anti-canine IgG polyclonal antibody (YijiaBiotech, Guangzhou, China) was coupled with acridinium ester (Sigma, St. Louis, MO, USA) to prepare an anti-canine IgG-acridinium ester luminous agent. Briefly, the anti-canine IgG antibody was mixed with acridinium ester (mass ratio: 3:1) and incubated with gently shaking at 37°C for 3 H. Then dialysis was performed against 0.1 M PBS to remove unbound acridinium ester. After purification by Sephadex G50 gel chromatography, the product with high luminescence intensity was collected and stored in a luminescent buffer (pH 6.3 PBS, 1% seaweed sugar, 0.5% Trion X-100) at 4°C in dark.

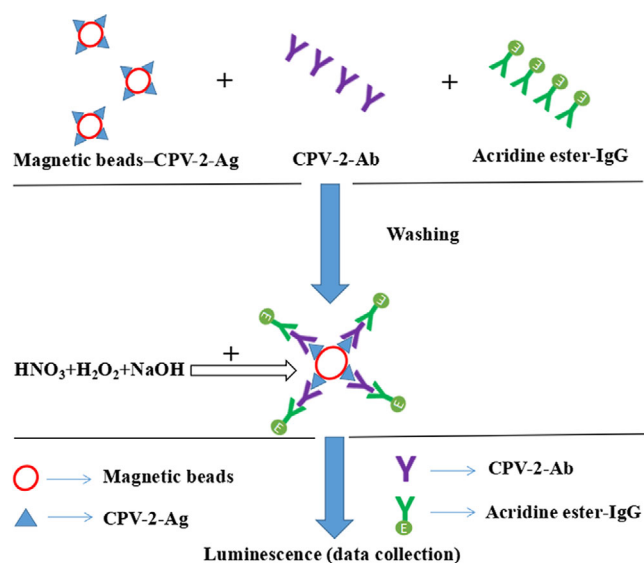


FIG. 1

The schematic of CPV-2-Ab detection.

2.3. Preparation of anti-parvovirus antibodies standards

Anti-parvovirus antibodies (CPV-2-Ab) standards were purchased from YijiaBiotech (Guangzhou, China). The standards were diluted into these final concentrations of 0, 1, 5, 10, 50, 100 ng/mL in the dilution buffer (PBS, 50 mmol/L, pH 7.4, with 1.5% BSA, and 0.15% NaN₃). Meanwhile, canine parvovirus antiserum was used as the quality control standard.

2.4. Test procedure

The schematic of CPV-2-Ab detection is shown in Fig. 1 and was operated by Keysmile's Automatic Chemiluminescence Instrument (Keysmile SMART6500, Chongqing, China). CPV-antigen-magnetic bead (50 µL) and anti-parvovirus antibodies standards or samples serum or quality control standard (50 µL) were added into the transparent test tubes (purchased from Keysmile, Chongqing, China) and incubated by gently shaking at 37°C for 30 Min. The CPV-2-Ag-magnetic bead and any specific captured substances were attracted by the magnets to the wall of the test tubes, and free substances were gently aspirated out by needle in the machine and then washed by using washing buffer (PBST, PBS + 0.05% Tween 20). Then, 100 µL anti-canine IgG-acridinium ester luminous agent was added, and the CPV-2-Ag-magnetic bead-CPV-2-Ab complex was resuspended and incubated for 30 Min. Finally, the automatic luminometer in machine successively pumped the substrate solution A (100 µL, 0.1 M HNO₃, 0.15% H₂O₂, 0.1% Trion X-100) and B (100 µL, 0.35 M NaOH, 2.5% Trion X-100) to the test tubes and read the luminosity (RLU) of each tube. The luminescence value is positively correlated with the antibody concentration in the specimen. The standard curve was drawn using the same protocol as the serum samples, and the precision and recovery experiments were carried out according to the conventional methods.

2.5. Specificity assays

For the specificity tests, different concentrations of anti-rabies virus antibodies (RBV-Ab), anti-canine distemper virus antibodies (CDV-Ab), anti-canine influenza virus antibodies (CIV-Ab), and anti-canine coronavirus antibodies (CCV-Ab) were determined.

