

Differential recognition of peptides within feline coronavirus polyprotein 1 ab by sera from healthy cats and cats with feline infectious peritonitis

Anastasia Chernyavtseva¹, Nick J. Cave, John S. Munday, Magdalena Dunowska*

School of Veterinary Science, Massey University, Palmerston North, New Zealand

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ABSTRACT

The aim of the study was to identify peptides within the polyprotein (Pp) 1 ab that are differentially recognised by cats with either enteric or systemic disease following infection with feline coronavirus. Overlapping 12-mer peptides ($n = 28,426$) across the entire Pp1ab were arrayed on peptide chips and reacted with pooled sera from coronavirus seropositive cats and from one seronegative cat. Eleven peptides were further tested in ELISA with individual serum samples, and three were selected for further screening. Two peptides (16433 and 4934) in the nsp3 region encoding the papain 1 and 2 proteases were identified for final testing. Peptide 4934 reacted equally with positive sera from healthy cats and cats with feline infectious peritonitis (FIP), while peptide 16433 was recognized predominantly by FIP-affected cats. The value of antibody tests based on these peptides in differentiating between the enteric and FIP forms of feline coronavirus infection remains to be determined.

1. Introduction

Feline enteric coronavirus (FECoV) infections are common among cats worldwide (Pedersen, 2009, 2014). Infected cats typically remain healthy or develop mild, self-limited enteritis. However, in a small proportion of FECoV-infected cats viral variants that have lost tropism for enterocytes and gained ability to replicate in macrophages emerge. This, combined with ineffective immune response to the virus, leads to development of a severe systemic disease termed feline infectious peritonitis (FIP) (Addie et al., 2009; Pedersen, 2014). Throughout this manuscript, the term “feline coronavirus (FCoV)” is used whenever distinction between the two pathotypes is not relevant. The terms FECoV and FIP virus (FIPV) are used when referring specifically to only one of the two pathotypes.

Clinically FIP is characterised by non-specific signs such as fever, loss of appetite and weight, jaundice or diarrhoea, that are accompanied by accumulation of protein-rich exudate in abdominal or chest cavities (wet form), or development of neurological deficits or ocular lesions (dry form) (Pedersen, 2009). There is no effective treatment and 95% of affected cats die within one year of the onset of disease (Legendre et al., 2017). While post-mortem diagnosis of FIP is fairly straight-forward due to the presence of characteristic histopathological lesions of widespread vasculitis (Kipar and Meli, 2014; Pedersen, 2009), the same is not true for *ante-mortem* assessment. A kitten from a multi-

cat environment that presents with compatible clinical signs is very likely to be affected by FIP (Pedersen, 2009). However, both attending veterinarians and owners of such cats often desire laboratory confirmation of the presumptive FIP diagnosis in order to facilitate an emotionally difficult decision to euthanize the cat. The fact that FIP usually affects young animals, combined with the variability in clinical and laboratory findings (Riemer et al., 2016) contributes to the problem.

As FIPV is highly macrophage-associated, detection of the virus *ante-mortem* requires invasive techniques and diagnostic sensitivity of the currently available tests is low (Pedersen et al., 2015; Tasker, 2018). In one study, the virus was detected in only approximately half of the effusion samples and none of the serum/plasma samples from FIP cats using a commercially available qPCR test (Felten et al., 2017). Cats exposed to FECV raise antibodies against structural proteins of the virus and the titer of these antibodies often rise to high levels after macrophage-tropic mutants arise and FIP disease begins (Pedersen, 2009). However, serology has been considered of limited diagnostic value due to inability to differentiate between immune responses to FECV and FIPV.

Feline coronaviruses are classified in the family *Coronaviridae* within the order *Nidovirales* (King et al., 2012). Other nidoviruses include members of *Arteriviridae*, *Roniviridae* and *Mesoniviridae* families. Typical for all nidoviruses, coronavirus non-structural genes are

* Corresponding author.

E-mail address: M.Dunowska@massey.ac.nz (M. Dunowska).

¹ Current address: Animal Health Laboratory, Ministry for Primary Industries, Wallaceville, New Zealand

Abbreviations

Ab	antibody
FCoV	feline coronavirus
FECV	feline enteric coronavirus
FIP	feline infectious peritonitis
neg	negative
nsp	non-structural protein
pos	positive
Pp1ab	polyprotein 1 ab

expressed soon after infection from two large open reading frames (ORF) 1a and 1b. The two polyprotein (Pp) products Pp1a and Pp1ab are then auto-cleaved into 16 non-structural proteins (nsps) that are essential for viral replication (Hagemeijer et al., 2012; Perlman and Netland, 2009). Thus, nsps are one of the first viral proteins abundantly produced within the infected cells. It is therefore logical to assume that cats infected with FCoV would raise an early immune response to at least some of FCoV nsps. However, while a number of previous studies focused on immune responses to structural proteins of the virus (Satoh et al., 2011; Takano et al., 2014), there are no data related to immune responses to nsps of FCoV. Similarly, studies with coronaviruses other than FCoV were designed to identify immunodominant epitopes within viral structural proteins, but not those present within nsps (Duan et al., 2005; Yu et al., 2007).

Several nsps have been identified as targets for adaptive humoral immune responses in nidoviruses other than coronaviruses. For example, a total of 10 non-linear B-cell epitopes were identified in nsp1, nsp2 and nsp4 of porcine respiratory and reproductive syndrome virus (PRRSV) (Oleksiewicz et al., 2001b) and sera from boars infected with PRRSV type I contained antibodies to both structural and non-structural proteins of the virus (Oleksiewicz et al., 2001a). In another study, sera from pigs infected with different PRRSV viruses reacted with nsp1, nsp2 and nsp7 (Brown et al., 2009). Johnson et al. (2007) described the presence of cross-reactive epitopes in nsp1 and nsp2 of various PRRSV strains, as well as type-specific epitopes within a hyper-variable region of nsp2. The latter provided a basis for development of serological assays able to differentiate between antibody responses due to infection versus vaccination. A number of nsps were also recognised by sera from horses infected with equine arteritis virus (EAV) (Go et al., 2011). Interestingly, there seemed to be a difference in the immune response to EAV nsps between horses that cleared the infection and those that became carriers (Go et al., 2011). There was also a difference between the antibody response to nsps of vaccinated horses and those experimentally infected with a virulent strain of EAV, suggesting that serological responses to nsps may be useful as a diagnostic tool to differentiate between infections with viruses of different virulence.

