Rapid Point-of-Care testing for feline and canine coronavirus (FCoV/CCoV) using the portable isothermal loop-mediated nucleic acid amplification PLUSLIFE platform

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Laboratory Study Report: Evaluation of sensitivity and specificity of a POC - FCoV/CCoV test based on nucleic acid identification

System investigated: Pluslife Mini Dock (Guangzhou Pluslife Biotech, Guangzhou, China)

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1. INTRODUCTION

The feline coronavirus (FCoV) and canine coronavirus (CCoV), members of the genus *Alphacoronavirus* of the subfamily *Coronavirinae*, family *Coronaviridae*, order *Nidovirales*, belong to a group of *envelope-bearing* viruses that possess a monocatenary positive-polarity RNA 27-32 kb in length. FCoV infection is widespread in overcrowded environments with high density of cats where transmission occurs mainly through the oro-fecal cycle. Most FCoV-infected cats develop asymptomatic infection or show mild enteritis through which they shed the virus externally but only a small proportion will develop Feline Infectious Peritonitis (FIP). The reason why FIP develops in some cases is because of one or more mutations that the virus undergoes in its replication process whereby it acquires the ability to replicate within macrophages in the presence of a favorable immune setup, characterized by a weak cell-mediated response and a strong humoral response.

CCoV is responsible for enteritis of varying severity in puppies between 6 and12 weeks of age, probably coinciding with a decrease in maternal antibody titer. In puppies it is responsible for vomiting and diarrhea, anorexia, dehydration, and sometimes death. The virus replicates in enterocytes, and its excretion with feces may continue for up to 150 days post infection. It is, therefore, necessary in both cases to obtain an etiologic diagnosis based primarily on the use of direct diagnostic tests, that is, aimed at finding the pathogen, antigen, or viral genome. The most widely used approach to date is based on qualitative or quantitative RT-PCR (*Reverse Transcriptase- Polymerase Chain Reaction*) methods (*Real-Time RT-PCR*, qRT-PCR), which are commonly used to identify target RNA sequences from fecal samples or effusions in the case of FIP. In addition, etiologic diagnosis is also critical for rapid intervention with proper prophylaxis to prevent the spread of infection.

2. OBJECTIVE OF THE STUDY

In this report, we report the results of a preliminary study aimed at evaluating the sensitivity and specificity of a POC - FCoV/CCoV test based on the identification of FCoV and CCoV RNA using Pluslife Mini Dock device (Pluslife FCoV/CCoV Card).

3. MATERIALS AND METHODS

3.1 Sampling

Nine samples of enteric origin, represented by rectal swabs, from 6 cats and 3 dogs were submitted for analysis during the period from March 2023 to March 2024. In addition, 7 ascitic fluid samples were collected from 7 cats presenting with abdominal effusion on clinical examination, during the period from July 2022 to June 2024. All animals were hospitalized at the University Veterinary Teaching Hospital (OVUD) of the Department of Veterinary Medicine of the University of Teramo, and at the time of sampling, an anamnestic survey form was completed for each animal under examination in which information regarding current symptomatology and any drug or vaccine treatments were collected.

3.2 Molecular screening by real time RT-PCR (qRT-PCR) for FCoV and qualitative RT-PCR for CCoV

A total of 200 µl of ascitic fluid or PBS in which each rectal swab had been resuspended was subjected to nucleic acid extraction using ZymoBIOMICS MagBead DNA/RNA commercial kit (Zymo Research, USA).

For FCoV identification, each RNA extract (2.5 µl) was tested using a previously validated qRT-PCR procedure for FCoV (Gut et al., 1999). The qRT-PCR reactions, set up using the TaqMan™ Fast Virus 1-Step Master Mix (Applied Biosystem, Foster, CA, USA), were carried out in the "StepOne" thermal cycler (Applied Biosystem, Foster, CA, USA) under the thermal conditions given in the reference bibliography. A standard curve was generated using logarithmic dilutions from 10^{10} to 10^{0} copies per reaction, cloning the fragment obtained in qualitative RT-PCR, within the pCR 2.1 vector (TA Cloning Kit, Invitrogen, Ltd, Milan, Italy).

