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REAL TIME PCR UTILITY, PROVED IN THIRTEEN FELINE INFECTIOUS PERITONITIS SUSPICION CASES

UTILITATEA REAL TIME PCR, DOVEDITĂ ÎN TREISPREZECE CAZURI DE SUSPICIUNE DE PERITONITĂ INFECȚIOASĂ FELINĂ

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ABSTRACT

Feline infectious peritonitis (FIP) is a worldwide disease of domestic and wild cats of all ages, with the outcome almost invariably fatal. In the aetiology of FIP, different strains of Feline coronavirus (FCoV) are involved. FIP is difficult to diagnose since some clinical indications are ambiguous and comparable to those described in other feline disorders, and laboratory tests available today cannot distinguish between FIP-causing strains and feline enteric coronavirus strains. Many cats do not acquire FIP illness even when infected with recognised FIP-causing strains. The variables that cause one cat to get ill while another remain healthy are unknown. Moreover, even modern polymerase chain reaction assays (PCR) designed to identify viral genetic material are incapable of distinguishing between coronavirus strains. However, the findings of PCR testing on fluid from a suspected FIP cat's belly might help to rule in or rule out the condition. PCR testing of blood from a suspected case is less definitive in diagnosing FIP, although it might be beneficial in specific cases for the practising veterinarian. In this paper, thirteen samples were analysed using RT-PCR from cats, different in age and breed. Our results support the utility of the PCR technique in FIP diagnostic protocols by providing evidence of FCoV infection on ascitic fluid or blood samples taken from cats with clinical suspicion of FIP.

Keywords: Feline Coronavirus, Real Time PCR, Feline Infectious Peritonitis

REZUMAT

Peritonita infecțioasă felină (PIF) este o boală răspândită pe tot globul care afectează pisicile domestice și sălbatice de toate vârstele, cu deznodământ aproape invariabil fatal. În etiologia PIF sunt implicate diferite tulpini de Feline coronavirus (FCoV). PIF este dificil de diagnosticat, deoarece unele indicații clinice sunt ambigue și comparabile cu cele descrise în alte boli ale pisicilor, iar testele de laborator disponibile astăzi nu pot face distincția între tulpinile care provoacă FIP și tulpinile de coronavirus enteric felin. Multe pisici nu dezvoltă PIF chiar și atunci când sunt infectate cu tulpini recunoscute că produc PIF. Variabilele care fac ca o pisică să se îmbolnăvească în timp ce alta rămâne sănătoasă sunt necunoscute. Chiar și testele moderne de diagnostic precum tehnica PCR, care au fost concepute pentru a identifica materialul genetic viral, sunt incapabile să facă distincția între diferitele tulpini de coronavirus. Cu toate acestea, rezultatele testării PCR pe probe de lichid ascitic prelevat de la pisici cu suspiciune de PIF, ar putea ajuta în diagnostic. Deși ar putea fi benefică în anumite cazuri, testarea prin PCR a probelor de sânge prelevate de la pisici cu suspiciune clinică de PIF este mai puțin utilă medicul veterinar practician. În această lucrare, treisprezece probe biologice prelevate de la pisici de diferite rase și vârste au fost analizate folosind RT-PCR. Rezultatele obținute susțin utilitatea tehnicii PCR în protocoalele de diagnosticare PIF, oferind dovezi ale prezenței FCoV în probele de lichid ascitic sau sânge prelevate de la pisici cu suspiciune clinică de FIP.

Cuvinte cheie: Feline Coronavirus, RT-PCR, peritonita infecțioasă felină

Even if, in the past few years, more and more cases of feline infectious peritonitis (FIP) have been reported, the disease remains one with many question marks regarding its diagnosis. Certain strains of the Feline Coronavirus, belonging to the *Nidovirales* order, the *Coronaviridae* family, and the *Alphacoronavirus* subfamily, have been linked to the disease known as FIP. Alphacoronoviruses include Transmisible Gastroenteritis Virus (TGEV), a swine pathogen virus, Porcine Respiratory Coronavirus (PRCV), Canine Coronavirus (CCoV), and Feline Coronavirus (FCoV). FCoV is widely spread and, in most cases, is responsible for mild enteritis symptoms, but in about 5% of cases, the virus spreads systemically and causes feline infectious peritonitis (11).