The aim of this study was to investigate humoral immune responses to FCoV nsps from Pp1ab in seropositive cats with different disease outcomes. We hypothesised that identification of immunodominant epitopes that are recognised by sera from the majority of healthy FCoV seropositive cats, but not by FIP-affected cats, would provide potential candidates for future development of vaccines against FIP. Immune responses raised by such vaccines may have the advantage of recognising FCoV infected cells early in infection, without the disadvantage of antibody-dependent enhancement of infection associated with humoral immune responses to structural proteins of the virus (Balint et al., 2014). Early clearance of FCoV infected cells would minimize the chances of *de novo* emergence of FIP-associated mutations and subsequent development of FIP. Identification of immunodominant epitopes that are recognised by sera from the majority of FIP-affected cats, but not by healthy FCoV seropositive cats, would provide targets for development of FIPV-specific serological test.

Table 1

Signalment data for cats with feline infectious peritonitis (FIP) and non-FIP cats enrolled into the study. Cats sampled on multiple occasions were considered as separate entries for the purpose of this comparison.

Category	FIP cats N (%)	Non-FIP cats N (%)
Age		
< 1	13 (43)	9 (15)
1	5 (17)	26 (42)
2	2 (7)	7 (11)
3–5	2 (7)	3 (5)
6–10	2 (7)	2 (3)
≥ 11	3 (10)	16 (26)
Not specified	3 (10)	0 (0)
Sex		
Female	2 (7)	19 (31)
Female spayed	12 (40)	9 (15)
Male	0 (0)	5 (8)
Male neutered	16 (53)	27 (44)
Unspecified	0 (0)	2 (3)
Breed		
Domestic short hair	19 (63)	49 (79)
Domestic medium hair	2 (7)	2 (3)
Domestic long hair	2 (7)	0 (0)
Siberian Forest	2 (7)	8 (13)
Ragdal	0 (0)	1 (2)
Burmese/Burmese x	1 (3)	1 (2)
Persian	2 (7)	0 (0)
Tonkinese	1 (3)	0 (0)
Birman	1 (3)	0 (0)
Source		
Colony cat	7 (23)	39 (63)
Diagnostic laboratory	0 (0)	9 (15)
Massey clinic/pathology	9 (30)	1 (2)
Private clinic	13 (43)	13 (21)
SPCA	1 (3)	0 (0)
Serology (ImmunoComb)^a		
≤ 1	0 (0)	7 (11)
1–1.5	0 (0)	4 (6)
2–2.5	1 (3)	4 (6)
3–3.5	1 (3)	10 (16)
4–4.5	14 (47)	20 (32)
5–5.5	14 (47)	17 (27)
Total	30	62

^a The results for different sample types collected from the same cat on the same sampling occasion never differed by more than 0.5. Hence, the result obtained with the serum sample was included in the table for cats from which multiple sample types were collected on the same sampling date.

2. Materials and methods

2.1. Cat enrolment and sampling

Cats with presumptive diagnosis of FIP were enrolled into the study from cases presented at Massey University Veterinary Teaching Hospital, as well as via local veterinary practices. The samples collected included serum/plasma, formalin-fixed tissue samples, and abdominal/thoracic effusion, if present. Fixed tissues were used for routine preparation of slides for histologic examination. Haematoxylin/eosin stained sections were assessed by a boarded pathologist (JM) for the presence of lesions characteristic of FIP (Kipar and Meli, 2014; Pedersen, 2009).

On occasion, immunohistochemistry (IHC) was performed to further support diagnosis of FIP. Following de-waxing and rehydration, slides were subjected to heat-induced antigen retrieval (98 °C in citrate buffer pH 6.0 for 20 min), loaded into Sequenza rack (Thermo Fisher Scientific), permeabilized (2 × 5 min in 0.25% TritonX in phosphate buffered saline (PBS) pH 7.0), washed with PBS containing 0.2% Tween 20 (PBST), blocked (Superblock with 0.1% Tween 20, Thermo Fisher Scientific) for 30 min, and incubated with primary antibody (FIPV3-70, Santa Cruz Biotechnology diluted 1:200 in PBST) at 4 °C overnight. The

Table 2

Sequences of peptides used in confirmatory ELISA assays for testing of individual samples (serum, plasma or effusion) from cats affected by feline infectious peritonitis (FIP) and from non-FIP cats for the presence of antibody (Ab) against feline coronavirus (FCoV).

Peptide ID	Sequence ¹	Amino acid position (AAY16374)	Fluorescence (arbitrary units)		
			Chip 1 control (FCoV Ab neg)	Chip 2 non-FIP (FCoV Ab pos)	Chip 3 FIP (FCoV Ab pos)
16431	VET SAKNDPWAA	1071–1082	0	0	5,076
16433	T SAKNDPWAAAV	1073–1084	0	0	6,952
4774	NGDLN HMGGVAR	1366–1377	12	1,460	0
4775	GDLN HMGGVARA	1367–1378	30	1,128	0
4929	CW INAICLALQR	1599–1611	20	2,378	0
4934	PTWKF PGVKGLW	1613–1624	18	1,485	0
22880	RGAVL GYIGATV	3874–3885	0	0	12,141
24480	VARRLL LGLQTQT	5474–5475	54	69	21,406
24481	ARRLL LGLQTQTV	5475–5486	13	0	14,830
25438	RCNLYNYGAQVR	6432–6443	92	2,648	0
28424	MVIGLLR KGKIL	6680–6691	4	284	18,403

¹ The amino acids present in the reference sequence (AAY16374) are shown in red.

following morning, slides were washed in PBST, quenched with 3% H₂O₂ in methanol for 10 min and washed again. The binding of primary antibody was detected using Mouse on Farma-HRP polymer (Biocare Medical) and Betazoid DAB chromogen kit (Biocare Medical) according to the manufacturer's instructions. After the final wash in water, the slides were counter stained with Gills haematoxylin.

Samples from non-FIP cats included archival serum/plasma samples from cats from Massey University Feline Nutrition Unit ("colony cats"), serum samples submitted to a diagnostic laboratory for unrelated reasons, and serum samples from healthy cats from households with FIP cats (Table 1). Samples collected for unrelated purposes (either routine yearly health checks or nutrition trials) were used whenever possible from colony cats to minimize the necessity for blood collection. Samples collected from the same cat, but at different dates, were labelled with the same sample ID, but different letter suffix (e.g. #57, #57a, #57b). The sampling protocol has been approved by the Massey University Animal Ethics Committee.

