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Research article

COMPARISON OF CLINICAL AND LABORATORY FINDINGS AT DIFFERENT CLINICAL STAGES IN CATS NATURALLY INFECTED WITH FELINE CORONAVIRUS

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Feline coronavirus (FCoV) infections occur commonly in cats, with entrocyte and monocyte-macrophage tropism. Most FCoV-infected cats remain asymptomatic, but up to 10% develop fatal feline infectious peritonitis (FIP). This study aims to investigate the diagnostic utility of clinical and laboratory examinations including serum and effusion AGP levels in cats either with symptomatic effusive FIP or asymptomatic feline enteric coronavirus (FECV). The study included 40 cats with effusive FIP and 10 cats with FECV infection. The FIP group was divided into two subgroups: abdominal (AE; n=30) and thoracic effusion (TE; n=10). Clinical and laboratory examinations, including serum or effusion AGP measurement, were performed. Among all the groups, TE group had higher body temperature, heart and respiratory rates ($P<0.000$). Compared with the FECV group, the FIP group had lower pH and HCO_3 levels and higher base excess and lactate levels ($P<0.05$). The leukocyte and lymphocyte counts were higher and the hematocrit was lower in the AE group among all the groups ($P<0.023$). MCV was lower in the FIP group compared to the FECV group ($P<0.002$). In the AE group, total protein level was the lowest and the AST, GGT, total bilirubin and cholesterol levels were the highest ($P<0.032$) among all the groups. Magnesium level was lower in the FIP group compared to the FECV group ($P<0.044$). Although the serum AGP level was highest in the TE group among all groups ($P<0.004$), the AGP levels of cats with FECV were similar to the AE group ($P>0.05$). Since FECV-positive cats will likely develop FIP, differences in clinical and laboratory findings in FECV-positive cats were identified. Among them, pH, HCO_3, base excess, lactate, MCV and magnesium were found to be important in the course of the disease, and AGP in the evaluation of the presence of an inflammatory state. It was concluded that clinical, laboratory and serum AGP evaluation could be used in the index of suspicion of development of FIP and FECV.

Keywords: Feline enteric coronavirus, feline infectious peritonitis, hematology, serum biochemistry, diagnosis

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INTRODUCTION

Feline coronaviruses (FCoV) are pleomorphic, enveloped, single-stranded and non-segmented RNA viruses [1]. There are two FCoV pathotypes, including feline enteric coronavirus (FECV), the ubiquitous enteric biotype, and feline infectious peritonitis virus (FIPV), the virulent biotype, causing feline infectious peritonitis (FIP) [1,2]. Inflammation plays a significant role in FIP infection, manifested by fibrinous serositis with highly proteinaceous body cavitiy effusions [2,3], disseminated pyogranulomatous formations and vasculitis [4]. In FECV infections, mild gastroenteritis [5,6] due to replication of FCoV in enterocytes [3] occurs. FIP has three major clinical forms, including the effusive (exudative), dry (non-effusive, non-exudative, granulomatous, parenchymatous) and mixed form. FIPV infection might affect many organs, including the intestines, liver, kidneys, eyes and central nervous system (CNS) [3,7] leading to variable clinical, laboratory and pathological manifestations, depending on presence of vasculitis, and the affected tissues [2,8].

The diagnosis of FIP is often challenging [1]. In effusive FIP, routine cytology and immunocytochemistry of effusion aspirates can be relatively easy to perform; in non-effusive forms, obtaining diagnostic samples is more challenging, requiring sonography-guided fine needle aspiration cytology or obtaining tissue biopsies when gross lesions are noted upon exploratory celiotomy. In the absence of a definitive diagnosis, FIP might be highly suspected based on signalment, clinical history, physical examination and laboratory findings [9]. Alhough routine laboratory tests will not confirm FIP, as abnormalities are often non-specific to FIP, such tests will help to exclude other conditions, and raise suspicion of FIP [8,10,11]. Lymphopenia was reported in 55-77% of FIP cases [10], while neutrophilia, in 39-55% [12]. Mild to moderate normocytic normochromic anemia occurs in 37-54% of FIP cases [1,13,14]. Serum chemistry abnormalities reported in cats with FIP include azotemia, hyperproteinemia (up to 60% of cases), hyperglobulinemia, hypoalbuminemia, hyperbilirubinemia (in 21-36% of effusive FIP cases) and increased liver enzyme activities [15-17]. In addition, alpha-1-acid glycoprotein (AGP), a positive acute phase protein that increases upon systemic inflammation and neoplasia, was reported to be diagnostically useful, albeit non-specific, in cats with experimentally-induced FIP [15,19]. Serum AGP concentration > 1.5 mg/mL is frequently encountered in cats with FIP [19].

Since FECV-positive cats will likely develop FIP, evaluation of clinical and laboratory findings during the different courses of FCoV-positive cats can provide information about the course of the disease. In addition, investigation of the inflammatory state in FECV-positive cats may allow for early intervention. Therefore, the present study was aimed to investigate the diagnostic efficacies of clinical and laboratory examinations including serum/effusion AGP levels that may be used to obtain a high index of suspicion of FECV and effusive FIP cases, especially in triage, which were confirmed by reverse transcription polymerase chain reaction (RT-PCR) either in feces or effusion samples.

MATERIALS AND METHODS

The study protocol was approved by the Faculty of Veterinary Medicine, Selcuk University Local Ethics Committee (Approval number: 2020/41). Informed consent was obtained by the owners of all cats enrolled in the study and permissions were obtained.

