

## Comparison of serologic techniques for the detection of antibodies against feline coronaviruses

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**Abstract.** The seroprevalence of feline coronavirus (FCoV) antibodies was studied in cats in southern Italy. One hundred twenty sera collected from cats belonging to catteries or community shelters and to households were tested for FCoV type I and II antibodies. The virus neutralization (VN) was performed and compared with indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). Ninety-six sera tested positive for FCoV antibodies by VN and ELISA. Interestingly, ELISA revealed 2 more positive sera than did the VN test and 3 more positive sera than did the IFAT. All results were confirmed by Western blotting. ELISA proved to be more sensitive and detected a seroprevalence of about 82%. Considering the cross-reactivity of FCoV type I and type II, ELISA was able to detect antibodies against both serotypes, allowing the use of the assay as a reference test for sera screening. The high prevalence of antibodies observed indicates that FCoVs are common in southern Italian cat populations.

**Key words:** Antibodies; cats; coronavirus; enzyme-linked immunosorbent assay; serotypes.

### Introduction

Coronaviruses, a genus of the family Coronaviridae, are large, enveloped, positive-stranded RNA viruses responsible for highly prevalent diseases in humans and domestic animals. They have the largest genome (27–32 kb) of all RNA viruses and replicate by a unique mechanism, which results in a high frequency of recombination. The S glycoprotein of coronaviruses, which forms the large petal-shaped spikes on the surface of the virion, is an important protein of the virus, and it mediates several important biologic functions.<sup>12</sup> Although a major immunological role has been attributed to the S protein, both the amino- and carboxyl-termini of the M protein elicit strong immune responses as well,<sup>10</sup> inducing antibody-dependent, complement-mediated, virus neutralization.<sup>31</sup>

Feline coronaviruses (FCoVs) exhibit a bimodal pathogenicity, producing subclinical or mild enteric infections in young kittens at one extreme and deadly feline infectious peritonitis (FIP) at the other. The low-virulence strains are referred to as feline enteric coronavirus and the highly virulent ones as feline infectious peritonitis virus. On the basis of neutralization tests *in vitro*, FCoV can be distinguished into 2 serotypes, FCoV type I and FCoV type II.<sup>17,18,24</sup> In the field, serotype II accounts for only 20% to 30% of

all FCoV infections.<sup>16,27</sup> Type I viruses represent the genuine FCoVs and grow poorly in cell culture. Type II viruses originated from double recombination events between FCoV type I and canine coronavirus (CCoV)<sup>15</sup>; they proliferate well in cell culture and are widely used for the study of FCoV infection, and consequently, acquisition of genetic data for type I viruses has been lagging behind that of type II viruses. Thanks to the advent of polymerase chain reaction technology, this gap has been largely filled with sequence data for type I viruses from both tissue culture-adapted strains and clinical samples. Comparison of the sequences of type I and II FCoVs and CCoV revealed that type II viruses have spike genes that are much more closely related to CCoV than to type I FCoVs. This explains the cross-reactivity patterns in the neutralization assays mentioned above, since the spike protein is the target for neutralizing antibodies.<sup>29</sup>

Feline enteric coronaviruses are ubiquitous in cat populations and have a particularly high prevalence in catteries and multiple-cat households. Both type I and type II are transmitted via the fecal-oral route and generally cause mild intestinal infections. Several authors have demonstrated that the virus persists in the intestine of healthy cats and may be excreted over a period of months or even years.<sup>11,13,14</sup> These cats represent the coronavirus reservoir, and some of them can develop a fatal immune-mediated disease called feline infectious peritonitis (FIP).<sup>8</sup>

Since FCoVs are ubiquitous pathogens, the evaluation of anti-FCoV antibodies can be useful to monitor infection in a breeding cattery and/or an