2.6. Antibody titer measurement and reference interval determination

Based on the canine's immune duration against parvovirus vaccine, before collecting their serum samples, it must be checked that canines were 1 month old after parvovirus vaccination. Following this requirement, 142 clinical serum samples from Guangzhou Fu Mao Pet Hospital were collected. And their antibody titers were measured by serum-neutralization (SN) assays following a microtitration method using flat-bottomed 96-well microtiter plates. Simultaneously, the concentrations of CPV-2-Ab in these clinical serum samples above were measured by the present method. The reference interval was established by comparing the antibodies titers with the corresponding CPV-2-Ab concentration.

2.7. Comparison and practical application of this magnetic bead-based chemiluminescence immunoassay

Following the sample collection requirement, the 70 clinical serum samples were collected and simultaneously detected using both this magnetic bead-based chemiluminescence immunoassay and a commercial ELISA kit (Jianglai Biotechnology, Shanghai, China). The commercial assays were performed according to the manufacturer's instructions.

2.8. Statistical analysis

Quantitative data are presented as mean \pm standard deviation (mean \pm SD). Comparisons between quantitative data were studied using the paired-samples *t* test, whereas correlations between groups were calculated with Spearman's rank correlation. SPSS 19.0 was used in the statistical analysis; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Assay optimization

A two-step chemiluminescence immunoassay was developed with the volume reaction system: conjugation of magnetic bead with antigen: serum: conjugation of acridinium ester with IgG antibody = 50 μ L:50 μ L:100 μ L, and total 1 H reaction time (0.5 H for sample incubation and 0.5 H for IgG incubation). Moreover, after analysis of the optimization results, the working conditions of magnetic bead-based chemiluminescence immunoassay were determined and screened with high S/N ratio (signal/noise) and without the hook effect as follows: The concentration of the conjugation of CPV-antigen to magnetic bead is 8.5 μ g/mL, and conjugation of acridinium ester to anti-canine IgG antibodies is 0.41 μ g/mL.

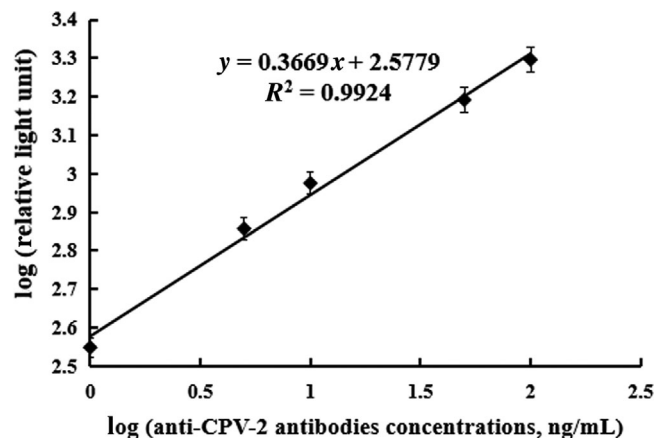


FIG. 2

The standard curve of the present CPV-2-Ab detection. The serial standard dilutions of anti-parvovirus antibodies standards (0, 1, 5, 10, 50, 100 ng/mL) were plotted on the *x* axis, and their corresponding RLU on the *y* axis, performed a linear fit, and draw a standard curve.

3.2. Standard curves

Log function values of the concentrations of anti-CPV-2 antibodies were plotted on the *x* axis, and the log function values of the corresponding RLU were plotted on the *y* axis to fit and draw the standard curve and obtain the standard equation: $y = 0.3669x + 2.5779$, $R^2 = 0.9924$ (Fig. 2), with the linear concentration range: 1.32–93.75 ng/mL and the R^2 of standard curve 0.9924. The mean concentration plus 1.645 standard deviations of the blank assayed in duplicate in 20 independent measurements was used as the definition of limit of blank (LOB). The sensitivity was determined by utilizing both the measured LOB and test replicates of a sample known to contain a low concentration of CPV-2-Ab. The lower limit of detection (LOD) is defined as $LOB + 1.645 (SD_{\text{low concentration sample}})$, and the LOD (sensitivity) is 0.36 ng/mL.