Animal selection and clinical examinations

The cats included in this study were all client-owned, 40 cats with a suspicion of effusive FIP (FIP group); 10 clinically healthy but FECV-positive cats (FECV group) presented to Selcuk University Veterinary Faculty Animal Hospital due to illness or routine health status screening. All cats underwent history taking, physical examination, laboratory testing and imaging examination (i.e., abdominal and thoracic ultrasonography and survey radiography). Cats with any comorbidity such as feline calicivirus (FCV), feline herpesvirus (FHV), feline immunodeficiency virus (FIV), feline leukemia virus (FeLV) and feline parvovirus (FPL) or with any other conditions that cause abdominal and/or thoracic effusions such as cardiac diseases, chyloabdomen and chylothorax or under treatment (antibiotics, corticosteroids, non steroidal anti-inflammatory drugs and immunomodulators) were excluded from the study. Cardiac disease, chyloabdomen and chylothorax were ruled out by examining the fluid's distinct appearances (modified transudate with a protein content usually ranges between 2.5 g/dl to 5.0 g/dl for cardiac disease, milky appearance for chyloabdomen and chylothorax). Viral diseases were ruled by rapid diagnostic test kits results performed according to the manufacturers' instructions which are explained below. The presence of FIP and FECV were confirmed by RT-PCR which is also explained below.

Clinical examination

After the physical examinations which include heart and respiratory rates, body temperature measurements and oral examination with capillary refill time (CRT) evaluation, all cats underwent both abdominal and thoracic ultrasonographic (Mindray Dc-6®, Shanghai, China) and radiographic (Medsinglong 630 mA 50kw X-ray Machine®, Guangdong, China) examinations. Ultrasonographic examinations were performed through the subxiphoid, splenorenal, hepatorenal and systocolic window for the abdomen; through the chest tube placement, bilateral dynamically focused pericardialand diaphragmatic region for the thorax using a 5.0 or 7.5 MHz microconvex probe. Radiographic examinations were performed using both laterolateral and ventrodorsal views and all sonographs and radiographs were reviewed by expert personnel.

Sampling and laboratory tests

Venous blood samples collected from all the cats via cephalic vein venipuncture, were placed in heparinized and K_3EDTA tubes and in tubes without anticoagulant

but with a gel separator. Thoracic and peritoneal effusion samples (5-10 mL) were collected with ultrasound-guidance with minimal animal restraint using a 25-gauge needle. Portions of the effusion samples were used for dipstick and refractometry analysis, and the rest was stored in eppendorfs at -80°C for RT-PCR analysis. Also, fecal samples were taken rectally in cases where there was no feces in the anus and perineum and stored at -80°C in phosphate buffered saline (PBS) (10% (w/v) faecal suspensions). Whole blood in K_3EDTA was used for complete blood count (CBC; MS4 CFE 279®, Haematology Analyzer, France) performed within 15 minutes from collection. Whole blood in heparin was used for blood gases and electrolyte analysis (ABL 90 Flex Blood Gas/Electrolyte Analyzer®, Model 5700 Radiometer, USA) and the tubes were sealed and stored in anaerobic conditions pending analysis which was performed within 5 minutes from collection. Blood in plain tubes was allowed to clot, centrifuged (10 minutes at 2000 g) and the harvested serum was used for biochemical analysis (Biotecnica BT 3000 Plus®, Italy), performed within 45 minutes from collection. Effusion samples were tested using dipsticks (URIT-31®, Accurex Biomedical, India) and refractometry (Aichose Brix refractometer®, Shenzhen, China). Serum and effusion AGP concentrations were measured using commercial feline-specific ELISA (Bioassay Technology Laboratory®, Zhejiang, China) following the manufacturer's instructions. According to the manufacturer, the assay has a detection range of 0.125-4 mg/mL, sensitivity: 0.31 mg/mL, intra-assay coefficient of variation: 4.8%, inter-assay coefficient of variation 9.8%). It was ensured that the whole physical examination and sampling time did not exceed 20 minutes [20,21].

Rapid serological tests

Additional serological tests were done on the serum and conjuctival and/or fecal swab samples, according the manufacturers' instruction, to minimize the likelihood of coinfection with additional viruses. These included feline calicivirus antigen (FCV Ag Asan Pharm®, Korea; sensitivity and specificity, 96% and 98% vs IFA, respectively), feline herpesvirus antigen (FHV Ag Asan Pharm®, Korea; sensitivity and specificity, 96.5% and 98% vs IFA, respectively), feline immunodeficiency virus antibody/ feline leukemia virus antigen (FIV Ab/FeLV Ag Asan Pharm®, Korea; sensitivity and specificity, 98% and 98.7% vs IFA, respectively) and feline parvovirus antigen (FPV Ag Asan Pharm®, Korea; sensitivity and specificity, 97.8% and 98.8% vs IFA, respectively) assays. All these tests were negative.

Viral RNA extraction using RT-PCR

All RT-PCR analyses were carried out in the central laboratory of Harran University (Figure 1). From cats with effusive FIP, using a QIAamp Viral RNA Kit (Qiagen®, Hilden, Germany), viral RNA was extracted from cell-free abdominal/thoracic effusions. In order to inactivate RNases and isolate the intact viral RNA, 140 µl aliquots of the samples were lysed under highly denaturing conditions. The silica membrane of the QIAamp® Mini spin column under modified buffering conditions grants optimal

binding. The RNA was eluted with 60 µl RNase-free buffer, washed twice with wash buffers and stored at -80°C. The one-step RT-PCR was carried out using the QuantiTect Probe RT-PCR kit (Qiagen®, Germany). All primers had a concentration of 0.8 µM, and 5'FAM/3'BHQ-1-labelled TaqMan probes (Applied Biosystems®, USA) had a concentration of 0.3 µM. In order to detect FCoV from effusion samples the procedure was performed as described previously [22,23]. In order to detect FCoV from cats in the FECV group, the same procedure was followed for RNA extraction using the QIAamp viral RNA mini kit which was described previously [23]. Viral RNA was extracted from 200 ml of a 10% faecal suspension in PBS using the QIAamp mini kit according to the manufacturer's instructions. Fecal samples were suspended in ASL buffer (Stool Lysis Buffer, Qiagen®, Germany) to remove inhibitory substances. After centrifugation (20 seconds at 16000 g) the substances were adsorbed. Following proteinase K (CAS 39450-01-6, BRENDA®) treatment, samples were bound to a silica-gel based capture membrane, washed, eluted in a low-salt buffer and stored at -80°C. 20 ml aliquot of extraction matrix was added to 10% fecal suspension, vortexed and suspended at room temperature for 15 minutes. The RNA-containing supernatant obtained following the boom method was evaluated with the Primer3 software package (University of Tartu, Estonia, access number DQ010921). Superscript II RNAse H^- reverse transcriptase (Invitrogen®, USA) was used to reverse transcribe viral RNA. Real-time PCR reactions were done in duplicate using Hot-StarTaq mastermix (Qiagen®, Germany) according to the manufacturer's instructions. Following reverse transcription the cDNA was diluted 1:1000 with AE buffer (10 mM Tris-Cl; 0.5 mM

