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Clinical Evaluation of a Rapid Diagnostic Test Kit for Canine Parvovirus and Coronavirus

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Abstract

Canine parvovirus type 2 (CPV-2) and canine coronavirus (CCoV) are major pathogens that can induce gastroenteritis in dogs. They are highly contagious and have a high morbidity rate. There are no specific treatments available for them to date. Therefore, rapid and accurate diagnosis becomes essential. The rapid diagnostic test (RDT) for animals can be used widely in the field because it is fast and easy to use for diagnosis. Thus, this study aimed to clinically evaluate and confirm the clinical utility of CPV-2/CCoV RDT. The parameters evaluated included the limit of detection (LoD), cross-reactivity, interference, sensitivity, specificity, negative likelihood ratio (NLR), and kappa value. The results revealed that the LoD values for CPV-2 and CCoV were 9.7×10 $TCID_{50}$/mL and 2.5×10^2 $TCID_{50}$/mL, respectively. There was no cross-reactivity with nine pathogens or interference by interfering materials. The RDT showed a sensitivity of 90.0%, a specificity of 100.0%, an NLR of 0.1, and a kappa value of 0.90 for diagnosing both viruses. In conclusion, the CPV-2/CCoV RDT is useful as a screening test because of its high sensitivity, specificity, kappa value, and low NLR.

Keywords

Canine parvovirus
Canine coronavirus
Rapid diagnostic tests
Sensitivity and specificity

1. Introduction

Canine parvovirus type 2 (CPV-2) and canine coronavirus (CCoV) are major pathogens that cause acute gastroenteritis in dogs [1,2]. Both viruses have high infectivity and transmission rates, affecting dogs of all ages, but puppies under 6 weeks to 6 months of age, as well as those with immunodeficiency and unvaccinated dogs, are particularly vulnerable [3-5]. Infection occurs through the fecal-oral route and spreads rapidly through direct contact or exposure to contaminated feces [3,6].

Coinfection with CPV-2 and CCoV is common and more lethal than single infections [1,2]. This is because the target cells of the viruses are the crypt cells of the small intestine and enterocytes, respectively, and coinfection disrupts the turnover of small intestinal cells [2,3,7]. Unlike bacterial infections, there is no specific treatment for viral infections, making rapid and accurate diagnosis even more critical [3,7-9].

Various diagnostic methods are available for CPV-2 and CCoV, including electron microscopy, virus culture, nucleic acid amplification techniques, and more [6,8]. Among them, polymerase chain reaction (PCR) for DNA viruses such as CPV-2 and reverse transcription PCR (RT-PCR) for RNA viruses such as CCoV are considered standard reference methods [4,6]. While these methods offer high sensitivity and accuracy, they require longer processing times, expensive equipment, and skilled personnel [6,10]. Rapid diagnostic tests (RDTs) are simple, cost-effective, and provide quick results [8,10], allowing for immediate decision-making and actions. Therefore, they are widely used in frontline clinical settings, in contrast to nucleic acid amplification techniques [8-11].

The animal RDT market is expected to grow further with the increasing pet population, the occurrence of zoonotic infections, and the rising consumption of animal-based food products [11,12]. However, according to a survey by the National Veterinary Research and Quarantine Service, there is a lack of trust in animal medical devices, with 48% reporting insufficient information about the devices, 21% citing issues with test results, and 3% expressing doubts about device quality [13].

Currently, there is less research on the performance evaluation of CCoV RDT compared to CPV-2 RDT, and there is almost no research on CPV-2/CCoV RDT, which can detect both viruses simultaneously. The sensitivity of RDTs is lower than nucleic acid amplification techniques, and the continuous variability among products poses limitations, necessitating further confirmation of the clinical utility of animal RDTs [4,8,10,14]. Therefore, this study aims to confirm the clinical utility of CPV-2/CCoV RDT through the performance evaluation of RDT for the diagnosis of CPV-2 and CCoV.