All serum, plasma and effusion samples were tested for the presence of antibody to structural proteins of FCoV using a commercially available ImmunoComb Feline Coronavirus (FIP) test (Biogal Laboratories), according to the manufacturer's instructions. The intensity of a blue colour of the sample dot was visually assessed on a scale 0 to 6, with the higher number indicating higher levels of FCoV antibody (Addie et al., 2015).

Table 3

Diagnostic sensitivity and specificity of ELISA tests with peptides 4934 and 16433 for detection of feline coronavirus antibody (4934 ELISA) or feline infectious peritonitis virus (FIPV) specific antibody (16433 ELISA).

ELISA	Threshold (corrected OD ₄₅₀)	Sensitivity (95% CI)	Specificity (95% CI)
4934	0.086 ^b	88.89 (79.95–94.79)%	80.00 (44.39–97.48)%
16433 (all cats) ^a	0.123 ^c	56.67 (37.43–74.54)%	91.94 (82.17–97.33)%
16433 (ImmunoComb + ve cats) ^a	0.123 ^c	56.67 (37.43–74.54)%	90.38 (78.97–96.80)%

^a Three colony cats that developed FIP two to four months following sampling (#70, #71 and #72) were excluded from this analysis, as they were healthy at the time of sampling.

^b The average corrected OD₄₅₀ value + 2SD obtained with samples from cats that were negative for feline coronavirus antibody by ImmunoComb. One outlier (#42) was excluded.

^c The average corrected OD₄₅₀ value + 2SD obtained with samples from all non-FIP cats, irrespective of their antibody status by ImmunoComb. Outliers (#34 and #56) were excluded.

2.2. Hybridisation of feline sera to peptide chips

Custom peptide chips were commercially synthesised (LLC Biosciences). Each library included 28,426 12-mer sequences covering all available variants of the entire Pp1ab of FCoV, with one amino acid walking distance. Each chip was then hybridised with the following samples:

Chip1: control serum from a cat negative for FCoV antibody (#66).

Chip 2: Pooled sera ($n = 5$) from FCoV antibody positive healthy colony cats (#45, #57, #67a, #68 and #69). The selected cats represented surviving siblings of cats that had died due to FIP. For each cat, archival blood sample from the same year as the death of the FIP-affected sibling was used in the study.

Chip 3: Pooled sera ($n = 5$) from FCoV antibody positive FIP cats (#04, #05, #07, #08, #09).

The level of fluorescence at each spot indicated the level of binding of the feline sera to a specific peptide. In order to visualise the level of fluorescence across Pp1ab, all peptide sequences as well as the reference sequence (AAY16374) were back-translated using a universal amino acid code. The back-translated nucleotide sequences were then mapped to the back-translated Pp1ab sequence, copied in the mapped order to an Excel spreadsheet, and then linked to the fluorescence data.

2.3. Peptide ELISAs

Selected peptides ($n = 11$, Table 2) were used as antigens in ELISA and tested with each individual serum sample that contributed to sample pools used for hybridisation to peptide chips. The selection of peptides was based on the presence of a comparatively stronger signal with a serum pool from FIP cats than with a serum pool from healthy FCoV seropositive cats (peptides 22880, 28424, 16431, 24480, 24481 and 16433), or vice-versa (peptides 4929, 4934, 25438, 4774 and 4775). Three peptides (4934, 25438, 16433) were further tested with an extended number of serum/plasma/effusion samples ($n = 50$) and two of those (4934, 16433) were further evaluated with additional samples ($n = 53$ for a total of 103 samples each).

CovaLink NH plates (Nunc, Thermo Fisher Scientific) were coated overnight at room temperature with 100 μ L of a relevant peptide/Sulfo-NHS solution (10 μ g/mL of peptide, 0.184 mg/mL of Sulfo-N-hydroxysuccinimide) in carbonate buffer (15 mM Na_2CO_3 , 35 mM NaHCO_3 , pH 9.6). Following three washes with distilled water plates were blocked with 300 μ L of 10% Goat Serum (Gibco, 16210-072) in carbonate buffer at room temperature for 2 h. The wells were then emptied and 100 μ L/well of each test sample diluted 1:10 in dilution buffer (phosphate buffered saline PH 7.2 (PBS) with 10% goat serum and 0.05% Tween 20) were added in duplicate to the plate. The plate was incubated at room temperature for 1 h, washed five times with CovaBuffer (2 M NaCl, 40 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05% Tween 20 in PBS). Horseradish (HRP)-conjugated Goat Anti-Cat IgG Fc (Abcam, ab112801) diluted 1:100000 in dilution buffer (100 μ L/well) at room temperature was then added to each well and the plate was incubated for 1 h at room temperature. After five washes with CovaBuffer, 100 μ L TMB ELISA Substrate (Highest Sensitivity) (Abcam, ab171522) was added to well and the plate was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 100 μ L of 1M H_2SO_4 to each well. The results were presented as corrected optical density values at 450 nm (OD_{450}), which were calculated by subtracting the OD_{450} of the “no peptide” well from the OD_{450} of the peptide-coated well tested with the same serum sample.

The diagnostic sensitivity and specificity of ELISA tests for detection of FCoV antibody positive cats (4934 ELISA) or FIP-affected cats (16433) were calculated using an on-line calculator available at https://www.medcalc.org/calc/diagnostic_test.php (Table 3). As similar corrected OD_{450} values were obtained when testing different sample types from the same cat (whenever available, see Fig. 5), values for only one sample type (in the order of preference: serum, plasma or effusion) were included in the analysis if more than one sample type was available from the same cat on the same sampling date.

3. Results

3.1. Cats

A total of 92 samples from 72 cats were available for the study (Table 1). The majority (28/42, 66.7%) of the non-FIP cats were clinically normal at the time of sampling. These comprised colony cats (39 samples from 19 cats), two kittens (#11 and #34) from the same households as FIP cases, and seven healthy cats/kittens from a breeding colony of Siberian Forest cats (#35 to #41). Two of the FIP-affected kittens (#09 and #30) from private households were Siberian Forest cats originally obtained from the same breeder. A small number (5/42, 11.9%) of non-FIP cats presented with clinical signs suggestive of FIP, but did not have histological lesions typical for FIP (#3, #6, #10, #12, #15). The remaining 9/42 (21.4%) non-FIP samples were opportunistically sourced from submissions to a diagnostic laboratory (#56 to #66). These were deemed to represent non-FIP cats based on the stated age of animals and the type of tests requested, but detailed clinical histories were not provided.