For CCoV identification, each RNA extract (2.5 µl) was tested using a previously validated method of nested RT-PCR for CCoV (Pratelli et al., 1999). The nested RT-PCR reactions, set up using the SuperScript™ III One-Step (Applied Biosystem, Foster, CA, USA) and GoTaq® Green Master Mix (Promega Italia S.r.l, Milan, Italy), were carried out in the "SimpliAmp" thermocycler (Applied Biosystem, Foster, CA, USA) at the thermal conditions indicated in the reference bibliography.

3.3 Analysis performed using POC "FCoV/CCoV Nucleic Acid test (PLuslife FCoV/CCoV Card)" and Pluslife Mini Dock device

For analysis using the Pluslife Mini Dock device, each swab was resuspended in the lysis buffer provided by the FCoV/CCoV Nucleic Acid Test Card kit (Pluslife FCoV/CCoV Card), and the procedures specified by the manufacturer were performed. Samples found to be invalid or for which further analysis was necessary were subjected to dilution. The following Pluslife Mini Dock devices were used to perform the analyses:

- Device 1 Serial No.: 2247040335;
- Device 2 Serial No.: 2247040336.

3.4 Data analysis

To evaluate the concordance between the results of RT-PCR, qRT-PCR and the results obtained at the POC Pluslife test, the following parameters were calculated:

- *Positive Percent* Concordance (*Positive Percent Agreement-PPA*, sensitivity), calculated by evaluating the number of positive samples in RT-PCR and qRT-PCR, which are also correctly detected as "positive" by the POC test;
- Negative *Percent* Concordance *Agreement-NPA* (specificity), calculated by evaluating the number of negative samples in RT-PCR and qRT-PCR, which are also correctly detected as "negative" by the POC test;
- Overall diagnostic accuracy was calculated by determining the number of samples in which both analyses were in agreement.

4 RESULTS

FCoV-specific qRT-PCR tests performed on the 13 samples of feline origin showed 9 samples (69.2%, 9/13) as positive for FCoV and 4 samples negative (4/13, 30.8%). Specifically, the positive samples showed threshold cycle (Ct) values ranging from 13.34 to 35.98 (mean Ct: 28.55), with viral loads ranging from 1.44×10^8 to 9.01×10^1 RNA copies/2.5 µl extract (mean 1.60×10^7 DNA copies/2.5 µl extract). In detail, the test performed showed 6 of 7 abdominal spills (85.7%) and 3 of 6 rectal swabs (50%) as positive.

The analysis of the 13 samples performed with the Pluslife "FCoV/CCoV Nucleic Acid" POC test identified 7 (53.7%, 7/13) positive samples, 5 (38.5%, 5/13) negative samples, and one sample (7.8%, 1/13) was found to be

invalid. Specifically, of the 7 effusions, 4 were positive (57.1%, 4/7), 1 invalid (14.3%, 1/7) and 2 negative (28.6%, 2/7), while of the 6 rectal swabs, 3 were positive (50%, 3/6).

The results thus obtained from the analysis with the Pluslife "FCoV/CCoV Nucleic Acid" POC test were compared with the results obtained in the screening performed with the specific qRT-PCR procedure.

In detail, it was found that of the 9 qRT-PCR positive samples, the Pluslife POC assay detected 7 positive samples with Ct values ranging from 13.34 to 35.98 (mean Ct: 27.43) and viral loads ranging from 1.44×10^8 to 9.01×10^1 RNA copies/2.5 µl of extract (mean 2.05×10^7 RNA copies/2.5 µl of extract). One sample (No. 5), with a Ct of 32.95 and viral load of 8.30×10^2 /2.5 µl of extract, was negative, while one sample (No. 6) with a Ct of 32.15 and viral load of 1.05×10^3 /2.5 µl of extract, was invalid, and in both cases it was effusion. Thus, the calculated sensitivity, expressed as PPA, was 77.8% (7/9).

Of the 4 negative samples in qRT-PCR, 4 were also negative in POC Pluslife test, so the calculated specificity, expressed as NPA, would be 100% (4/4).

The results of tests performed by qRT-PCR and Pluslife "FCoV/CCoV Nucleic acid" POC test for FCoV RNA identification are shown in Table 1.