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FCoV has two serotypes: FCoV1 and FCoV2. The second variant is a combination between FCoV1 and CCoV (8). Feline coronavirus has a microscopic appearance similar to other viruses in this family, and it is described as a spherical, pleomorphic particle, enveloped, roughly 100 nm in diameter, with peplomers that give it a crown microscopic look. The genetic material consists of one RNA strand, positive sense, produced by roughly 30000 nucleotides (6, 7).

FCoV is usually spread through faeces that can contaminate water, feed, and the environment (6, 18). Ingestion of the virus from environment is possible, enterocytes are linked to the early stages of infection. The virus replicates in macrophages and monocytes. This will cause widespread infection, at which time mutations may emerge. Viremia in the plasma can be decreased within a few days (6, 17, 18). The antibodies enhance the viral absorption and the replication of the virus leads to a antibodies-mediated reaction. The immune system is very important, as the reactivity is different and for that cats that carry the virus without any clinical sign may shed the virus in tissular macrophages, lymph nodes and the viral RNA was found even in the Kupffer cells in the liver (10, 14, 17). Seven days after infection, cats begin to eliminate the virus and may remain viral reservoirs and eliminators for the rest of their lives (1, 2, 12, 13, 19).

There are two types of feline infectious peritonitis: effusive and non-effusive (dry or pyogranulomatous). The most severe and common kind is the effusive (wet) form. It is found in around 70% of instances. Fever (more or less persistent and consistent), dehydration, lethargy, and the formation of a protein-rich exudate in the abdomen and/or thoracic cavities are the symptoms. The patient eventually becomes cachectic, with dyspnoea and tachypnoea. The elevated protein content in the exudates causes exudative inflammation of the serous membranes and adhesion (4, 5, 7, 13). Identifying the dry-form (non-effusive) can be challenging due to the absence of specific clinical markers. Patients with the dry-form typically present with a range of symptoms, including fever, loss of appetite, anorexia, diarrhoea and vomiting, anaemia, icterus, and cachexia. Vasculitis and pyogranulomatous inflammation are common lesions. The most frequently observed anatomopathological abnormalities are granulomatous inflammation in the liver, spleen, renal epiploon, lungs, and lymph nodes. Cats may also develop ocular or neurologic forms of the disease, with uveitis being recorded in cats with the ocular type and white precipitates observed in the anterior chamber of the eye. Ocular symptoms are present in around 50% of cats with non-effusive types, while neurological symptoms can be detected in approximately 25% of cats with the dry-form. The main symptoms in the neurological form are depression, seizures, ataxia, nystagmus or paresis (1-5, 7, 13, 16).

Diagnosing FIP can be challenging, as some symptoms are similar to those seen in other feline disorders. Additionally, current laboratory tests cannot differentiate between FIP-causing strains and other types of coronaviruses in cats. Interestingly, some cats do not develop FIP even when infected with known FIP-causing strains, and the reasons behind this are still unknown. While modern PCR tests can identify viral genetic material, they are not always able to distinguish between different coronavirus strains. However, PCR testing on fluid from a cat's belly may provide valuable insight into whether FIP is present or not, whereas blood testing is less conclusive but may still be useful in certain cases. In this paper, thirteen samples from cats with clinical suspicion of FIP are analysed using RT-PCR.