With the exception of one case (#18), a diagnosis of FIP was

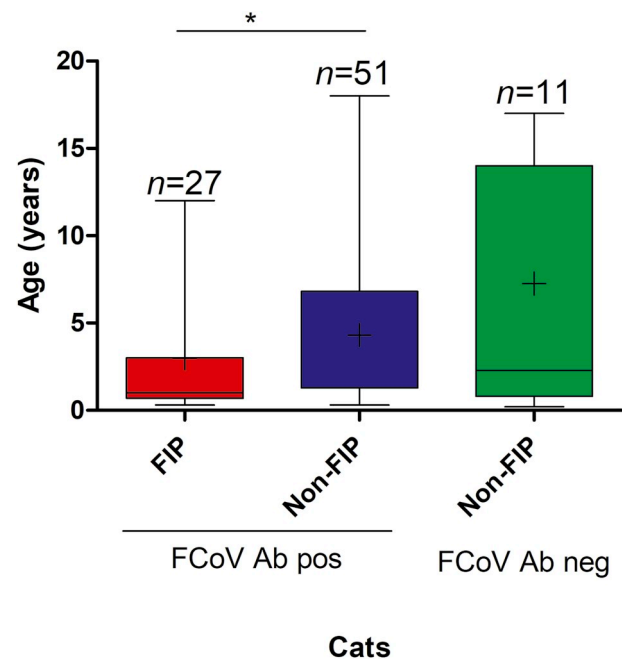


Fig. 1. Age distribution of FIP-affected and non-FIP cats at the time of sampling. Samples collected from the same cat on multiple sampling times were included as separate entries. Cats for which age was not available ($n = 3$) were excluded. The whiskers in box and whiskers graphs represent minimum and maximum values. “+” represents mean values.

confirmed histologically. The #18 cat was considered FIP-affected based on clinical history alone, as tissues were not available for the post-mortem examination. Finally, archival blood samples from three FIP-affected colony cats (#70, #71, #72) were collected while the cats appeared clinically normal, two to four months before they were euthanised due to FIP.

The range of ages for FIP (0.3–12 years), seropositive non-FIP (0.2–18 years) and seronegative non-FIP (0.2–17 years) cats were similar. However, the average age of FIP cats (3.0 years) was lower than the average age of seropositive non-FIP cats (4.3 years) ($p = 0.047$, Fig. 1). The distribution of sexes within FIP and non-FIP groups was also similar, with 54.8% and 52.5% of males (including both entire and castrated males) in each group, respectively.

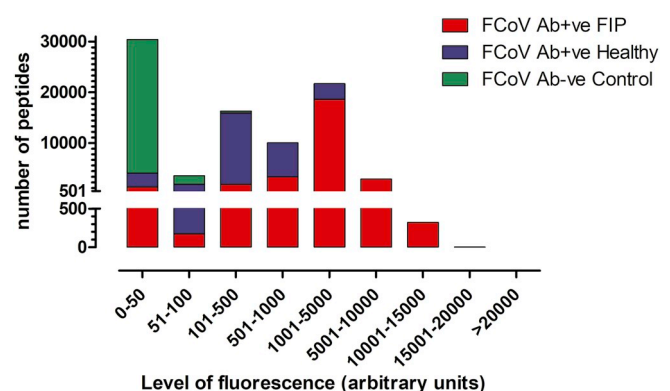


Fig. 2. Distribution of fluorescence between the three peptide chips. Each chip was coated with 28,426 peptides (12 amino acids each) covering all available variants of the entire polyprotein 1 ab of feline coronavirus (FCoV) and hybridised with three different samples comprising feline sera from cats with different FCoV antibody (Ab) and health status, as indicated. The presence of fluorescence indicates binding of antibody to a specific peptide. The level of fluorescence is presented in arbitrary units – higher numbers indicate higher fluorescence (= stronger binding). FIP = feline infectious peritonitis.

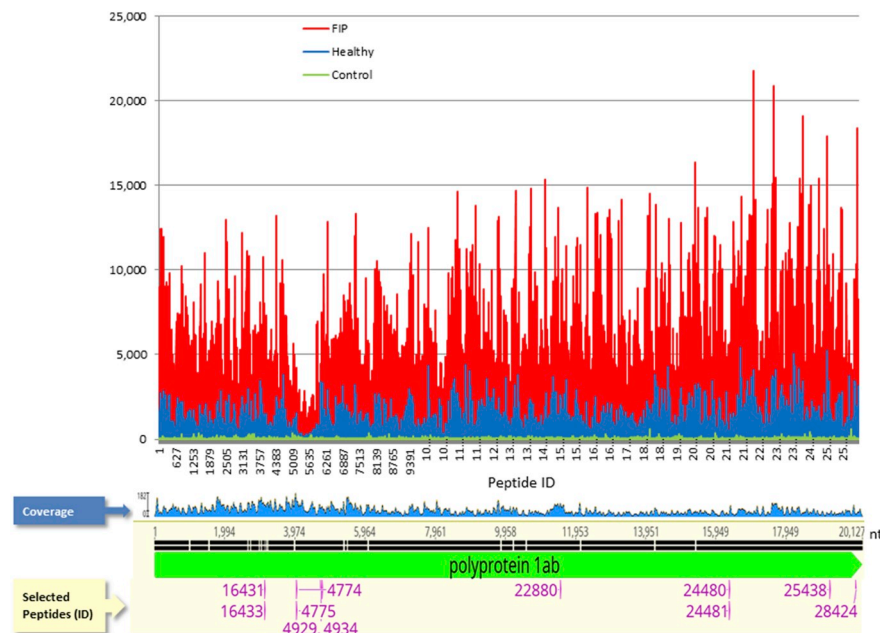


Fig. 3. Distribution of fluorescence plotted against polyprotein 1 ab. The position and ID of 11 peptides selected for further evaluation is indicated.

3.2. Hybridisation to peptide chips

The results of the testing are visually presented in Figs. 2 and 3. There was minimal binding of the negative control serum to Chip1 (average fluorescence per peptide 8 units, range 0–616), with clear binding detected to Chip 2 (average fluorescence per peptide 487 units, range 0–5424) and Chip 3 (average fluorescence per peptide 2562 units, range 0–21,745). Sera from FIP cats (Chip 3) appeared to recognise more antigens with stronger binding to selected peptides than sera from healthy cats (Chip 2).

One region, spanning about 34 amino acids within nsp3 (aa 1017 to 1051 in the reference sequence AAY16374) showed a comparatively low level of binding to sera from seropositive cats. The average fluorescence per peptide ($n = 228$) in that region was 415, 45 and 11 for chips 3, 2 and 1, respectively.