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FCoV-free household. Assessment of antibodies by the gold standard FCoV indirect fluorescent antibody test (IFAT),<sup>1</sup> or by indirect enzyme-linked immunosorbent assay (ELISA),<sup>2</sup> provides evidence of the exposure of a cat to FCoV. The anti-FCoV antibody frequency in the feline population may vary considerably. In a California study, an occurrence of 20% positive cats in samples collected at random was reported.<sup>22</sup> In a study conducted in Austria, type I was found to be present in 62% of the cats studied.<sup>25</sup> A recent survey was performed on 292 cats attending clinics and practitioners in the Czech Republic and 292 cat sera collected in Austria to evaluate the prevalence of antibodies against FCoV type I. A total of 58% of the Czech cats and 64% of the Austrian cats tested seropositive (Moestl K, Knotek Z, Toman M, Faldyna M: 2002, Seroprevalence of feline coronaviruses in two central European countries: Czech Republic and Austria. Proceedings of the 2nd International Feline Coronavirus/Feline Infectious Peritonitis Symposium, August 4–7, Glasgow Scotland). In general, antibodies against FCoVs are found in 80% to 90% of the animals living in catteries or multiple-cat households and in up to 50% of solitary cats; only 1% to 5% of these seropositive cats eventually develop FIP.<sup>28</sup> The aim of the present investigation was to evaluate the prevalence of FCoV-specific antibodies in the sera of cats from catteries, community shelters, or households by using the virus neutralization (VN) test and then comparing its results with those of IFAT and ELISA.

## Materials and methods

### Serum samples

A total of 120 serum samples were collected from clinically healthy cats living in catteries and/or community shelters (101 samples) and in single-cat households (19 samples) in the region of Apulia in southern Italy. The age of the cats ranged from 6 to 12 months (37 samples) and more than 1 year (83 samples). All the samples were stored at  $-20^{\circ}\text{C}$  prior to analysis.

### Viruses, cells, and monoclonal antibodies

FCoV type II was used throughout the study, while serotype I was employed in only 1 assay. FCoV type II strain 25/92 was isolated from a cat with FIP in 1992.<sup>5</sup> The virus was isolated and cultivated on Crandell feline kidney (CrFK) cells propagated in Eagle's minimal essential medium<sup>a</sup> supplemented with 10% fetal calf serum (FCS). Sequence analysis of the gene encoding for the S protein characterized the strain 25/92 as FCoV type II (data not shown). The FCoV type II antigen was prepared for ELISA and Western blot starting from the supernatants of CrFK cell cultures infected with FCoV type II strain 25/92 or mock infected cultures. Both infected and mock-infected cells were harvested 48 hours postinfection and clarified at

$3,000 \times g$  for 20 minutes at  $4^{\circ}\text{C}$ . Subsequently, the supernatants were centrifuged for 1 hour at  $140,000 \times g$  at  $4^{\circ}\text{C}$ . The pellets were suspended in phosphate-buffered saline (PBS; pH 7.2) and used as viral and control antigens. The viral titer of the positive pellet was determined in 96-well microtitration plates<sup>b</sup> using a 50% tissue culture infectious dose assay ( $\text{TCID}_{50}/50 \mu\text{l}$ ) on CrFK cells. The infectivity titer was  $10^{4.50} \text{TCID}_{50}/50 \mu\text{l}$ . The FCoV type I strain Black<sup>c,21</sup> was cultivated on feline cell whole fetus (fcwf-4) and propagated in Leibovitz L-15 medium<sup>d</sup> supplemented with 20% FCS. Two monoclonal antibodies (MoAbs)<sup>e</sup> specific for FCoV type I (*SS FPV TN 406-1010 B11F*, lot. *DK05094*) and FCoV type II (*SS FPV 1146 019 E8D*, lot. *14/04/97*) with an IFAT titer  $>1:800$  each were included as controls for the specificity of the assay.