Table 1

Samples used from cats with FIP suspicion in the RT-PCR testing

Sample #	Biological sample	Breed	Age	Sex
1	Blood	Siamese	1 year	Male
2	Ascitic liquid	European	5 years	Male
3	Blood	British Shorthair	1 year	Male
4	Ascitic liquid	European	2 years	Male
5	Blood	European	1 year	Male
6	Blood	European	1 year	Male
7	Blood	Sphynx	2 years	Male
8	Blood	British Shorthair	6 months	Female
9	Blood	European	3 months	Female
10	Blood	European	4 years	Male
11	Blood	British Shorthair	2 years	Female
12	Blood	Maine Coon	2 years	Female
13	Blood	European	6 months	Male

MATERIALS AND METHODS

Animals and Biological samples

The attending veterinarian collected and labelled samples from all 13 cats. The blood was collected in sterile EDTA tubes, while the ascitic liquid samples were collected in vials without additives. The cats had varying breeds, with seven being European, three British Shorthair, one Siamese, one Sphynx, and one Maine Coon. Their ages ranged from three to 6 months for three of the patients, while four cats were one year old, three cats were two years old, one cat was four years old, and the last one was five years old (Table 1). The samples were refrigerated until primary processing.

Real Time PCR

Real-time PCR is an incredibly precise and sensitive diagnostic method. It involves amplifying a specific sequence of nucleotides using particular primers unique to each pathogen.

The RNA extraction

RNA extraction was carried out manually using QIAGEN'S QIAamp Cador Pathogen Mini Kit (9). This step is non-specific, meaning that all RNA categories in the biological sample are isolated. The most commonly used RNA extraction technologies rely on selectively binding silica membranes or the adsorption of nucleic acids on the surface of paramagnetic particles. At least 200 µl of EDTA blood or ascitic liquid is needed to perform the test. QIAGEN'S QIAamp Cador Pathogen Mini Kit is used to extract DNA and RNA from blood, plasma, other fluids, and tissues. It includes various components such as collection tubes, K-proteinase, VXL buffer, ACB buffer, AW1 and AW2 buffers, and AVE buffer. Additional materials used include an Eppendorf centrifuge, incubator, thermoshaker, vortex, and -80°C freezer. To conduct real-time PCR, QIAGEN'S Fast Cycling Master Mix, 5x-Q Solution, and forward and reverse primers were utilized (Table 2).

These methods provide numerous advantages, such as fast acquisition of nucleic acids with high purity levels suitable for most applications, an efficient and user-friendly workflow, and the added convenience of automated extraction. To carry out the reaction, the SmartCycler Analyzer was utilized. The PCR process entails three crucial steps: denaturation for 5 minutes at 95°C, amplification for 30-40 cycles, involving denaturation (5 seconds at 96°C), annealing (5 seconds at 60°C) to facilitate primer binding to the targeted sequence, and extension (3 seconds at 68°C) to enable Taq polymerase to create new RNA strands by adding nucleotides. Finally, during one cycle lasting 1 minute at 72°C, extension occurs.

RESULTS AND DISCUSSIONS

Case #1: A 1-year-old male Siamese cat has been displaying symptoms of apathy, fever, and weakness for several weeks. As no effusions were detected, a blood sample was submitted for laboratory testing. The real-time PCR result revealed a CT value of 25.7 (Fig. 1).

Case #2: A 5-year-old male European feline presented with fever, vomiting, and abdominal distension. Ascitic fluid was submitted and tested with RT-PCR, with a Ct value of 24.64 (Fig. 1).

Case #3: A 1-year-old male British shorthair feline had a coronavirus genome detected in its EDTA-blood sample with a low CT value of 25.10 (Fig. 1).

Case #4: A 6-month-old female British shorthair with fever, dyspnea, and anaemia had an EDTA blood sample collected, and no viral genome was detected.

Case #5: A 2-year-old male European cat has been showing signs of fever, lethargy, abdominal distention, anorexia, prostration, and anaemia. The ascitic liquid has been sent to the laboratory for testing, and a CT value of 27.7 has been obtained (as shown in Fig. 2). This value indicates a medium viral charge in the biological sample submitted.

Case #6: A 1-year-old male European cat is exhibiting clinical signs of fever, lethargy, anorexia, and slight anaemia. A blood sample was analysed for the RT-PCR test since there were no effusions present.