3.3. Peptide ELISAs

All 11 peptides tested showed some level of binding to FCoV-antibody positive sera and no binding to the control FCoV-antibody negative serum in ELISA. However, there was considerable variability between cats (Fig. 4). None of the peptides tested reacted exclusively with sera from FIP or serologically-positive non-FIP cats. Out of three peptides selected for further testing, one (25438) produced inconsistent binding patterns and was discarded. The ELISA results for the remaining two (16433 and 4934) are shown in Figs. 5 and 6. Both peptides were located within nsp3 of FCoV: peptide 16433 within the N-terminus of papain 1 protease domain (PLP1) and peptide 4934 within PLP2 domain. There was no difference in the mean corrected OD₄₅₀ values obtained with sera from either FIP-affected (0.52) or non-FIP seropositive cats (0.51) when tested against peptide 4934 (Fig. 6), although there was a considerable variability in the level of positivity between individual cats (Fig. 5). Peptide 16433 was recognized predominantly by FIP-affected cats (Fig. 5), with the mean corrected OD₄₅₀ values for samples from FIP cats (0.52) significantly higher than mean corrected OD₄₅₀ of sera from either FCoV-antibody positive non-FIP cats (0.09, $p < 0.0001$) or seronegative cats (0.01, $p < 0.0001$) (Fig. 6). The immunogenicity of this peptide appeared to be linked to the presence of threonine (Thr) at the first position (Fig. 7).

The diagnostic sensitivity and specificity of ELISA tests with

peptides 4934 and 16433 for detection of FCoV antibody positive cats or FIP-affected cats, respectively, are shown in Table 3.

4. Discussion

The study was designed to test two hypotheses. Firstly, cats infected with FCoV develop humoral immune responses to selected nsps from Pp1ab of the virus. Secondly, the targets for such immune responses differ between cats that develop FIP and cats that do not. We have shown that infection with FCoV induces antibodies that recognise nsps of FCoV. This conclusion was based on results from two different tests: hybridisation of pooled sera to custom-made peptide chips, and ELISA with selected peptides used as antigens against individual serum samples from cats with different FCoV antibody- and health-status. The agreement between the results of the two tests was poor (Fig. 4). This is not necessarily unexpected, as the tests were run using different binding conditions and different detection methods. In addition, the chip data would have been affected by the make-up of the pooled serum samples, while individual serum samples were used in ELISA. In addition, the development of peptide ELISAs was hindered by problems associated with the presence of high background due to non-specific binding of feline sera to the plates. Normalisation of the data to OD₄₅₀ values from “no peptide” wells allowed for the correction for non-specific binding, but this may have also reduced sensitivity of the test and hence, may have contributed to the differences observed between the chip and ELISA data for some combinations of peptides and clinical samples.

Irrespective of these shortcomings, it is clear that cats' immune system is able to recognise Pp1ab following infection with FCoV, as sera from FCoV-antibody positive cats (both FIP-affected and non-FIP) showed higher fluorescence (for chip data) or higher OD₄₅₀ values (for ELISA) than sera from FCoV antibody negative cats. However, we were unable to map specific immunodominant epitopes within Pp1ab based on the chip data. This is likely to reflect the considerable variability in the Pp1ab sequences available in the public databases, combined with the use of pooled sera.

We expected to see spikes in reactivity at few selected locations of Pp1ab. Instead, the reactivity of feline sera from FCoV seropositive cats was fairly constant across the entire Pp1ab, with only one region of apparently low reactivity. The low immunogenic region was located in the N-terminus of nsp3, within the hypervariable Glu-rich domain that