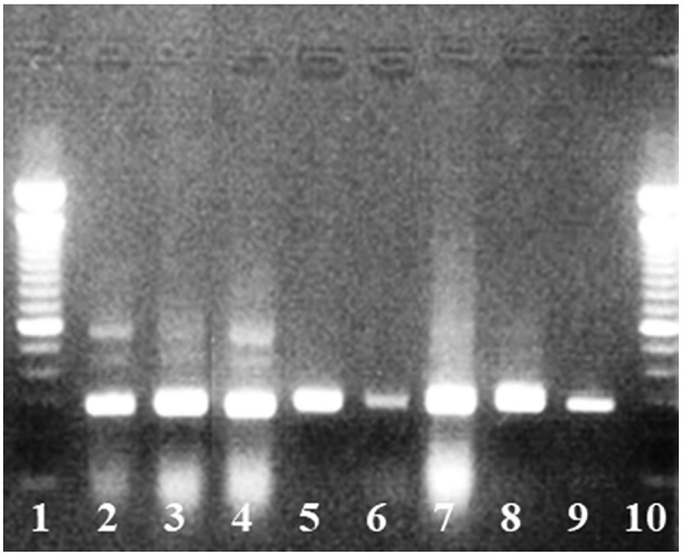

Figure 1. Amplification of FCoV mRNA by RT-PCR. 223 bp product size, CT levels were lower than 29 cycles, Lane 1: 100 Marker, Lane 10: 100 Marker, Lane 2, 3, 7: FECV, Lane 4, 5, 6, 8, 9: FIPV, Primer: P205, Sequence: GGCAACCCGATGTTTAAAACTGG, Orientation: Sense, Target: 3'-UTR, Product size: 223 bp.

EDTA; pH 9.0) and 5 ml was used as template in the real-time PCR assay. Cats with FECV yielded a low C_T value as expected which indicates high concentration of viral RNA as previously described [23, 24].

Statistical analysis

Statistical analysis was done using a statistical software package (SPSS 21.00 for Windows®, USA). One sample Kolmogorov-Smirnov test was used to examine the distribution pattern of data. Parametric data were presented as mean ± SD and non parametric data were presented as median and range. Data analysis of three groups were performed with one-way ANOVA or Kruskal-Wallis test depending on the distribution pattern. Receiver operator characteristic (ROC) analysis, with the area under the curve (AUC), was performed in order to examine the diagnostic efficacies of serum and effusion AGP concentration. The optimal cut-off points were those with the least number of misclassifications, and for these the sensitivity and specificity were determined. Observed power (Op or post hoc power) was obtained by performing general linear model univariate analysis. An insignificant result possibly indicates a correct statistical decision or might result from Type-2 error. Since low Op cannot make this distinction, Op was considered <0.5 (<50%) for insignificant results. A $P<0.05$ was considered significat in all analyses.

RESULTS

Anamnestic data

Cats with effusive FIP and cats with FECV were aged between 1-2 years, all were intact and mixed breed and of these, 22 were male, 18 were female in the FIP group and 6 were male, 4 were female in the FECV group. All the cats were unvaccinated indoor cats fed on commercial dry food with no other pets at home. Also, anamnestic data revealed that the complaints such as loss of appetite, pyrexia, abdominal distension and stagnation in cats with suspected abdominal effusion had been present for 11.5 (5, 22) days, and complaints such as loss of appetite, pyrexia, constant sleepiness, inactivity and labored breathing in cats with suspected thoracic effusions had been present for 8.5 (5, 18) days.

Clinical examination findings

As a result of clinical examinations, it was observed that body temperature ($P<0.000$), respiration rate (RR) ($P<0.000$), heart rate (HR) ($P<0.000$) and capillary refill time (CRT) ($P<0.019$) values were highest in the TE group compared to other groups, and body temperature and RR levels were statistically different in cats with FIP compared to the cats with FECV ($P<0.000$). Clinical examination findings are shown in Table 1.

Table 1. Physical examination findings of 40 cats with feline infectious peritonitis and 10 cats with feline enteric coronavirus infection at presentation

Parameters	FECV Group (n:10) (mean ± SD)	AE Group (n:30) (mean ± SD)	TE Group (n:10) (mean ± SD)	P value
Rectal temperature (°C)	38.54 ± 0.29^{c}	39.04 ± 0.36^{b}	39.58 ± 0.30^{a}	0.000
Respiratory rate (breaths/min)	38.8 ± 5.75^{c}	68.07 ± 13.24^{b}	93.60 ± 11.26^{a}	0.000
Heart rate (bpm)	131.6 ± 10.31^{b}	126.47 ± 12.52^{b}	159.60 ± 7.64^{a}	0.000
Capillary refill time (sec)	2.40 ± 0.51^{b}	2.87 ± 0.77^{ab}	3.30 ± 0.48^{a}	0.019

a, b, c: abc test was used to explore the relationship between two datasets. Statistical difference was observed between a, b and c but no difference between a and ab and b and ab ($P<0.05$; Kruskal-Wallis test). FECV: Feline enteric coronavirus, AE: Abdominal effusion, TE: Thoracic effusion

Laboratory findings

Blood gas and electrolyte analyte findings

Statistical differences were observed in pH, lactate, base excess (BE) and HCO_3 levels after blood gas and electrolyte analyses ($P<0.05$). Mild metabolic acidosis was observed in cats with FIP compared to cats with FECV ($P<0.05$). The lactate level was highest in the AE group among all the groups ($P<0.026$). The BE value was higher in the FIP group compared to the FECV group ($P<0.021$). The HCO_3 value was lower in the FIP group compared to the FECV group ($P<0.012$). Blood gas and electrolyte analyte findings are shown in Table 2.