### Virus neutralization

Virus neutralization was used to test neutralizing antibodies against FCoV type II. Each serum sample was heat inactivated by incubation at  $56^{\circ}\text{C}$  for 30 minutes prior to use in the test. In duplicate 96-well microtitration plates,<sup>b</sup> serial 2-fold dilutions of each serum sample starting from 1:2 were mixed with 100  $\text{TCID}_{50}/50 \mu\text{l}$  of FCoV type II strain 25/92. The plates were kept at room temperature for 90 minutes. Subsequently, 20,000 freshly trypsinized CrFK cells in 100  $\mu\text{l}$  were added to each well, and the plates were incubated for 5 days at  $37^{\circ}\text{C}$ . The VN antibody titer was expressed as the reciprocal of the highest serum dilution that completely inhibited viral cytopathic effect. If the sera 1:2 diluted did not neutralize the virus, the titre was considered 1:2 (final dilution).

### Indirect fluorescent antibody test

Fcwf-4 and CrFK cells infected with FCoV type I strain Black and FCoV type II strain 25/92, respectively, were placed in multichamber culture slides<sup>f</sup> and then fixed with acetone 80%. Before testing, fixed cells were immersed briefly into PBS (pH 7.2). Serial 2-fold dilutions of each serum (1:50 to 1:800) were performed in PBS. Twenty microliters of diluted sample was placed into duplicate wells and allowed to incubate for 30 minutes at  $37^{\circ}\text{C}$  in a moist incubator. Slides were washed 3 times in PBS and blotted dry, and 20  $\mu\text{l}$  of pretitrated goat anticat IgG conjugated to fluorescein isothiocyanate<sup>d</sup> diluted 1:60 were added to each well. The slides were then incubated for 30 minutes at  $37^{\circ}\text{C}$ , once again washed 3 times in PBS, counterstained with Evans blue,<sup>g</sup> and visualized under a fluorescent microscope. Only about three fourths of the cells in each well were infected, thus providing an internal negative control for the nonspecific binding of antibodies to the cell monolayer. The titer was the highest serum dilution that still produced detectable levels of fluorescence in the foci of virus-infected cells. Antibody titers lower than 1:50 were scored as negative.

### Enzyme-linked immunosorbent assay

Microtiter NUNC-polysorp immunoplates<sup>h</sup> were coated with 25  $\mu\text{g}/\text{ml}$  of FCoV type II antigen diluted in carbonate buffer (15 mM  $\text{Na}_2\text{CO}_3$ , 35 mM  $\text{NaHCO}_3$ , 0.02%  $\text{NaN}_3$

**Table 1.** Assessment of anti-feline coronavirus (FCoV) antibodies in cats by enzyme-linked immunosorbent assay (ELISA): comparison with virus neutralization (VN) and indirect fluorescent antibody test (IFAT) performed with FCoV type II strain 25/92 and type I strain Black, respectively, taken as the gold standard.\*

ELISA	VN			IFAT FCoV II			IFAT FCoV I		
	+	−		+	−		+	−	
+	96	2	98	95	3	98	86	12	98
−	0	22	22	0	22	22	0	22	22
	96	24	98.3	95	25	97.5	86	34	90

\* The concordant sera and overall agreement are reported in bold.

[pH 9.6]; final dilution 1:40) and incubated overnight at 4°C with shaking. The plates were washed in PBS containing 0.05% Tween 20 (PBST), then treated with blocking solution (0.2% gelatin in carbonate buffer) for 90 minutes at 37°C and washed with PBST. Each cat serum, diluted 1:50 in PBST, was added in duplicate, and the plates were incubated for 90 minutes at 37°C. After a washing cycle, rabbit anticat IgG conjugated to horseradish peroxidase,<sup>i</sup> diluted 1:5000 in PBST, was added to each well, and the plates were incubated for 1 hour at 37°C. After another washing cycle, freshly prepared substrates were placed into each well. The solution consisted of 10 mg ABTS (2,2'-Azino-di-[3-ethylbenzthiazoline sulfonate] diammonium salt)<sup>d</sup> in 50 ml 0.05 M phosphate citrate buffer (pH 5.0) containing 25 µl/100 ml 30% hydrogen peroxide. After adding the stop buffer solution (1% sodium dodecyl sulfate [SDS]), the optical densities (ODs) were determined at 405 nm using an automatic ELISA plate reader.<sup>j</sup> The adjusted OD values of each sample were obtained by subtracting the absorbance of the mock antigen-coated well from that of the corresponding virus antigen-coated well.