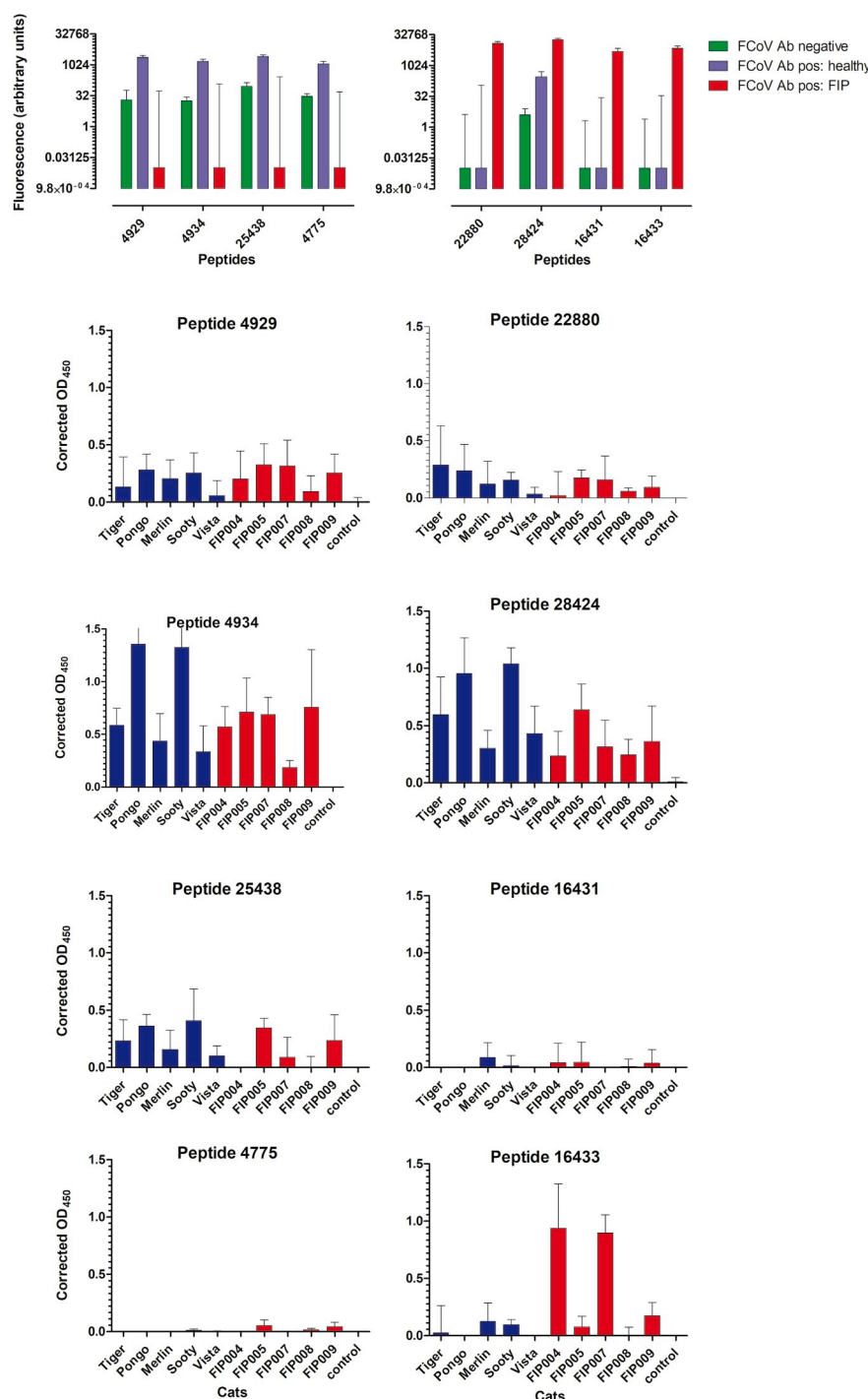


Fig. 4. Comparison of the level of antibody (Ab) to four selected peptides from feline coronavirus (FCoV) polyprotein 1 ab as detected using either the peptide chip (top two graphs) or peptide ELISA (bottom eight graphs).

is present in all coronaviruses. The function of this region is currently unknown (Lei et al., 2018).

We hoped that identification of immunodominant peptides that are recognised by sera from all FCoV infected cats combined with those that are selectively recognised by sera from FIP-affected cats only would provide potential targets for future development of specific diagnostic tests for FIP. Two of the peptides identified in the current study (4934 and 16433), both located within nsp3, showed promising binding pattern with extended collection of feline sera from FIP and non-FIP cats. There was, however, a considerable level of variability in the ability of sera from individual cats to recognise the two selected peptides (Fig. 5).

The reasons for the observed differences remain to be established, but may include factors such as age, genetic make-up, previous exposure to similar antigens, or timing of sample collection with relation to FCoV infection. They may also be virus-related and reflect variability between field viruses circulating among cats (Kipar and Meli, 2014). The fact that the reactivity of the feline sera from FIP affected cats seemed to be linked to the presence of Thr at residue 1073 of Pp1ab seems to support the importance of viral sequence on the selective recognition of this peptide by some of the FIP affected cats. As the majority of FCoV sequences available in public databases had Val at this position, it would be of interest to investigate the effect of Val₁₀₇₃ to Thr₁₀₇₃ change on

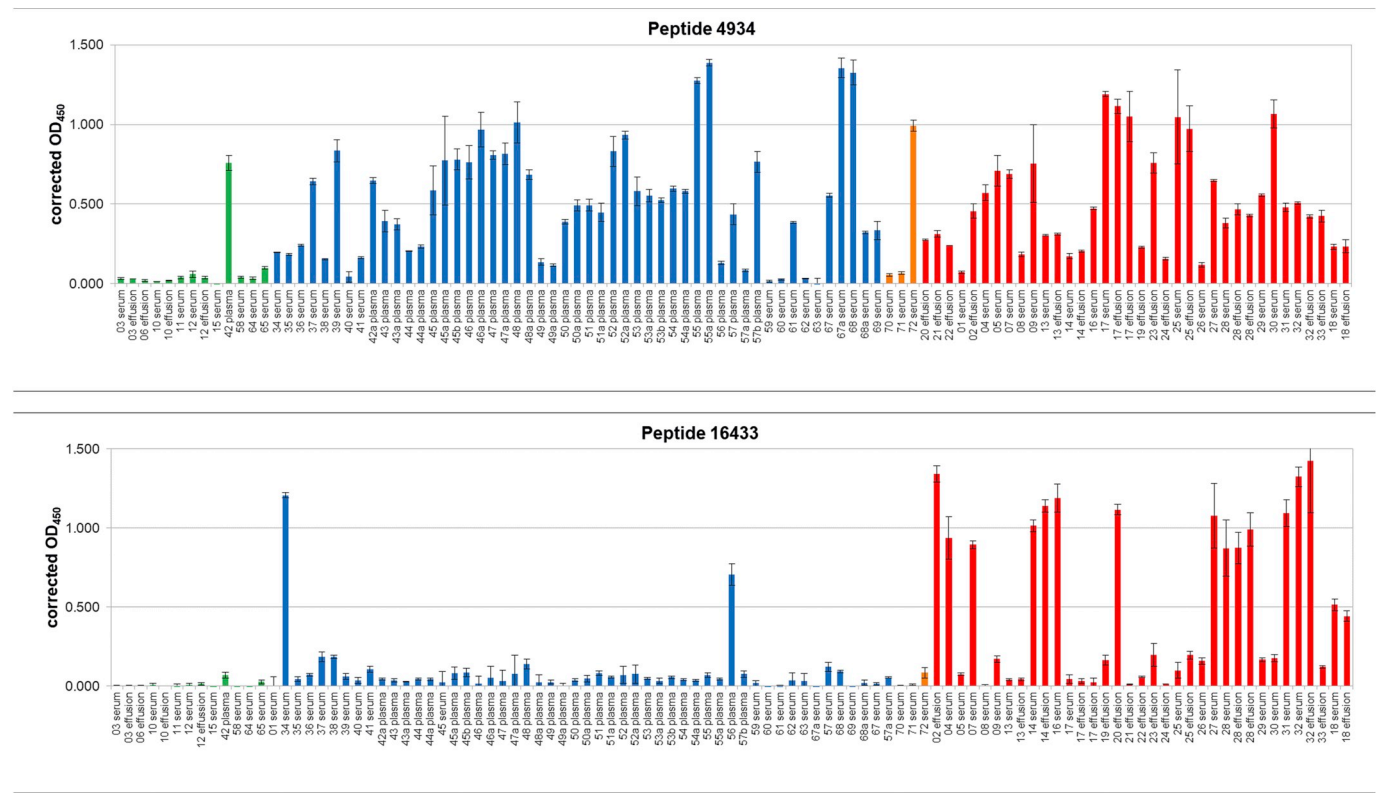


Fig. 5. Peptide ELISA results with two selected peptides. Red colour indicates samples from feline infectious peritonitis (FIP) cats, orange samples from cats that were healthy at the time of sampling but succumbed to FIP two to four months later, blue samples from non-FIP cats positive for feline coronavirus (FCoV) antibodies on ImmunoComb FIP (Biogal) test, and green non-FIP cats negative for FCoV antibody on ImmunoComb FIP test.