Table 2. Venous blood gas and electrolyte analysis results of 40 cats with feline infectious peritonitis and 10 cats with feline enteric coronavirus infection

Parameters	FECV Group (n:10) (mean ± SD)	AE Group (n:30) (mean ± SD)	TE Group (n:10) (mean ± SD)	P value
pH	7.36 ± 0.52^{b}	7.30 ± 0.94^{a}	7.26 ± 0.14^{a}	0.050
pCO_2 (mmHg)	36.35 ± 9.36	35.69 ± 10.14	38 ± 8.24	0.839
pO_2 (mmHg)	37.57 ± 8.19	34.64 ± 8.42	34.09 ± 8.24	0.577
$SatO_2$ (mmHg)	56.70 ± 18.17	45.47 ± 15.16	43.30 ± 20.92	0.150
Potassium (mmol/L)	3.51 ± 0.21	3.73 ± 0.65	3.51 ± 0.39	0.394
Sodium (mmol/L)	155.20 ± 6.01	157.80 ± 7.70	160 ± 7.16	0.347
Chloride (mmol/L)	117.70 ± 6.20	117.90 ± 7.33	121.30 ± 5.92	0.372
Lactate (mmol/L)	1.52 ± 0.62^{b}	2.72 ± 1.29^{a}	2.20 ± 1.27^{ab}	0.026
Base excess (mmol/L)	-4.12 ± 2.33^{a}	-7.58 ± 3.87^{b}	-7.62 ± 2.56^{b}	0.021
HCO_3^- (mmol/L)	20.27 ± 1.82^{a}	17.55 ± 2.74^{b}	17.54 ± 2.03^{b}	0.012

a, b, c: abc test was used to explore the relationship between two datasets. Statistical difference was observed between a, b and c but no difference between a and ab and b and ab ($P<0.05$; Kruskal-Wallis test). pH: Power of hydrogen, pCO_2: Partial pressure of carbon dioxide, pO_2: Partial pressure of oxygen, $SatO_2$: Oxygen saturation, HCO_3: Bicarbonate, FECV: Feline enteric coronavirus, AE: Abdominal effusion, TE: Thoracic effusion

Complete blood count findings

Leukocyte (WBC) and lymphocyte levels were higher in the AE group compared to the other groups (P<0.010). The MCV value was lower in the FIP group compared to the FECV group (P<0.002). The hematocrit (Hct) level was lowest in the AE group among all the groups (P<0.023). CBC findings are shown in Table 3.

Table 3. CBC findings of 40 cats with feline infectious peritonitis and 10 cats with feline enteric coronavirus infection

Parameters	FECV Group (n:10) (mean ± SD)	AE Group (n:30) (mean ± SD)	TE Group (n:10) (mean ± SD)	P value
Leukocytes ($x10^3/mm^3$)	12.80 ± 3.11[b]	24.61 ± 12.36[a]	17.50 ± 10.56[ab]	0.010
Lymphocytes ($x10^3/mm^3$)	4.30 ± 1.31[a]	10.14 ± 8.51[b]	5.57 ± 3.31[a]	0.003
Mononcytes ($x10^3/mm^3$)	1.01 ± 0.52	1.63 ± 1.28	1.80 ± 1.18	0.264
Granulocytes ($x10^3/mm^3$)	7.48 ± 2.74	12.83 ± 7.54	10.14 ± 7.88	0.105
Red blood cells (M/mm^3)	9.75 ± 2.37	9 ± 2.49	10.47 ± 2.82	0.270
Mean corpuscular volume (fl)	50.71 ± 8.71[a]	42.72 ± 5.08[b]	43.62 ± 4.68[b]	0.002
Hematocrit (%)	48.44 ± 9.87[a]	38.16 ± 10.60[b]	44.56 ± 10.83[ab]	0.023
MCH (pg)	13.69 ± 3.62	12.52 ± 1.56	12.69 ± 1.44	0.320
MCHC (g/dL)	26.89 ± 4.68	29.51 ± 2.95	29.42 ± 1.65	0.079
Hemoglobin (g/dL)	12.79 ± 2.08	11.08 ± 2.84	13.17 ± 3.24	0.072
Platelets ($x10^3/mm^3$)	176.30 ± 88.98	166.60 ± 99.54	178.40 ± 153.70	0.944

a, b, c: abc test was used to explore the relationship between two datasets. Statistical difference was observed between a, b and c but no difference between a and ab and b and ab (P<0.05; Kruskal-Wallis test). MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, FECV: Feline enteric coronavirus, AE: Abdominal effusion, TE: Thoracic effusion

Serum biochemistry findings

Total protein level was lowest in the AE group among all the groups (P<0.003). The highest AST and GGT levels were detected in the AE group among all the groups (P<0.002). Cholesterol level was highest in the AE group among all the groups (P<0.032). Total bilirubin level was also highest in the AE group (P<0.031). Magnesium level was lower in the FIP group compared to the FECV group (P<0.044). Serum biochemistry findings are shown in Table 4.

Dipstick and refractometer test findings of effusion samples

Dipstick analysis was used to evaluate the pH and total WBC levels of the effusion samples. WBC levels were higher in the TE group than that of the AE group (P<0.042). Specific gravity (Sg) and total protein levels of the effusion samples were evaluated by refractometry. No statistical differences were observed between AE and TE groups in both parameters (P>0.728). Nevertheless, physical examination of the effusion samples such as exudative structure; yellowish, sticky, dense and Sg>1.020, total protein>3 g/L were consistent with the previous findings [9,11].