3.3. Precision

The intraassay precision was calculated by analyzing each concentration 10 times in a single batch. Moreover, three batches of products were analyzed to confirm the interassay precision. As shown in Table 1, the results show that the inter- and intraassay coefficient of variation (CV) are all below 15%, which indicate that the reproducibility of this magnetic bead-based chemiluminescence immunoassay is very high.

3.4. Specificity

Four proteins at different concentrations were tested as potential interferents. As presented in Table 2, no obvious cross-reactivities are observed, which indicate that this magnetic bead-based chemiluminescence immunoassay exhibits high specificities for the detection of CPV-2-Ab.

3.5. Recovery

Three clinical serum samples with known concentrations of CPV-2-Ab were used in this study. The CPV-2-Ab was added to

TABLE 1

Precision of this magnetic bead-based chemiluminescence immunoassay (n = 10)

	Concentrations	Average (ng/mL)	Standard deviation	CV (%)
Interassay (n = 10)	Low	2.01	0.22	10.95
	Median	50.13	4.31	8.60
	High	99.72	8.92	8.95
Intraassay (n = 10)	Low	2.20	0.21	9.55
	Median	50.43	5.11	10.13
	High	97.82	11.27	11.52

TABLE 2

Cross-reactivities of this magnetic bead-based chemiluminescence immunoassay (n = 10)

Interferents	Concentrations (ng/mL)	Determined (ng/mL)	Standard deviation	Cross-reactivity (%)
CPV-Ab	30.00	29.80	1.62	99.33
RBV-Ab	50.00	0.40	0.22	0.80
CDV-Ab	20.00	0.15	0.12	0.75
CIV-Ab	50.00	0.35	0.19	0.70
CCV-Ab	50.00	0.39	0.21	0.78

TABLE 3

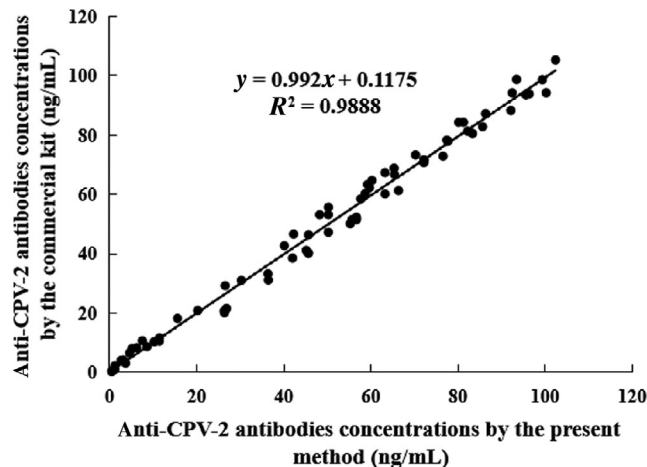
Recoveries of this magnetic bead-based chemiluminescence immunoassay (n = 10)

Fortified (ng/mL)	Determined (ng/mL)	Recovery (%)	Average recovery (%)
5.00	5.21	104.20	100.89
50.00	48.62	97.24	
100.00	101.23	101.23	

the serum samples, and the final concentrations of CPV-2-Ab are 5.0, 50.0, and 100.0 ng/mL. As presented in Table 3, the recoveries rates is between 97.24% and 104.20% with the average recoveries rate 100.89%. These results indicate that this assay is free from serum interferences.

3.6. Reference interval determination

By comparing the relationship between CPV-2-Ab titer and concentration, the reference interval of this method has been

**FIG. 3**

Correlation between the magnetic bead-based chemiluminescence immunoassay and commercial ELISA kit. The CPV-2-Ab concentrations of 70 clinical serum samples were detected with parallel tests using this magnetic bead-based chemiluminescence immunoassay with a commercial ELISA kit.

established: titer < 4 vs. < 0.5 ng/mL; 4 < titer < 16 vs. 0.5–2.3 ng/mL; 16 < titer < 256 vs. 2.3–8.5 ng/mL; 256 < titer < 1024 vs. 8.5–19.7 ng/mL; 1024 < titer < 4096 vs. 19.7–35.2 ng/mL; titer > 4096 vs. > 35.2 ng/mL.