### Western blotting

FCoV type II antigen diluted in Laemmli sample buffer<sup>i</sup> was heated at 95°C for 4 minutes, subjected to electrophoresis in SDS-polyacrylamide mini gel (5%–20%), and transferred onto nitrocellulose membrane Immobilon P (pore size = 0.45 µm)<sup>j</sup> with a Bio-Rad Transblot Cell apparatus at 10 mA for 2 hour. Nonspecific binding sites were blocked overnight at 4°C with 5% nonfat dry milk, Blotting Grade Blocker<sup>j</sup> in Tris-buffered saline (TBS; Tris 25 mM, NaCl 200 mM, pH 7.4) containing 0.05% Tween 20 (TBS-TM). After a washing cycle with TBST, the membrane was probed with feline serum samples diluted 1:100 in TBST for 2 hours with shaking at room temperature. The membrane was then washed with TBST and incubated for 2 hours with rabbit anticat IgG conjugated to horseradish peroxidase<sup>i</sup> with shaking at room temperature. After being washed extensively in TBST, DAB (3,3'-diaminobenzidine tetrahydrochloride)<sup>d</sup> in TBS containing 0.08% hydrogen peroxide was used in the chromogenic reaction.

### Statistical analysis

The results were then compared using the Cohen kappa test for agreement and repeatability.<sup>6</sup> The kappa test is

a measure of association between 2 measurements of the same element. Mathematically, kappa is defined as the improvement upon change agreement, divided by the maximum possible improvement upon change agreement  $(AO - AA)/(1 - AA)$ , where AO is the observed agreement and AA is the expected agreement by change. Values less than 0.4 indicate low association, values between 0.40 and 0.75 indicate medium association, and values greater than 0.75 indicate high association between the 2 rates.

### Results

All the serum samples were preliminarily tested with the VN assay for the detection of FCoV antibodies. Of the 120 samples tested, 24 were negative and 96 were positive. Twenty-two of the 24 negative sera were also found to be free of FCoV-specific antibodies by Western blotting. Of these 24 cats, 23 were kittens aged 6 to 12 months (95.8%), and only 1 (4.1%) was an adult cat living in a household. The sera that were negative by VN test and Western blotting were subsequently examined by ELISA and used to adjust the ELISA cutoff value (3 standard deviations higher than the arithmetic mean of the absorbance of these negative samples). Samples with values exceeding 0.049 were considered to be positive. To verify if the ELISA was able to recognize responses against FCoV types I and II, 2 specific MoAbs were included. Both antibodies, diluted 1:1000 as suggested by the supplier,<sup>e</sup> gave positive results (OD = 0.412 and OD = 0.389, respectively).

Two samples that had tested negative by VN gave a positive ELISA signal, and FCoV-specific antibodies were also found in the Western blotting test. These sera were recorded to be accordant positive in the ELISA. Using the VN test as the gold standard test, ELISA had a sensitivity of 100% and a specificity of 92%, with an overall agreement of 98.3% (Table 1). The kappa test indicated a high association between the 2 tests (0.93). The positive predicted value (PPV) was 0.97, and the negative predicted value (NPV) was 0.91.

All the samples and the 2 MoAbs were also tested by IFAT employing both the acetone-fixed CrFK cells infected with FCoV type II strain 25/92 and



**Table 2.** Results of the evaluation of antibodies to feline coronavirus (FCoV) types I and II in serum samples of cats employing different techniques.\*

	VN		IFAT		ELISA	
	POS	NEG	POS	NEG	POS	NEG
FCoV type I	ND	ND	86	34	ND	ND
FCoV type II	96	24	95	25	98	22

\* VN = virus neutralization test; IFAT = indirect fluorescent antibody test; ELISA = indirect enzyme-linked immunosorbent assay; POS = positive serum samples; NEG = negative serum samples; ND = not determined.