Table 4. Serum biochemistry findings of 40 cats with feline infectious peritonitis and 10 cats with feline enteric coronavirus infection

Parameters	FECV Group (n:10) (mean ± SD)	AE Group (n:30) (mean ± SD)	TE Group (n:10) (mean ± SD)	P value
BUN (mg/dl)	13.26 ± 3.26	22.77 ± 19.50	19.66 ± 7.99	0.264
Urea (mg/dl)	0.95 ± 0.34	1.51 ± 1.34	1.55 ± 0.94	0.367
Glu (mg/dl)	137.30 ± 36.98	130.40 ± 41.50	151.20 ± 38.72	0.371
T.pro (g/dl)	8.02 ± 0.89^{a}	6.55 ± 1.40^{b}	7.59 ± 0.71^{a,b}	0.003
Alb (g/dl)	3.37 ± 0.40	3.26 ± 1.12	3.19 ± 0.70	0.913
AST (U/L)	34.4 ± 14.47^{a}	84.66 ± 66^{b}	34.7 ± 16.34^{b}	0.000
ALT (U/L)	74.70 ± 27.12	73.60 ± 54.06	56.20 ± 17.17	0.539
ALP (U/L)	53.3 ± 51.16	172.7 ± 240.31	70.1 ± 30	0.129
GGT (U/L)	4 ± 2.49^{b}	7.06 ± 3.33^{a}	3.70 ± 2.35^{b}	0.002
Chol (mg/dl)	130.50 ± 30.07^{a}	209.83 ± 104.92^{b}	150.30 ± 33.39^{ab}	0.032
Trig (mg/dl)	56.1 ± 12.54	86.13 ± 53.99	59 ± 17.3	0.203
T. bil (mg/dl)	0.51 ± 0.32^{b}	2.08 ± 2.57^{a}	1.1 ± 0.77^{a,b}	0.031
Cal (mg/dl)	10.91 ± 1.01	10.68 ± 2.40	10.55 ± 1.80	0.927
Phos (mg/dl)	5.41 ± 1.08	5.80 ± 2.50	5.82 ± 2.03	0.877
Mag (mg/dl)	2.91 ± 0.94^{a}	2.06 ± 1.03^{ab}	1.89 ± 0.90^{b}	0.044

a, b, c: abc test was used to explore the relationship between two datasets. Statistical difference was observed between a, b and c but no difference between a and ab and b and ab ($P<0.05$; Kruskal-Wallis test). BUN: Blood urea nitrogen, Crea: Creatinine, Glu: Glucose, T.pro: Total protein, Alb: Albumin, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma glutamyl transferase, Chol: Cholesterol, Trig: Triglyceride, T. bil: Total bilirubin, Cal: Calcium, Phos: Phosphorous, Mag: Magnesium, FECV: Feline enteric coronavirus, AE: Abdominal effusion, TE: Thoracic effusion

AGP measurements and ROC-based diagnostic performance analysis results

The serum AGP level of the FIP group was found to be higher compared to the FECV group, while the highest serum AGP level was found in the TE group among all the groups ($P<0.04$). The effusion AGP level of the TE group was higher ($P<0.05$) compared to the AE group. The results are shown in Table 5. As a result of ROC-based diagnostic performance analysis between the three groups of serum AGP levels, it was observed that at the cut-off value of 2.37 mg/mL serum AGP level had excellent specificity (100%), very good sensitivity (85%) and AUC (0.893) values in differentiating cats with effusive FIP from cats with FECV. However, in the ROC-based diagnostic performance analysis between the AE and TE groups of serum and effusion AGP levels, it was observed that serum AGP level had good sensitivity (70%) and AUC (0.675) values at the cut-off value of 3.34 mg/mL, while the effusion AGP value had excellent sensitivity (90%) and good AUC (0.692) value at the cut-off value of 3.02 mg/mL in demonstrating the severity of the acute phase response.

Optimal cut-off values were selected based on Youden's index. ROC-based diagnostic performance analysis results are shown in Table 6. In addition, ROC curves of serum and effusion AGP levels in comparison of FECV and FIP and AE and TE groups are shown in Figure 2.

Table 5. Serum and Effusion AGP measurement results

Parameters	AE Group (n:30) (mean ± SD)	TE Group (n:10) (mean ± SD)	FECV Group (n:10) (mean ± SD)	P value
Serum AGP (mg/mL)	3.42 ± 1.73[ab]	4.76 ± 2.43[a]	2.02 ± 0.52[b]	0.004
	AE Group (n:30) (mean ± SD)	**TE Group (n:10) (mean ± SD)**	**P value**	
Eff. AGP (mg/mL)	3.20 ± 0.72	3.69 ± 0.63	0.050	

a, b, c: abc test was used to explore the relationship between two datasets. Statistical difference was observed between a, b and c but no difference between a and ab and b and ab ($P<0.05$; Kruskal-Wallis test). Eff: Effusion, AGP: Alpha 1-acid glycoprotein, FECV: Feline enteric coronavirus, AE: Abdominal effusion, TE: Thoracic effusion

Table 6. Receiver operator characteristic-based analysis of serum and effusion AGP levels to distinguish cats with FIP from cats with FECV

Parameters	AUC	Std. Error	P value	Asymp. %95 CI		Cut-off	Sens	Spec	Op
				Lower Bound	Upper Bound				
Serum AGP↓	0.893	0.046	0.000	0.803	0.982	2.37	85%	100%	87.8%
Serum AGP*	0.675	0.106	0.101	0.468	0.882	3.34	70%	66.7%	86%
Effusion AGP*	0.692	0.088	0.072	0.518	0.865	3.02	90%	43.3%	62%

↓ Indicates the comparison between the FECV and FIP groups, * indicates the comparison between AE and TE groups. AUC: Area under curve, Std error: standard deviation, CI: confidence interval, Sens: Sensitivity, Spec: Specificity, Op: observed power, FECV: Feline enteric coronavirus, AE: Abdominal effusion, TE: Thoracic effusion.