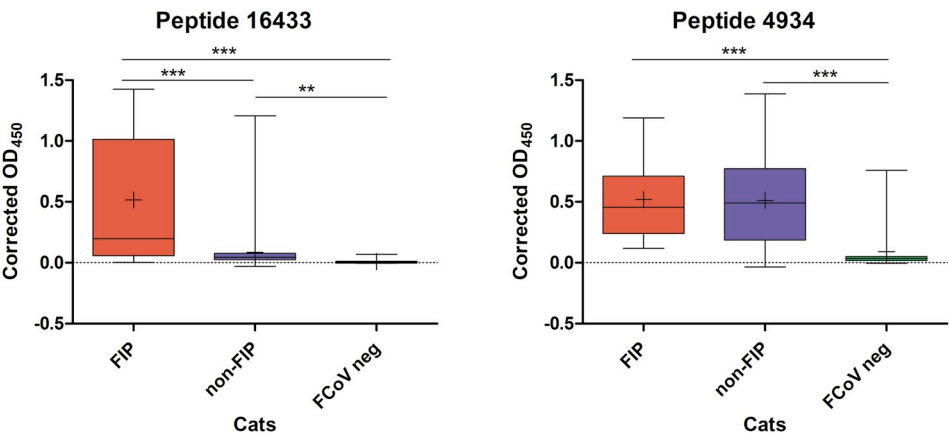


Fig. 6. Summary of data presented in Fig. 5. Corrected OD₄₅₀ values obtained using ELISA with peptides 16433 (left) and 4934 (right). The whiskers in box and whiskers graphs represent minimum and maximum values. “+” represents mean values. Significance (one way ANOVA) is indicated by ** (p < 0.01) or *** (p < 0.001).

the pathogenicity of the virus in future studies.

Although corrected OD₄₅₀ values of sera from FCoV-antibody positive cats that did not show any clinical signs of FIP at the time of sampling were generally low in peptide 16433 ELISA (Fig. 6), sera from two healthy cats were highly positive. One of these two cats (#34) was a young cat residing in the same household as a FIP-affected kitten (#31). The other cat (#56) was an adult (13 years old) healthy colony cat. None of these two cats developed FIP within half a year of sample collection: cat #34 died due to chronic kidney failure and cat #56 remained healthy.

In general, sera that were negative for FCoV antibody based on the ImmunoComb FIP assay showed low corrected OD₄₅₀ values when tested by peptide ELISA with either 16433 or 4934 peptide, supporting the view that high corrected OD₄₅₀ values were indicative of the presence of FCoV-specific antibody in feline sera. One exception was serum from the cat #42, which was negative by ImmunoComb FIP testing, but

highly positive by ELISA with peptide 4934. The same cat tested highly positive both by ImmunoComb (5/6) and 4934 ELISA a week later (#42a in Fig. 5). One may hypothesise that the immune response to nsps (tested by ELISA) precedes the immune response to the structural proteins of the virus (detected by ImmunoComb FIP). If so, the likely explanation for these results is that the blood sample from the cat #42 was collected soon after infection with FCoV, before antibodies to structural proteins of FCoV were raised.

In conclusion, two selected peptides were tested with an extended numbers of feline serum/plasma/effusion samples. While peptide 4934 was recognized by the majority of FCoV infected cats irrespective of their FIP status, diagnostic sensitivity and specificity of peptide 4934 ELISA was low when compared with the commercially available ImmunoComb test. Hence, the use of this peptide alone for serological diagnosis of FCoV infection is probably of a limited value. In contrast, peptide 16433 was preferentially detected by FIP-affected cats and not