Table 1

CAT ID.	QUANTITATIVE RT-PCR (Gut et al., 1GG).		PLUSLIFE DEVICE
	Ct	Quantity	
ASCITIC FLUID			
1	27.0	6,50E+03	POS
2	26.17	1,19E+04	POS
3	35.98	9,01E+01	POS
4	13.34	1,44E+08	POS
5	32.95	8,30E+02	NEG
6	32.15	1,05E+03	INVALID
7	NEG	NEG	NEG
RECTAL SWAB			
8	31.18	1,30E+03	POS
9	27.1	6,49E+03	POS
10	31.25	1,29E+03	POS
11	NEG	NEG	NEG
12	NEG	NEG	NEG
13	NEG	NEG	NEG

CCoV-specific RT-PCR tests performed on the 3 dog enteric samples revealed one sample (33.3%, 1/3) as positive for the presence of CCoV RNA and 2 negative samples (2/3, 66.7%).

Analysis of the 3 samples performed with the Pluslife "FCoV/CCoV Nucleic Acid" POC test identified one (33.3%, 7/13) positive sample and 2 (66.7%, 2/3) negative samples.

The results thus obtained from the analysis with the Pluslife "FCoV/CCoV Nucleic Acid" POC test were compared with the results obtained in the screening performed with the specific RT-PCR procedure. The positive sample in RT-PCR for CCoV was also positive in the Pluslife POC test (1/1), which also confirmed the negativity of the remaining 2 negative samples in RT-PCR (2/2), so the calculated sensitivity, expressed as PPA, and the calculated specificity, expressed as NPA, were both 100%.

The results of the tests performed by RT-PCR and Pluslife "FCoV/CCoV Nucleic acid" POC test for the identification of CCoV RNA are shown in Table 2

Table 2

DOG ID.	QUANTITATIVE RT-PCR (Pratelli et al., 1GG)	PLUSLIFE DEVICE
14	POS	POS
15	NEG	NEG
16	NEG	NEG

Finally, the overall diagnostic accuracy, calculated on the basis of the number of samples in which both analyses agreed, was 87.5 percent (14/16), respectively.

5. DISCUSSIONS

In this study, the sensitivity and specificity of a POC - FCoV/CCoV test based on the identification of FCoV and CCoV RNA using Pluslife Mini Dock device was evaluated. For this purpose, the results obtained from the analysis of 13 samples of feline origin performed by specific qRT-PCR procedure for FCoV, 3 enteric samples of dog investigated by using qualitative RT-PCR for CCoV, and the results obtained from the analysis of the same by Pluslife Mini Dock device and POC test "FCoV/CCoV Nucleic Acid" (PLuslife FCoV/CCoV Card) were compared.

In total; of the 16 samples tested, the Pluslife POC test identified 8 samples (7 of feline origin and 1 of canine origin) as positive. Of these, the 7 samples of feline origin were positive in qRT-PCR for FCoV while the sample of canine origin was positive in RT-PCR for CCoV. Considering the total number of positive samples detected in both methods and excluding the feline-origin sample found to be invalid by Pluslife POC, the sensitivity, understood as PPA, of the POC test would be 87.5% (7/8).

Regarding the samples that tested negative, there were 6 total negative samples in the molecular procedures, specifically 4 cat samples were negative at qRT-PCR for FCoV and 2 dog samples were negative at RT-PCR for CCoV. Thus, considering the total negative samples identified in both methods with the Pluslife POC, the specificity, understood as NPA, of the POC test would be 100% (6/6).

In conclusion, based on the results obtained and excluding the invalidated bell, the overall diagnostic accuracy of the POC test "FCoV/CCoV Nucleic Acid" (PLuslife FCoV/CCoV Card) calculated on the basis of the number of samples in which both tests are in agreement was 93.3% (14/15), respectively.

6. BIBLIOGRAPHY

Gut M, Leutenegger CM, Huder JB, Pedersen NC, Lutz H. One-tube fluorogenic reverse transcription- polymerase chain reaction for the quantitation of feline coronaviruses. J Virol Methods. 1999 Jan;77(1):37-46. doi: 10.1016/s0166-0934(98)00129-3.

Pratelli A, Tempesta M, Greco G, Martella V, Buonavoglia C. Development of a nested PCR assay for the detection of canine coronavirus. J Virol Methods. 1999 Jun;80(1):11-5. doi: 10.1016/s0166-0934(99)00017-8.