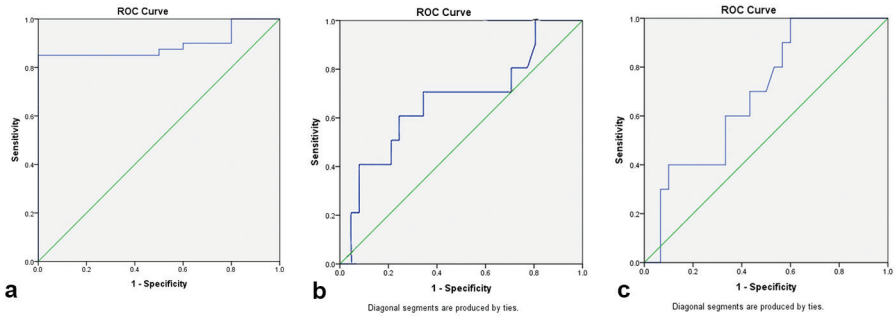

Figure 2. ROC curves of serum and effusion AGP levels in comparison of FECV and FIP and AE and TE groups. a) ROC curve of serum AGP level when distinguishing cats with FIP from cats with FECV. Diagonal segments are produced by ties. (AUC: 0.893), b) ROC curve of serum AGP level when demonstrating the inflammatory state between cats with abdominal and thoracic effusion. Diagonal segments are produced by ties. (AUC: 0.675), c) ROC curve of effusion AGP level when demonstrating the inflammatory state between cats with abdominal and thoracic effusion. Diagonal segments are produced by ties. (AUC: 0.692).

DISCUSSION

In this study, the diagnostic efficacies of clinical and laboratory analyses including CBC, blood gas and electrolyte, serum biochemistry, effusion dipstick and refractometer tests along with serum and effusion AGP levels were investigated. Most of the alterations were observed in vital signs such as body temperature, respiratory and heart rates, CRT, and serum biochemistry analytes. In addition, it was observed that serum AGP level could increase not only in FIPV-infected cats but also in FECV-infected cats. It was determined that in the diagnosis and differentiation of effusive FIP, serum AGP level in the course of the disease, pH, HCO_3, base excess, lactate, MCV and magnesium levels could provide important diagnostic information, especially in the triage process before advancing to further laboratory tests.

Unlike FIPV, which causes severe systemic infection with lesions in many organs and tissues, FECV infection appears to be mainly restricted to the intestinal tract where the virus replicates in the villous epithelial cells [25]. Therefore, mild vomiting and diarrhea may be observed in FECV-infected cats [18,26]. However, it was reported that every FCoV infection goes through a systemic phase and there is only a 5% chance of FIP developing [8,18,27]. Clinical and pathological laboratory findings have been reported to be related to the location and severity of vascular damage and the affected organs and tissues [28]. Many cats with FIP develop effusions with an incidence ranging from 17-62% [3,7]. In previous studies, it was reported that the localization of effusions was abdominal with an incidence of 62%, thoracic with 17%, and both thoracic and abdominal with 21% [15]. Effusion localization was abdominal at a higher incidence (75% abdominal versus 15% thoracic) in the present study, also. This may be related to the presence of aminopeptidase N, a cell surface glycoprotein released from monocyte lineage progenitors used by coronaviruses as their cellular receptor, mostly in renal, digestive epithelial cells and less in respiratory epithelial cells [6,14]. Compared to cats with FIP with abdominal effusion, cats with thoracic effusions sometimes have prominent cyanotic mucous membranes with dyspnea, tachypnea or panting. Pyrexia, vomiting and diarrhea have also been reported [3,29]. In this study, abnormal physical examination findings such as pyrexia, elevated respiration and heart rate along with prolonged CRT were observed in comparison between the FECV and FIP groups ($P<0.019$). In a recent study, evaluating referral feline cases with history of pyrexia, FIP was the most common diagnosis [30]. Also, it was reported that clinical symptoms such as pyrexia, elevated heart and respiration rate and prolonged CRT could be related to decreased/depletion of T-cell counts [2], anemia [28] and an increased viral load in cases of FIP. However, it should be kept in mind that clinical findings may change over time (such as development of a small amount of effusion), and the presence of pyrexia and evaluation of other vitals may help complete the clinical picture in the diagnosis of FIP [9]. Also, when compared to the AE and FECV groups, considering the duration of clinical symptoms, worse vitals of the TE group in the present study ($p < 0.019$) may be related to the fact that owners of the cats with thoracic effusions

notice increased respiratory rate and dyspnoea earlier than the owners of the cats with abdominal effusions.

Blood gas and electrolyte disorders, which can be the cause or consequence of various life-threatening disorders, are frequently found in critically ill feline patients with diagnostic and prognostic relevance [31,32]. The association between acid-based disorders and mortality has been reported in various critical conditions in cats such as advanced chronic kidney disease and gastrointestinal obstruction [33,34] but not in cases of FECV and FIP. In the present study, mild metabolic acidosis (considering the HCO_3 and BE values) was more prominent in the FIP compared to the FECV group ($P<0.05$). These findings were interpreted as a result of loss of HCO_3 through the intestines and/or kidneys and accumulation of lactic acid [35,36] in cats with effusive FIP.