acetone-fixed fcwf-4 cells infected with FCoV type I strain Black. As expected, the acetone-fixed CrFK-infected cells were positive only with the MoAb specific for FCoV type II, and the acetone-fixed fcwf-4-infected cells were positive only with the MoAb specific for FCoV type I. Only 25 samples, which included the 24 sera found to be free of FCoV-neutralizing antibodies, yielded a negative result when tested with acetone-fixed CrFK cells infected with FCoV type II strain 25/92, while 95 sera tested positive (serum dilution 1:100–1:200). Considering the IFAT performed with FCoV type II as the gold standard, ELISA had a sensitivity of 100% and a specificity of 88%, with an overall agreement of 97.5% (Table 1). The kappa value showed a good agreement between the 2 tests (0.92), while the PPV and the NPV were 0.96 and 0.88, respectively.

In contrast, the IFAT performed on the acetone-fixed fcwf-4 cells infected with FCoV type I strain Black yielded 34 negative sera and 86 positive sera. The 34 negative serum samples included the 24 sera found to be free of FCoV-neutralizing antibodies. Eleven of the 86 positive samples exhibited a low titer (1:50), while the remaining 75 had titers ranging from 1:100 to 1:800. The 22 sera that were negative by VN test and Western blotting were also found to be free of FCoV-specific antibodies by both the IFATs (Table 2). Considering the IFAT performed with FCoV type I strain Black as a gold standard, ELISA has a sensitivity of 100% and a specificity of 65%, with an overall agreement of 90% (Table 1). The kappa test indicated a medium association between the 2 tests (0.72), and the PPV and the NPV were 0.87 and 0.64, respectively.

### Discussion

Although the humoral immune response of cats to coronaviruses has been studied by means of several methods including IFAT, ELISA, VN, and Western blotting, antibody titer measurement is a valid and effective diagnostic tool only when performed in a suitable manner and with appropriate techniques. It is known that antibody measurements by different methods do not always yield similar results, and there

is often a lack of agreement among different tests used for FCoV antibody detection. Hence, it is important to develop a uniform testing method for the detection of antibodies in cats. Whereas ELISA and Western blotting detect antibodies against all major viral proteins, the VN test measures only neutralizing antibodies and may thus lack sensitivity and provide misleading information on the epidemiological features of FCoV infection in cat populations. In a recent study, the antibody responses of 3 naturally infected dogs against the major structural proteins of CCoV were evaluated using Western blotting at different time points postinfection.<sup>9</sup> The sera exhibited a strong and persistent reactivity to the M and N proteins, also in the early stages of disease when the antibody response is not yet fully developed, while reactivity to the S protein was poor. The S protein is the most important inductor of neutralizing antibodies and has undergone antigenic variations presumably involving the epitopes recognized by reactive antibodies.<sup>30</sup> Thus, the apparently poor reactivity to the S protein may partly be due to a weak correlation between serum antibodies and the viral antigen used in a previous study. By contrast, the M and N proteins have more conserved amino acid sequences and are the predominant antigens produced in coronavirus-infected cells,<sup>7</sup> which makes them the major viral targets and can account for the higher sensitivity of ELISA. On the other hand, although IFAT has been broadly used thus far in clinical veterinary serology,<sup>4</sup> ELISA has shown excellent potential as a rapid and sensitive method for the detection of FCoVs antibodies in cats.

Thus, to verify whether ELISA could be used to detect FCoV antibodies, its sensitivity and specificity were evaluated against the VN and IFATs considered as gold standards. ELISA has proven to have a good specificity and sensitivity in measuring FCoV responses, as it had in the past for the detection of anti-CCoV antibodies in dog sera<sup>26</sup>; it can therefore be used effectively for antibody screening in cats. When ELISA was compared with IFA testing, performed with FCoV type I strain Black taken as the gold standard, its sensitivity was 100% and specificity was

65%, with an overall agreement of 90%. Considering that FCoV type I is characterized by lower replication kinetics, most likely leading to a lower antigenic mass within the cells, the data observed can explain why the IFAT developed with FCoV type I revealed a higher number of seronegative samples. The relationship between the age of the cats and the presence of FCoV antibodies was determined, and an evident rise in seroprevalence with age was observed, in agreement with previous studies.<sup>19,23</sup>