3.7. Comparison with commercial assays

In total, the anti-CPV-2 antibodies concentrations of 70 clinical serum samples were simultaneously analyzed with the magnetic bead-based chemiluminescence immunoassay and commercial ELISA kit. And the concentration data of anti-CPV-2 antibodies from the present method are plotted on the *x* axis, the concentrations of anti-CPV-2 antibodies from commercial kit are plotted on the *y* axis to fit a curve, whose curve and its calibration equation are presented in Fig. 3. Here the calibration equation is $y = 0.992x + 0.1175$ with the correlation coefficient 0.9888, which means strong correlations between the methods are obtained in clinical samples. Thus these results indicate that the magnetic bead-based chemiluminescence immunoassay is a reliable method for the determination of CPV-2-Ab with clinical samples.

4. Discussion

It's one of the main tasks for a veterinarian to determine the vaccination frequency of the vaccine used [13]. Generally, the veterinarian should determine the vaccination frequency based on the various factors such as the safety, effectiveness and duration of immunity, exposure risk, animal susceptibility, and so on [5, 13]. In clinical tests, annual revaccination has almost become the standard vaccination specification. However, some researchers have begun to oppose this reference time for the revaccination. Abdelmagid et al. found that the dogs responded with very high SN titers after 1 month of vaccination,

the immunity to CPV lasted for at least 55 months, and the immunity persisted for more than 4 years in some dogs after vaccination [14]. Annual revaccination looks to be unnecessary, so the detection of antibody titer after vaccination and before revaccination looks very important, which could provide the reference time and effect for revaccination [7, 15, 16].

At present, the regular methods for the detection of canine virus antibodies are colloidal gold immunochromatography and ELISA in clinical samples. Colloidal gold immunochromatography is one of the fastest and most commonly used qualitative detection method, whose disadvantage is the inability to perform quantitative detection. Moreover, the ELISA method could realize quantitative detection, but its detected sensitivity is not high enough to cover the sample with low concentration CPV-2-Ab. Therefore, it remains a big challenge to find a new approach to improve the detection sensitivity of infectious viral antibodies [17]. However, polymer magnetic bead-aided immunoassays, especially chemiluminescence immunoassay, have attracted more and more attention in the infectious viral antibodies detection because of its high sensitivity and specificity [10–12]. Among the various chemiluminescent labels (e.g., the classic luminescent reagent luminol and lucigenin), acridinium esters are particularly attractive because they have high quantum yields with detection limits in the atto mole range [18, 19]. Moreover, they exhibit fast light emission with simple chemical triggers and their small size permit simple labeling protocols for proteins and nucleic acids [20]. Some earlier studies have reported the applications of acridinium phenyl esters in immunoassays and nucleic acid assays and pointed toward their potential as useful labels in clinical diagnostics [21, 22]. In this study, we have established magnetic bead-based chemiluminescence immunoassay using acridinium phenyl esters as chemiluminescent labels for detecting the concentration of anti CPV2-Ab in sample.

To date, hemagglutination-inhibition test and SN assays for antibody titers are regarded as “the gold standard” for quantitative determination of CPV2 antibodies in the sera of dogs. Most of the previous methods represented the antibody concentrations in titers, and they are only the semiquantitative method in the strict sense [23–25]. This assay could detect quantitatively the concentration of CPV-2-Ab within 1 H, and the sensitivity is 0.36 ng/mL. There is no obvious cross-reaction happens with potential interferents. Also, compared with the ELISA commercial kits, high correlation coefficient ($R^2 = 0.9888$) is obtained. Additionally, we also studied the reference interval between CPV-2-Ab titer and concentrations, which increased user acceptability in the case of complete quantification.

In conclusion, a magnetic bead-based chemiluminescence immunoassay was developed for the determination of CPV-2-Ab. And compared with the commercial ELISA kit, the established magnetic bead-based chemiluminescence immunoassay provides a new idea for rapid, convenient, specific and sensitive determination in CPV-2-Ab level, which could be used for real-time detection of CPV-2-Ab after vaccination.

5. Acknowledgements

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6. Conflicts of Interest

The authors declare that they have no conflicts of interest.

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