Lactic acid production increases in organ failure and in the absence of HCO_3 or non HCO_3 buffers, which results in lactic acidosis. Therefore, hyperlactatemia occurring without acidosis or acidemia is observed if the perfusion and compensation mechanisms have a sufficient titer that can balance the blood pH [37,38]. Consequently, in the present study, the lactate level was higher in the AE group than that of the TE and FECV groups ($P<0.026$) whereas BE and HCO_3 levels were lower in the TE group among all the groups ($P<0.021$). The mild metabolic acidosis of the FIP group of the present study can also be explained by the insufficient activation of compensation mechanisms [39]. Higher lactate level in the AE group compared with the TE group was interpreted as a result of hypoperfusion and probably more severe liver damage (Table 4). Also, in comparison with the FECV group, low BE and HCO_3 levels were observed in the FIP group resulting from loss of appetite and inadequate compensation response due to intestinal damage and hyperlactatemia [38].

The diagnostic difficulty of cats with findings such as persistent pyrexia with a fluctuating course, unresponsiveness to antibiotics [40] is the choice between direct or indirect tests for definitive diagnosis. Although the sensitivity and specificity of indirect tests are highly variable, routine CBC, blood gas and electrolyte and serum biochemistry analytes are important in supporting diagnosis of FIP [4]. The common abnormal CBC findings in cats with effusive FIP are reported to be anemia, microcytosis with or without anemia, lymphopenia, band neutrophilia and thrombocytopenia [4,28]. However, in cases of FECV infections, normal peripheral lymphocyte levels were reported [25]. In this study, in comparison with the AE group, the low lymphocyte level ($P<0.05$) of the TE group was consistent with previous reports in cats with acute FIP lesions [2,41]. This situation can be explained by the exacerbation of the disease due to low lymphocyte counts and the role of lymphocytes in the modulation of disease development considering the duration of symptoms in the TE group. Although the observed differences ($P<0.05$) between the groups in terms of total WBC and lymphocyte counts can be explained by the early involvement of the immune system in the disease process, it should be noted that the low antibody titer in acute FIP cases is due to the formation of immune complexes [13]. Also, MCV

(P<0.002) and hematocrit levels (P<0.023) were determined to be lower in the FIP group compared with the FECV group. These abnormal changes are interpreted due to the effects of FIP on the hematopoietic system [3,4]. FECV is known as a low or non-virulent pathogen. Also, FECV is tropic for cells of the mature apical epithelium of the intestinal villi, targeting cells from the caudal part of the duodenum to the cecum during acute infection [15]. In chronically infected animals the lower part of the gastrointestinal tract was identified as a major site of viral replication [25]. Therefore, the normal CBC findings of FECV-infected cats may be related to the low virulence and/or tropism of the virus [8,25].

The vast majority of changes observed in blood analyses of cats with FIP are detected in the serum biochemistry [4]. Specifically, reported abnormalities are: hyperproteinemia, hyperglobulinemia, hypoalbuminemia, azotemia and increased liver enzyme levels, which vary depending on the extent of organ and tissue involvement [15]. Although these abnormalities can be found in combination with each other, they are neither pathognomonic nor specific for FIP [2]. Nevertheless, evaluation of serum biochemistry findings can be used to support the diagnosis of FIP and/or increase the index of suspicion [11]. In this study, lower total protein (P<0.003) and higher AST (P<0.000) and GGT (P<0.002) levels were determined in the AE Group compared with the TE and FECV groups. These findings can be explained by the fact that the amount of protein-rich effusion was more in the abdominal effusion form and the development of infection-related abdominal organ damage is more severe [4].

It is known that some viruses use cholesterol while infecting the host cell [42]. In a study in which a cationic amphiphilic drug was administered to cats affected with FIP, it was reported that FIPV proliferation was prevented by inhibition of intracellular cholesterol transport in the feline cell line. Besides, it was observed that cholesterol accumulation is transported into the cytoplasm of monocytes into the peripheral blood [43]. In this study, high colesterol levels were observed in the FIP group compared to the FCEV group and determined to be highest in AE group (P<0.032). This finding can be explained by the presence of a high viral load and the possible role of cholesterol in the development of FIP [40]. In addition, considering that the dominant type in the field is type I FCoV, it was interpreted that investigating the effect of cholesterol levels on FCoV infectivity in cats infected either with FECV and/or FIP may shed light on the studies on the subject [44].

Magnesium is the most abundant cationic mineral in the body which has many roles in the immune system mechanisms [45]. It has been reported that magnesium deficiency plays a role in the development of some severe and chronic viral infections [46]. In the present study, magnesium level was determined to be lower in the TE group compared with the FECV group (P<0.044). Although the magnesium level of the AE group was determined to be numerically lower than the FECV group, no statistical difference was observed. This finding can be explained by the remodeling of inflammatory cells accompanying infectious or inflammatory conditions and significant T cell dysfunction [47]. However, it couldn't be determined whether the low magnesium

levels of the FIP group were the result of FIP infection or a predisposing cause. Therefore, considering that chronic magnesium deficiency results in oxidative stress and increases predisposition to infectious diseases [46], investigation of magnesium levels in cats infected with FECV and/or FIP may help demonstrating the function of this mineral in the formation and course of the disease.