Table 2

Final Volumes for RT-PCR reaction

Reactive	Volume	Final Concertation
QIAGEN Fast Cycling PCR Master Mix	20µl	Hotstart Plus AND polymerase, 1x fast Cycling Buffer, Mg^2 optimized contraction
5x Q Solution	4 µl	1x
10x Coral Load Dye (optional)	2 µl	1x
Primer forward	Variable	0.1-1.0 µM
Primer reverse	Variable	0.1-10 µM
Rnase-Free water	Variable	
Sample	Variable	

The CT value after the PCR was performed was 29.9, indicating a low viral quantity in the sample.

Case #7: A 3-month-old female European cat had diarrhoea,fever,and dehydration.An EDTA blood sample was taken for RT-PCR, but the result was negative.

Case #8: A 4-year-old male European feline presented with lethargy, fever, vomiting, dyspnea, and dehydration. Blood samples were taken, but the results were negative.

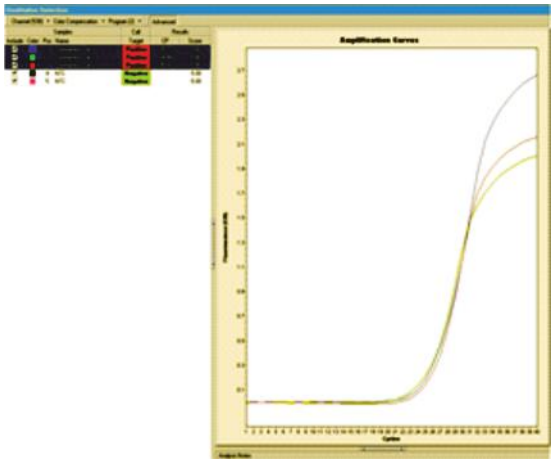

Fig. 1. Ct values of felines #1, #2, and #3 (Ct value #1= 25.7, Ct value #2= 24.64, and Ct value # 3= 25.10)

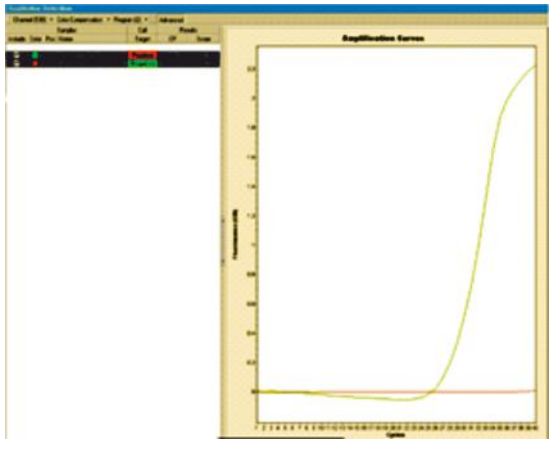

Fig. 2. Ct values of felines # 5 (Ct value # 5 = 27.70)

Case #9: A 1-year-old male European cat presented with lethargy, fever, vomiting, dyspnea, and dehydration. The viral genome was detected, but in low quantity (22.67 value) (Fig. 3).

Case #10: A 2-year-old female British Shorthair cat presented with lethargy, severe fever, vomiting, dyspnea, and dehydration. RT-PCR analysis was negative.

Case #11: A 2-year-old female Maine Coon presented with lethargy, severe fever, and dehydration. The RT-PCR test was negative for coronavirus RNA.

Case #12: A 2-year-old male European feline presented with a severe fever, lethargy, and dehydration. RT-PCR testing was negative, and no coronavirus RNA was detected.

Case #13: A 2-year-old male Sphinx cat presented with apathy, anorexia, fever, and slight dehydration. An EDTA blood sample was tested by RT-PCR, and a low viral level of the coronavirus genome was detected with a CT value of 32.48.