2. Materials and Methods

2.1. Virus strains and materials

The virus strains used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA). CPV-2 (VR-953, 2.0×10^5 50% tissue culture infectious dose [$TCID_{50}$]/mL) and CCoV (VR-2068, 2.5×10^5 $TCID_{50}$/mL) were used for the limit of detection (LoD), cross-reactivity, and interference testing. CCoV (VR-809, 106.5 $TCID_{50}$/mL) was used for CCoV spiking sample preparation. Cross-reactivity testing included the use of canine distemper virus (CDV), bovine parvovirus (BPV), porcine parvovirus (PPV), canine parainfluenza virus (CPIV), canine adenovirus type 1 (CAV-1) and type 2 (CAV-2), canine herpesvirus (CHV), *Escherichia coli* (*E. coli*), *Salmonella* Paratyphi (*S.* Paratyphi). The concentrations used were 5.0×10^3 $TCID_{50}$/mL for viruses and 108 colony-forming units (CFU)/mL for bacteria. Interfering substances included blood (1%), bilirubin (342 μM, Sigma-Aldrich, St. Louis, MO, USA), lipids (1.5 mg/mL, Sigma-Aldrich), and cholesterol (500 mg/dL, Sigma-Aldrich).

2.2. Clinical specimens

From May to November 2018, fecal samples were collected from 60 dogs (50 CPV-2 and 10 CCoV positive) that presented with symptoms of CPV-2 and CCoV at animal hospitals in five regions, including Jeonju, Iksan, Gunsan, Bu-an in Jeollabuk-do, and Yongin in Gyeonggi-do, South Korea. Additionally, fecal samples from 90 dogs that tested negative were collected. Among these, fecal samples from 40 negative dogs were used for CCoV spiking.

2.3. CCoV spiking

CCoV, inactivated to a 0.1% concentration using 4% formalin, was diluted to a total of 500 μL in buffer solution provided in the kit at ratios of 1/5 and 1/10.

Frozen negative fecal samples were thawed, and a swab was used to spread the feces' surface and interior in the diluted solution.

2.4. Rapid diagnostic test (RDT)

A single strip, the CCV/CPV Ag test (Genbody, Cheonan, Korea), which simultaneously detects CPV-2 and CCoV, was employed. The manufacturer's instructions were followed. Before testing, frozen fecal samples were left at room temperature for 15–30 minutes. The swab provided in the kit was used to vigorously mix the surface and interior of feces in 1 mL of buffer solution. Four drops of the diluted sample (approximately 100 μL) were applied to the test area, and the results were read after 10 minutes. A red band appearing only at the control line (C) indicated a negative result, while the appearance of test line 1 along with the C line indicated CPV-2 positive, and the appearance of test line 2 along with the C line indicated CCoV positive. If no band appeared at the C line, a retest was performed.

2.5. Nucleic acid amplification method

Clinical specimens were thawed at room temperature 15–30 minutes before testing. Fecal samples collected with swabs were placed in 600 μL phosphate-buffered saline (PBS) and vortexed for 10 minutes. The mixture was then centrifuged at 3,000 rpm for 15 minutes. A total of 200 μL of the supernatant was used for nucleic acid extraction with the QIAamp® *cador*® Pathogen Mini Kit (Qiagen, Germany). PCR was performed using HelixAmp™ Direct PCR [3G] (Nanohelix, Daejeon, Korea), and the primers (Cosmogenetech, Seoul, Korea) were diluted to a concentration of 10 pmol/μL (**Table 1**). The PCR protocol included an initial denaturation at 50°C for 5 min and 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 53°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. A result of 583 bp band after gel electrophoresis indicated a positive result for CPV-2. Complementary deoxyribonucleic acid (cDNA) synthesis was performed using TOPscript™ cDNA Synthesis Kit (Enzynomics, Daejeon, Korea), and RT-PCR was carried out using the synthesized cDNA with HelixAmp™ Direct PCR [3G] (Nanohelix, Daejeon, Korea) (**Table 1**). A result of a 409 bp band after gel electrophoresis indicated a positive result for CCoV.

2.6. Limit of detection (LoD)

LoD is the lowest detectable concentration that confirms the ability to detect a specific substance, used to assess the validity of the RDT. The experiment followed the National Committee for Clinical Laboratory Standards (NCCLS, currently known as Clinical and Laboratory Standards Institute [CLSI]) EP17-A guidelines and serially diluted the confirmed antibody-positive CPV-2 and CCoV in the kit with buffer solution (2-fold serial dilution) and performed measurements twice [15]. From the concentration at which no further positive reaction was visible to the naked eye and five steps above that, measurements were repeated 20 times a day for three days, setting the LoD of the RDT at a concentration that showed 95% or