Consensus	060	1,070	1,090	1,090	FIP cats			Non-FIP cats		
					Mean	SD	p	Mean	SD	p
6566	PDEQLSSVEEKVEISAKNDPWAAA	DEKVEISAKNDP			747	483	4.10E-02	81	245	7.00E-01
7553	EEFVDSAKNDP	EEFVDSAKNDP			578	633	3.90E-01	26	70	5.30E-01
8532	EEKVEISAKNDP	EEKVEISAKNDP			989	380	1.20E-03	184	126	1.20E-01
9699	EEKVDVSAKNDP	EEKVDVSAKNDP			199	267	5.50E-01	50	69	6.00E-01
12339	EEKVEITAKNDP	EEKVEITAKNDP			1,220	413	4.10E-04	112	119	2.90E-01
14989	EEKVEISAKNDP	EEKVEISAKNDP			198	182	3.80E-01	160	161	3.60E-01
16428	EEKVEITAKNDP	EEKVEITAKNDP			3,840	1,682	2.00E-02	0	62	1.00E+00
18084	MTKAEISAKNDP	MTKAEISAKNDP			263	369	3.50E-01	134	62	1.10E-01
20074	EKKDEISAKNDP	EKKDEISAKNDP			544	433	1.50E-01	118	45	6.20E-02
1606	EKVEISAKNDPW	EKVEISAKNDPW			991	469	1.40E-01	23	34	9.60E-01
7554	EEFVDSAKNDPW	EEFVDSAKNDPW			3,163	1,277	1.40E-02	1	89	8.30E-01
8533	EEKVEISAKNDPW	EEKVEISAKNDPW			3,500	918	4.60E-05	537	349	1.10E-01
9700	EKVDVSAKNDPW	EKVDVSAKNDPW			1,497	463	1.60E-03	884	361	1.60E-02
11058	EKVEISAKNDPW	EKVEISAKNDPW			1,182	471	6.10E-02	244	119	6.10E-02
12340	EKVEITAKNDPW	EKVEITAKNDPW			2,246	984	1.20E-02	739	295	1.10E-02
14990	EEKVEISAKNDPW	EEKVEISAKNDPW			1,248	509	1.70E-02	211	86	2.20E-02
16429	EKVEITAKNDPW	EKVEITAKNDPW			6,782	1,888	2.80E-04	176	101	1.60E-01
17453	EKVEISAKNDPW	EKVEISAKNDPW			2,735	638	2.30E-05	288	214	1.50E-01
18085	TKAEISAKNDPW	TKAEISAKNDPW			1,423	736	4.10E-02	214	166	2.80E-01
20075	EKKDEISAKNDPW	EKKDEISAKNDPW			1,871	632	2.00E-03	477	194	2.20E-02
1607	EKVEISAKNDPWAA	EKVEISAKNDPWAA			438	187	4.50E-01	0	18	1.00E+00
7555	EEFVDSAKNDPWAA	EEFVDSAKNDPWAA			2,682	1,129	1.90E-02	11	90	7.50E-01
8534	EKVEISAKNDPWAA	EKVEISAKNDPWAA			2,050	617	2.10E-04	557	278	4.00E-02
9306	NAEISAKNDPWAA	NAEISAKNDPWAA			362	584	5.80E-01	71	65	3.70E-01
9701	EKVDVSAKNDPWAA	EKVDVSAKNDPWAA			709	520	2.00E-01	398	161	1.70E-02
11059	EKVEISAKNDPWAA	EKVEISAKNDPWAA			483	318	5.60E-01	65	48	3.60E-01
12341	EKVEITAKNDPWAA	EKVEITAKNDPWAA			1,096	531	1.20E-02	324	168	4.50E-02
14991	NAEISAKNDPWAA	NAEISAKNDPWAA			1,011	484	4.40E-02	223	179	2.40E-01
16430	EKVEITAKNDPWAA	EKVEITAKNDPWAA			4,449	1,114	4.80E-05	0	51	1.00E+00
17454	EKVEISAKNDPWAA	EKVEISAKNDPWAA			1,005	397	1.40E-02	0	40	1.00E+00
18086	EKVEISAKNDPWAA	EKVEISAKNDPWAA			538	518	2.30E-01	54	81	8.10E-01
20076	EKVEISAKNDPWAA	EKVEISAKNDPWAA			1,551	426	1.30E-04	223	163	2.50E-01
27316	EKVEISAKNDPWAA	EKVEISAKNDPWAA			1,647	891	7.00E-02	469	274	7.50E-02
1608	EKVEISAKNDPWAA	EKVEISAKNDPWAA			336	328	9.10E-01	0	353	1.00E+00
8535	EKVEISAKNDPWAA	EKVEISAKNDPWAA			1,845	605	5.80E-04	325	171	4.70E-02
9307	AEISAKNDPWAA	AEISAKNDPWAA			0	261	1.00E+00	85	70	3.00E-01
9702	VDVSAKNDPWAA	VDVSAKNDPWAA			681	282	2.30E-02	296	170	9.60E-02
11060	VEISAKNDPWAA	VEISAKNDPWAA			306	284	9.80E-01	98	75	3.00E-01
12342	VEITAKNDPWAA	VEITAKNDPWAA			949	899	1.90E-01	213	118	5.50E-02
14992	AEISAKNDPWAA	AEISAKNDPWAA			477	288	1.30E-01	182	147	2.50E-01
16431	VEITAKNDPWAA	VEITAKNDPWAA			5,076	2,005	1.00E-02	0	28	1.00E+00
18087	AEISAKNDPWAA	AEISAKNDPWAA			508	460	2.00E-01	14	74	1.00E+00
20077	DEISAKNDPWAA	DEISAKNDPWAA			1,125	515	1.90E-02	98	61	2.90E-01
27317	VEISAKNDPWAA	VEISAKNDPWAA			1,062	1,255	4.10E-01	183	99	4.20E-02
1609	EASAKNDPWAAA	EASAKNDPWAAA			0	153	1.00E+00	0	18	1.00E+00
8536	EISAKNDPWAAA	EISAKNDPWAAA			0	175	1.00E+00	287	192	1.20E-01
9308	EISAKNDPWAAA	EISAKNDPWAAA			98	206	7.70E-01	168	91	8.80E-02
9703	DVSAKNDPWAAA	DVSAKNDPWAAA			314	291	3.40E-01	322	294	2.90E-01
12343	EVITAKNDPWAAA	EVITAKNDPWAAA			243	228	3.40E-02	176	124	1.20E-01
16432	EVITAKNDPWAAA	EVITAKNDPWAAA			4,767	1,131	1.80E-05	0	15	1.00E+00
18088	EVSANNDPWAAA	EVSANNDPWAAA			438	495	2.90E-01	84	63	4.30E-01
20078	EVSANNDPWAAA	EVSANNDPWAAA			621	438	1.10E-01	0	34	1.00E+00
27318	EGSAKNDPWAAA	EGSAKNDPWAAA			1,579	774	4.60E-02	325	288	2.30E-01
1610	ASAKNDPWAAA	ASAKNDPWAAA			443	226	5.20E-01	86	48	1.80E-01
8537	ISAKNDPWAAA	ISAKNDPWAAA			0	192	6.20E-01	748	490	1.20E-01
9309	VSANNDPWAAA	VSANNDPWAAA			384	255	1.70E-01	206	96	4.40E-02
12344	VTAKNDPWAAA	VTAKNDPWAAA			1,489	439	8.40E-05	282	277	2.90E-01
16433	TSANNDPWAAA	TSANNDPWAAA			6,952	2,256	1.80E-03	0	35	1.00E+00
18089	VSANNDPWAAA	VSANNDPWAAA			0	291	1.00E+00	0	40	1.00E+00
20079	VSANNDPWAAA	VSANNDPWAAA			811	499	7.40E-02	99	86	4.50E-01
27319	GSANNDPWAAA	GSANNDPWAAA			3,281	1,286	1.20E-02	0	8	1.00E+00
9310	SAKNDPWAAV	SAKNDPWAAV			833	370	3.10E-02	411	167	1.70E-02
12345	TAKNDPWAAV	TAKNDPWAAV			2,816	858	3.70E-04	217	125	6.50E-02
18090	SAKNDPWAAV	SAKNDPWAAV			573	621	2.90E-01	114	204	6.90E-01
20080	SAKNDPWAAV	SAKNDPWAAV			3,286	1,140	3.10E-03	261	104	2.90E-02

Fig. 7. Alignment of 65 peptides surrounding peptide 16433, with corresponding fluorescence values obtained with pooled sera from cats affected by feline infectious peritonitis (FIP cats) and feline coronavirus seropositive healthy cats (non-FIP cats). The consensus sequence is shown at the top. Arrows point to peptides with threonine (T) at position 1073, which appears to be linked to increased binding of sera from FIP cats, but not from healthy seropositive cats.

be FCoV antibody positive non-FIP cats. Although only 57% of FIP affected cats were positive in peptide 16433 ELISA, the test appeared reasonably specific, with approximately 90% of negative cats being either healthy or affected by diseases other than FIP at the time of sampling. This is the first description of a serological test that appears to have some discriminatory power between FCoV infected cats that remain healthy versus those that develop FIP. Availability of such test would be of a great benefit to companion animal veterinarians worldwide. Hence, it would be of value to investigate factors that influence development of antibodies to peptide 16433 in FCoV infected cats. Future research should also involve search for additional peptides with similar properties to improve diagnostic performance of the test. Altogether, identification of peptide 16433 provides a proof-of-concept that development of a serological assay to support diagnosis of FIP may be feasible.

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