Acute phase proteins (APPs) are synthetized in the liver in response to cytokines released from macrophages and monocytes in many inflammatory and non inflammatory diseases [9]. AGP is an APP, and its measurement can be helpful in the diagnosis of FIP [28]. Although AGP elevations (>0.48 mg/mL) per se are not specific for FIP, markedly elevated AGP levels (>1.5 mg/mL) are often seen in FIP cases. Therefore, the magnitude of the increase may be helpful in aiding diagnosis of FIP, with higher levels being more useful in raising the index of suspicion [48]. It was reported that when the pretest probability of FIP was high such as supportive history and clinical findings of FIP, moderate serum AGP levels (1.5–2 mg/mL) could discriminate cats with FIP from cats without FIP, but only higher serum AGP levels (>3 mg/mL) could support a diagnosis of FIP in cats with a low pretest probability of disease when history and clinical findings are not supportive of FIP [49]. However, it should be kept in mind that serum AGP levels may also increase in neoplasia and inflammatory disorders [19,49]. In this study, measurements of serum and effusion AGP levels were performed both to support the diagnosis of FIP and to investigate its diagnostic efficacy in cases of FECV and FIP. In the comparison of the three groups, the serum AGP level was highest in the TE group ($P<0.004$). In addition, the effusion AGP level was higher in the TE group compared to the AE group ($P<0.05$). These findings were interpreted as a more severe acute phase response develops in the presence of thoracic effusion than abdominal effusion. Although the serum AGP level of the FIP group was higher compared to the FECV group, the serum AGP level of the FECV group was higher than the reported serum AGP levels of healthy cats (>0.5 mg/ml) [50]. This finding may indicate that clinically healthy but FECV-positive cats also develop inflammatory response and serum AGP measurement in FECV-positive cats may be useful in predicting development of FIP infection with de novo by spontaneous mutations from FECV. As a result of ROC-based diagnostic performance analysis of serum AGP level of the comparison between the three groups, it was observed that at the cut-off value of 2.37 mg/mL serum AGP level had excellent specificity, very good sensitivity and AUC values in differentiating cats with effusive FIP. However, in comparison between the AE and TE groups, it was observed that serum AGP level had good sensitivity and AUC values at the cut-off value of 3.34 mg/mL, while the effusion AGP value had excellent sensitivity and good AUC values at the cut-off value of 3.02 mg/mL (Table 6). These findings were interpreted as the serum AGP level at a cut-off value of 2.37 mg/mL in cases of effusive FIP may be a useful marker with 100% specificity in the presence of findings consistent with FIP.

This study has some limitations such as the limited number of animals and the lack of histopathological evaluation of the involved organs and tissues. Although the small

sample size scenario is common in medical tests, a comprehensive study of small sample size properties of various methods for the construction of the confidence/credible interval (CI) for AUC has been by large missing in the literature. As previously reported [51] it was observed that the larger the true AUC value and the smaller the sample size, the larger the discrepancy among the results of different approaches. Therefore, among the limitations aforementioned, the major limitation of this study is the limited number of animals which may influence the results of the ROC-based diagnostic performance analyses results. Hence, the authors recommend evaluating the promising results of this study with a larger number of naturally developed FECV and effusive FIP cases.

CONCLUSION

It was observed that alterations in laboratory tests such as CBC, blood gas and electrolyte and serum biochemistry analytes along with elevations in serum AGP levels can also be observed not only in cats with FIP but also in cats with FECV. Also, as a result of ROC-based diagnostic performance analyses, it was observed that serum AGP evaluation could be used to evaluate the index of suspicion of FIP and FECV, and evaluation of the effusion AGP level may indicate a more severe inflammatory response. Thus, it was concluded that pH, HCO_3, base excess, lactate, MCV and magnesium levels were found to be important in the course of the disease, and AGP in the evaluation of the presence of an inflammatory state, especially in triage in order to enable early intervention, in cats naturally infected with FCoV.

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Authors' contributions
EG and MO involved in drafting the manuscript or revising it critically for important intellectual content and contributed to conception and design of the study. MKD and YEE collected the specimens and data. KÜ and TMP performed the analysis. EG performed the statistical analysis. All authors read and approved the final manuscript.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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POREĐENJE KLINIČKIH I LABORATORIJSKIH NALAZA U RAZLIČITIM KLINIČKIM FAZAMA KOD MAČAKA PRIRODNO INFICIRANIH KORONAVIRUSOM

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Koronavirusne infekcije mačaka (FCoV) su česte, pri čemu je naglašen tropizam ka monocitima i makrofagima. Većina FCoV inficiranih mačaka su asimptomatske, međutim kod do 10% životinja, razvija se fatalni infektivni peritonitis (FIP). Cilj ove studije je bio da se ispita dijagnostička primenljivost kliničkih i laboratorijskih ispitivanja uključujući serumske i efuzione vrednosti AGP kod mačaka bilo sa simptomatskim efuzivnim FIP ili kod asimptomatski inficiranih mačaka enteralnim koronavirusom (FECV). U okviru studije, obuhvaćeno je 40 mačaka sa efuzivnim oblikom FIP i 10 mačaka sa FECV infekcijom. FIP grupa je bila podeljena u dve podgrupe: abdominalna (AE; n=30) i torakalna efuzija (TE; n=10). Obavljena su klinička i laboratorijska ispitivanja, uljučujući serumska i efuziona AGP merenja. U okviru svih grupa, TE grupa životinja je imala najvišu telesnu temperaturu, kao i srčani i respiratorni puls (P<0,000). U poređenju sa FECV grupom FIP grupa je imala niže pH i HCO3 vrednosti kao i višak baza i vrednosti laktata (P<0,05). Brojevi leukocita i limfocita bili su povišeni, a hematokrit je bio manji u AE grupi u okviru svih grupa (P>0,023). MCV je bio smanjen u FIP grupi u poređenju sa FECV grupom (P<0,002). Kod životinja AE grupe, ukupni nivo proteina bio je najmanji, a AST, GGT kao i vrednosti ukupnog bilirubina i holesterola su bile najveće između svih grupa (P<0,032). Nivo magnezijuma ja bio manji u FIP grupi u poređenju sa FECV grupom (P<0,044). Iako je nivo AGP seruma bio najveći u TE grupi u odnosu na sve grupe (P<0,004), AGP vrednosti kod mačaka sa FECV su bile slične onima u AE grupi (P<0,05). Identifikovane su razlike u kliničkim i laboratorijskim rezultatima ispitivanja kod FECV-pozitivnih mačaka s obzirom da će one verovatno razviti i FIP. Od navedenih rezultata ispitivanja, ustanovljeno je da su vrednosti pH, HCO3, višak baza, laktata, MCV i magnezijuma bile od značaja za tok oboljenja. Od značaja za evaluaciju prisustva i stanja zapaljenske reakcije bila je i vrednost AGP. Zaključeno je da klinička, laboratorijska i evaluacija AGP seruma, mogu da se koriste kao indeks sumnje tokom razvoja FIP i FECV.