Table 1. Primer sequences used for PCR and RT-PCR

Primer		Sequences (5'→3')
CPV-2	Forward	5' – CAG GAA GAT ATC CAG AAG GA – 3'
	Reverse	5' – GGT GCT AGT TGA TAT GTA ATA AAC A – 3'
CCoV	cDNA (Reverse)	5' – TCT GTT GAG TAA TCA CCA GCT – 3'
	Forward	5' – TCC AGA TAT GTA ATG TTC GG – 3'
	Reverse	5' – TCT GTT GAG TAA TCA CCA GCT – 3'

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; CPV-2, canine parvovirus type 2; CCoV, canine coronavirus; cDNA, complementary deoxyribonucleic acid.

more positive results. Concentrations were rounded to the first decimal place.

2.7. Cross-reactivity and interference

Cross-reactivity was checked for nine pathogens that have a similar structure to CPV-2 and CCoV or similar symptoms. The experiment was conducted three times with the same kit lot, once a day. If the results were negative according to the result criteria, it was considered to have no cross-reactivity. Interferences were checked to determine whether physiological components that may be present in the sample, such as blood, bilirubin, lipids, and cholesterol, affect the results. The experiment followed CLSI EP7-A2 guidelines [16]. In the experiment, the negative standard was PBS, and the low-concentration positive standard material was the lowest concentration, which was confirmed visually during the LoD measurement, i.e., CPV-2 1.6×10^3 $TCID_{50}$/mL and CCoV 3.9×10^3 $TCID_{50}$/mL. The experiment was conducted three times with the same kit lot, once a day. If there was no difference when comparing the results between the control group without interfering substances and the experimental group with interfering substances, interference was considered not to have occurred.

2.8 Statistical analysis

Sensitivity, specificity, likelihood ratio (LR), kappa value (κ), and 95% confidence interval (95% CI) were used to evaluate the accuracy of the test method. Statistical analyses were performed using GraphPad Software (San Diego, CA, USA) and MedCalc Software Diagnostic test evaluation calculator version 20.019 (Ostend, Belgium).

3 Result

3.1. Limit of detection (LoD)

The concentrations at which no positive reactions were visually observed after stepwise dilution of standard materials were CPV-2 4.9×10 $TCID_{50}$/mL and CCoV 1.2×10^2 $TCID_{50}$/mL. From these concentrations up to five steps higher (CPV-2 7.8×10^2 $TCID_{50}$/mL and CCoV 2.0×10^3 $TCID_{50}$/mL), measurements were repeated 20 times a day for three days. Both viruses showed consistent results upon repeated measurements. The final LoD for CPV-2 detection by RDT was determined to be 9.7×10 $TCID_{50}$/mL, and the final LoD for CCoV detection by RDT was determined to be 2.5×10^2 $TCID_{50}$/mL (**Tables 2** and **3**).

3.2. Cross-reactivity and interference

No cross-reactivity was observed with nine pathogens, including CDV, BPV, PPV, CPIV, CAV-1, CAV-2, CHV, *E. coli*, and *S.* Paratyphi, which have similar structures or show similar symptoms to CPV-2 and CCoV (**Table 4**). Interfering substances such as blood, bilirubin, lipids, and cholesterol did not interfere with the detection of CPV-2 and CCoV (**Table 5**).

3.3. Sensitivity, specificity, negative likelihood ratio (NLR), and kappa statistic (κ)

The sensitivity of the RDT for CPV-2 detection was 90.0% (95% CI, 78.31–96.7), with 45 out of 50 PCR-positive samples detected as RDT-positive. The specificity was 100.0% (95% CI, 92.9–100.0), as all 50 PCR-negative samples were correctly identified as RDT-negative. For CCoV detection, the RDT exhibited a sensitivity of 90.0% (95% CI, 78.1–96.7) with 45 of 50 RT-PCR-positive samples being RDT-positive. The specificity is 100.0% (95% CI, 92.9–100.0), as all 50 RT-PCR negative samples were correctly identified as RDT-negative. The NLR for both viruses was 0.1 (95% CI, 0.04–0.23), and κ was 0.90 (95% CI, 0.81–0.99) (**Table 6**).