The results presented in the present study, although derived from a limited number of sera, confirmed that FCoV seropositivity is high in catteries or community shelters where cats live in crowded conditions as well as in many single-cat households. FCoV type II strains have hardly been found in Europe and have been detected only incidentally in the United States.<sup>3,16,20,24,25,29</sup> Furthermore, a study<sup>20</sup> has recently observed that a serological differentiation between antibodies to FCoV types I and II may not be possible in the early phase of infection, when cross-reacting epitopes of both types are responsible for comparable titers, but only after the infection has resulted in an increase of antibody concentration and specificity. In experimentally FCoV type I infected cats, the antibody development against both types was followed, and it was observed that only after an observation period of more than 17 weeks did antibodies to FCoV type I show a significant increase over those to FCoV type II.<sup>20</sup> The sera employed in the present study were collected from clinically healthy cats, and it was not possible to determine the time of the infection and antibody development. Hence, there is good reason to believe that in the FCoV type II-positive cats, the infection was started with FCoV type I virus, but overall, it became clear that the true prevalence of type I and type II infection cannot be determined precisely when the anamnesis of the animals is unknown. Endemic infection in the facility seems to be maintained by chronically infected asymptomatic carriers that harbor FCoV population long after weaning, rather than through the repetitive occurrence of novel FCoV variants that escape immunity. Immune response to FCoV, though inadequate to sterilize the infection (FCoV-specific antibody titers were modest and remained low throughout the chronic infection), is vigorous enough to enforce immune system and to prevent infection by antigenically related FCoVs. To support this observation, a previous study<sup>14</sup> regarding coronavirus epidemiology, persistence, and evolution in a closed cat-breeding facility with an endemic FCoV infection reported that the virus involved was a serotype I strain and that some cats, previously identified as shedders, still had detectable viral RNA in their feces.

To determine if the cats were subject to frequent reinfection or were chronically infected asymptomatic carriers and to determine the relationship between the FCoV types in the breeding facility, a genetic analysis was performed on FCoV samples. It was observed that each FCoV sample constituted a cloud of variants with related, yet nonidentical, genomes. All of these data point to the unlikelihood of defining a clear distinction on the epidemiological prevalence between FCoV types I and II.

Several data report that FCoV type II does not occur very frequently in the field but is mostly used in laboratories because of its ease of cultivation *in vitro*. In contrast, FCoV type I replicates to high viral load *in vivo* and hardly stimulates the immune system, inducing higher antibody titers. Therefore, FCoV type I may become frequently associated with FIP, and this can be explained by the better adaptation of type I to cats.<sup>20</sup> Moreover, it is important to underline that FCoV type II originates from recombination events between FCoV type I and CCoV.<sup>15</sup> Because of the retrospective nature of the study, no data are available on the presence of dogs in the environment of the tested cats, but this hypothesis cannot be excluded, as witnessed by the higher number of FCoV type II positive cats detected. The results reported in the present study, supported by further analysis, allow for the consideration of ELISA, which was able to detect antibodies against both FCoV serotypes, as the gold standard for the screening of cat sera.

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### Sources and manufacturers

- a. BioWhittaker, Cambrex Bio Science, Verviers, Belgium.
- b. Falcon-Becton, Dickinson Labware, Franklin Lakes, NJ.
- c. Generously donated by Professor H.-J. Thiel, Justus Liebig Universität, Giessen, Germany.
- d. Sigma Chemicals, St. Louis, MO.
- e. Generously donated by Dr. Gilles Chappuis, Merial, Lyons, France.
- f. LAB-TEK Chamber Slide System, Nalge Nunc International Corp., Naperville, IL.
- g. bioMérieux sa, Marcy l'Etoile, France.
- h. NUNC, A/S, Roskilde, Denmark.
- i. Labogen, Cortex-Biochem, San Leandro, CA.
- j. Bio-Rad, Hercules, CA.

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