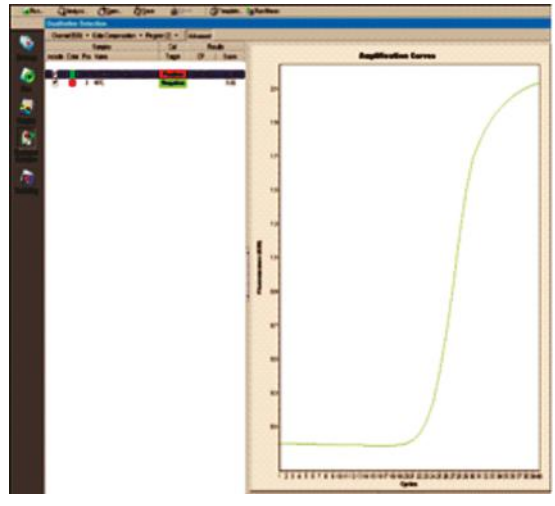

Fig. 3. Positive result: Ct value 22.67

According to the study's results, a noteworthy percentage of the felines examined, more than 76%,have surpassed their first year of life. Furthermore, approximately 23% of the cats under observation belonged to the age bracket of three months to one year (Fig. 4).

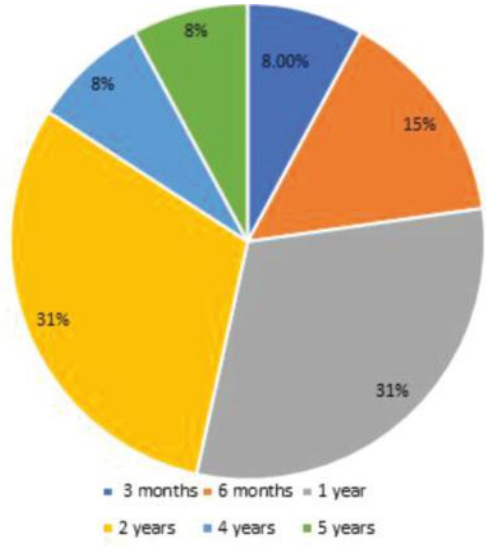

Fig. 4. Age of cats tested for feline coronavirus infections by RT PCR

The European cat breed was involved in 54% of the cases, followed by the British shorthair breed, which

made up 23% of the cases. Other breeds accounted for 7% each. After the testing, it was observed that 54% of the results were positive, and the remaining 46% had a negative result. Of all the cases, 80% had a dry form of FIP, and 15% had an effusive form of the disease.

When it comes to diagnosing FIP, the test's specificity is more important than sensitivity. This is because a high specificity helps to prevent misdiagnosis of FIP in cats, which can lead to unnecessary euthanasia. In Porter et al. (2014) study, the specificity of real-time RT-PCR in effusion was found to be 95.8%. However, it's worth noting that one control cat with chronic kidney disease tested positive for the FIPV pathotype in an effusion sample. This could be due to the presence of FCoV spike protein mutations that have previously been identified as markers for the systemic spread of the virus, rather than the FIP phenotype. These mutations have been found in tissue samples of healthy cats that were infected with FCoV (15). Our findings suggest that in cats exhibiting symptoms consistent with feline infectious peritonitis (FIP), a positive FCoV RT-PCR result in their fluids or tissues may indicate an active FIP infection. However, if a cat is clinically healthy but tests positive for FCoV RT-PCR in their tissues, it only suggests that they have been exposed to FCoV at some point.

CONCLUSIONS

The findings of the study indicate that a significant proportion of the felines tested, over 76%, were observed to have surpassed the first year of their life. Additionally, it was noted that around 23% of the cats under investigation fell within the age range of three months to one year. Out of the thirteen cats examined, nine tested positive for Coronavirus,representing 54% of the animals evaluated. The remaining four cats tested negative utilizing the real-time PCR method. If a cat is showing symptoms that match FIP, then a positive FCoV RT-PCR result in their fluids or tissues could mean that they have active FIP. However, if a clinically healthy cat has FCoV RT-PCR positive results in their tissues, it only indicates that they have been infected with FCoV.

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