4. Discussion

With the increase in single-person households and an aging population worldwide, there is a growing interest in pet health [17]. Among pets, dogs represent the largest proportion [12], and digestive system diseases

Table 2. Limit of detection of rapid diagnostic test for CPV-2 and CCoV

CPV-2			CCoV		
Concentration ($TCID_{50}$/mL)	**Test 1**	**Test 2**	**Concentration ($TCID_{50}$/mL)**	**Test 1**	**Test 2**
2.0×10^5	Pos	Pos	2.0×10^5	Pos	Pos
1.0×10^5	Pos	Pos	1.3×10^5	Pos	Pos
5.0×10^4	Pos	Pos	6.3×10^4	Pos	Pos
2.5×10^4	Pos	Pos	3.1×10^4	Pos	Pos
1.3×10^4	Pos	Pos	1.6×10^4	Pos	Pos
6.2×10^3	Pos	Pos	7.9×10^3	Pos	Pos
3.1×10^3	Pos	Pos	3.9×10^3	Pos	Pos
1.6×10^3	Pos	Pos	2.0×10^3	Pos	Pos
7.8×10^2	Pos	Pos	9.8×10^2	Pos	Pos
3.9×10^2	Pos	Pos	4.9×10^2	Pos	Pos
1.9×10^2	Pos	Pos	2.5×10^2	W+	W+
9.7×10	W+	W+	1.2×10^2	Neg	Neg
4.9×10	Neg	Neg			

Abbreviations: CPV-2, canine parvovirus type 2; CCoV, canine coronavirus; $TCID_{50}$, 50% tissue culture infectious dose; Pos, positive; Neg, negative; W+, weakly positive.

Table 3. Measurement of five concentrations to confirm the limit of detection for CPV-2 and CCoV

CPV-2				CCoV			
Concentration ($TCID_{50}$/mL)	**Day 1**	**Day 2**	**Day 3**	**Concentration ($TCID_{50}$/mL)**	**Day 1**	**Day 2**	**Day 3**
7.8×10^2	Pos (20/20)	Pos (20/20)	Pos (20/20)	2.0×10^3	Pos (20/20)	Pos (20/20)	Pos (20/20)
3.9×10^2	Pos (20/20)	Pos (20/20)	Pos (20/20)	9.8×10^2	Pos (20/20)	Pos (20/20)	Pos (20/20)
1.9×10^2	Pos (20/20)	Pos (20/20)	Pos (20/20)	4.9×10^2	Pos (20/20)	Pos (20/20)	Pos (20/20)
9.7×10	W+ (20/20)	W+ (20/20)	W+ (20/20)	2.5×10^2	W+ (20/20)	W+ (20/20)	W+ (20/20)
4.9×10	Neg (20/20)	Neg (20/20)	Neg (20/20)	1.2×10^2	Neg (20/20)	Neg (20/20)	Neg (20/20)

Results were repeated 20 times per day for 3 days. Abbreviations: See **Table 2**.

Table 4. Cross-reactivity of rapid diagnostic test for CPV-2 and CCoV

	CDV	**BPV**	**PPV**	**CPIV**	**CAV-1**	**CAV-2**	**CHV**	***E. coli***	***S.* Paratyphi**
CPV-2	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
CCoV	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Abbreviations: CPV-2, canine parvovirus type 2; CCoV, canine coronavirus; CDV, canine distemper virus; BPV, bovine parvovirus; PPV, porcine parvovirus; CPIV, canine parainfluenza virus; CAV-1, canine adenovirus type 1; CAV-2, canine adenovirus type 2; CHV, canine herpesvirus; *E. coli*, *Escherichia coli*; *S.* Paratyphi, *Salmonella* Paratyphi; Pos, positive; Neg, negative.

Table 5. Interference of rapid diagnostic test for CPV-2 and CCoV

	Whole blood		**Bilirubin**		**Lipid**		**Cholesterol**	
	+	-	+	-	+	-	+	-
PBS	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
CPV-2	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
CCoV	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Abbreviations: CPV-2, canine parvovirus type 2; CCoV, canine coronavirus; PBS, phosphate buffered saline; +, including interference materials; -, excluding interference materials; Pos, positive; Neg, negative.

Table 6. Comparison of diagnosis results for CPV-2 and CCoV from RDT with PCR and RT-PCR

CPV-2/CCoV	PCR/RT-PCR		Total
	Positive	Negative	
RDT			
Positive	45	0	45
Negative	5	50	55
Total	50	50	100

Abbreviation: CPV-2, canine parvovirus type 2; CCoV, canine coronavirus; PCR, polymerase chain reaction; RT-PCR, reverse-transcription polymerase chain reaction; RDT, rapid diagnostic test.

are the primary cause of dog clinic visits and deaths [18,19]. Symptoms of digestive system diseases, such as diarrhea and vomiting, are particularly common in dogs under the age of three [18]. Severe diarrhea can lead to fatal dehydration, making it essential to identify the causative pathogen for proper treatment [1,3]. CPV-2 and CCoV are major pathogens of acute gastroenteritis with a significant association with diarrhea [1,2], emphasizing the importance of evaluating the clinical utility of RDT for CPV-2/CCoV diagnosis. According to previous studies, CPV-2 was detected in clinical samples at a concentration of approximately 106 $TCID_{50}$/mL [20], and CCoV was detected at an equivalent virus titer of 1×10^{6} $TCID_{50}$/mL [21]. While the detection of a small quantity of virus may be challenging depending on the sample collection method or the disease stage [22,23], the LoD evaluation results suggest that the virus titers in clinical samples are sufficiently detectable.

RDTs that can detect more than one virus on a single strip are more cost-effective and efficient in terms of sample volume, cost, and time compared to RDTs designed for the detection of a single virus [24,25]. However, RDTs with multiple test lines, excluding the control line, within a single strip may potentially pose issues related to cross-reactivity and interference due to the narrow spacing between test lines, as they rely on antigen-antibody interactions [24,25]. In this study, cross-reactivity between CPV-2 and CCoV was not observed despite using high concentrations of both viruses during the LoD experiment. This result suggests that the use of antibodies specific to the virus antigen being targeted for detection, including those used in this study, prevents cross-reactivity between CPV-2, CCoV, and other pathogens.

The results of RDTs may be influenced by the presence of a large number of antigens required for visual confirmation and may be affected by the host's immune response, leading to virus clearance or dilution due to diarrhea [10,22]. In contrast, PCR and RT-PCR, utilizing the principle of nucleic acid amplification, enable the detection of trace amounts of nucleic acids [14]. Moreover, in cases of viral infections, infected cells usually contain the nucleic acid of the respective virus, allowing relatively higher sensitivity of nucleic acid amplification compared to RDTs [22]. Several studies comparing nucleic acid amplification and RDTs have shown that CPV-2 RDT had sensitivity ranging from 22.2% to 95.4% and specificity from 71.4% to 100.0% [8-10]. CCoV RDT, on the other hand, exhibited a sensitivity of 93.1% (95% CI, 83.3–98.1) and specificity of 97.5% (95% CI, 92.9–99.5) [26]. It is believed that the differences in antigen-antibody binding affinity due to the antibodies used in the RDTs may have influenced the sensitivity of the RDT [27].

The LR is a statistic that indicates the accuracy of a diagnostic test and is not influenced by the prevalence of the disease [9,28]. The NLR is considered to be very useful if it is less than 0.1 and less useful if it is greater than 0.5 [8,9]. In this study, the RDT exhibited an NLR of 0.1, indicating good performance. The positive likelihood ratio (PLR) is considered very useful if it is greater than 10 and less useful if it is less

than 2. However, as the RDT used in this study has a specificity of 100%, making the denominator zero, PLR cannot be calculated [8,9]. The κ statistic measures the agreement between two diagnostic methods, and a value of 0.60 or higher is generally considered good agreement [8,9,29]. According to previous studies, for CPV-2 RDT, PLR ranged from 0 to 10.18, NLR from 0.07 to 0.78, and κ from 0.03 to 0.67 [8-10], while for CCoV RDT, PLR was 37.2 (95% CI, 12.26–15.05), NLR was 0.07 (95% CI, 0.03–0.18), and κ was 0.91 (95% CI, 0.85–0.98) [26].

In conclusion, the Genbody CCV/CPV RDT demonstrated high sensitivity, specificity, κ values, and low NLR for the detection of both viruses. Therefore, it is considered useful as a screening test for the early detection and subsequent management of these infections. However, there are several limitations to this study. First, the limited number of CCoV-positive samples necessitated the use of spiking samples for evaluation. When the number of clinical samples is insufficient, performance evaluation can be conducted using spiking samples [30]. Nevertheless, further research with an adequate number of clinical samples is needed. Second, the viral titers of the clinical samples used for the evaluation were not confirmed. Future studies utilizing samples with a variety of disease stages and confirmed viral titers would enhance the reliability of the test.

Disclosure statement

The authors declare no conflict of interest.

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