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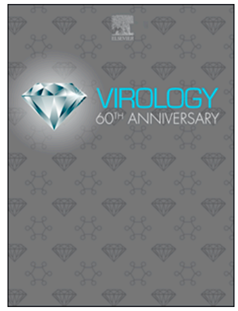

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Differential recognition of peptides within feline coronavirus polyprotein 1ab by sera from healthy cats and cats with feline infectious peritonitis.

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Keywords: FIP, feline coronavirus, feline infectious peritonitis, non-structural proteins, antibody response, feline enteric coronavirus.

Abbreviations:

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

Abstract

The aim of the study was to identify peptides within the polyprotein (Pp) 1ab 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 five 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.

Highlights

- Cats develop antibodies to polyprotein 1ab (Pp1ab) of feline coronavirus.
- This is most evident for cats with feline infectious peritonitis (FIP).
- Differences exist in responses to selected peptides between FIP and non-FIP cats.
- Such differences may be utilised for development of a serological test for FIP.

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 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 et al., 1977). 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 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 immunodominat 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 FECoV 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.

Materials and Methods

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 minutes), loaded into Sequenza rack (Thermo Fisher Scientific), permeabilized (2 x 5 minutes 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 minutes, and incubated with primary antibody (FIPV3-70, Santa Cruz Biotechnology diluted 1:200 in PBST) at 4 °C overnight. The following morning, slides were washed in PBST, quenched with 3% H_2O_2 in methanol for 10 minutes 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).

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.

Peptide ELISAs

Selected peptides (*n*=11, Table 2) were used as antigens in ELISA-based format 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). Five peptides (25438, 26242, 28423, 25438, 16433) were further tested with an extended number of serum/plasma/effusion samples (*n*=50) and two of those (25438, 16433) were further evaluated with additional samples (*n*=53 for a total of 103 samples each).

CovaLink NH plates (Nunc, Thermo Scientific) were coated overnight at room temperature with 100 µL of a relevant peptide/Sulfo-NHS solution (10 µg/mL of peptide, 0.184 mg/mL of Sulfo-Nhydroxysuccinimide) 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 µL of 10 % Goat Serum (Gibco, 16210-072) in carbonate buffer at room temperature for two hours. The wells were then emptied and 100 µL/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 one hour, washed five times with CovaBuffer (2 M NaCl, 40 mM $MgSO_4 \cdot 7H_20$, 0.05% Tween 20 in PBS). Horseradish (HRP)-conjugated Goat Anti-Cat IgG Fc (Abcam, ab112801) diluted 1:100000 in dilution buffer (100 µL/well) at room temperature was then added to each well and the plate was incubated for one hour at room temperature. After five washes with CovaBuffer, 100 µL 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 µL 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 Figure 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.

Results

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 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 to 12 years), seropositive non-FIP (0.2 to 18 years) and seronegative non-FIP (0.2 to 17 years) cats were similar. However, the median age of FIP cats (3.0 years) was lower than an average age of seropositive non-FIP cats (4.3 years) ($p=0.047$, Figure 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.

Hybridisation to peptide chips

The results of the testing are visually presented in Figures 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 - 5,424) and Chip 3 (average fluorescence per peptide 2,562 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 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 1, 2 and 3, respectively.

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 (Figure 4). None of the peptides tested reacted exclusively with sera from FIP or serologically-positive non-FIP cats. Out of five peptides selected for further testing, three (25438, 26242, 28423) produced inconsistent binding patterns and were discarded. The ELISA results for the remaining two (16433 and 4934) are shown in Figures 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 OD_{450} values obtained with sera from either FIP-affected (0.52) or non-FIP seropositive cats (0.51) when tested against peptide 4934 (Figure 6), although there was a considerable variability in the level of positivity between individual cats (Figure 5). Peptide 16433 was recognized predominantly by FIP-affected cats (Figures 5), with the mean corrected OD_{450} values for samples from FIP cats (0.52) significantly higher than mean corrected OD_{450} of sera from either FCoV-antibody positive non-FIP cats (0.09, $p<0.0001$) or seronegative cats (0.01, $p<0.0001$) (Figure 6). The immunogenicity of this peptide appeared to be linked to the presence of threonine (T) at the first position (Figure 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.

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 (Figure 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 OD_{450} 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 OD_{450} 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 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 (Figure 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 Val_{1073} to Thr_{1073} change on the pathogenicity of the virus in future studies.

Although corrected OD_{450} 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 (Figure 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 OD_{450} values when tested by peptide ELISA with either 16433 or 4934 peptide, supporting the view that high corrected OD_{450} 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 Figure 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 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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Figure legends:

Figure 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.

Figure 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 1ab 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.

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

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

Figure 5: Peptide ELISA results with two selected peptides. Red colour indicates samples from 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 FCoV antibodies on ImmunoComb FIP (Biogal) test, and green non-FIP cats negative for FCoV antibody on ImmunoComb FIP test.

Figure 6: Summary of data presented in Figure 5. Corrected OD_{450} 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 *** ($p<0.001$).

Figure 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.

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)
Burnese/Burnese 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)[1]		
≤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**

[1] 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.

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	VETSAKNDPWAA	1071-1082	0	0	5,076
16433	TSAKNDPWAAAV	1073-1084	0	0	6,952
4774	NGDLNHMGGVAR	1366-1377	12	1,460	0
4775	GDLNHMGGVARA	1367-1378	30	1,128	0
4929	CWINAICLALQR	1599-1611	20	2,378	0
4934	PTWKFPGVKGLW	1613-1624	18	1,485	0
22880	RGAVLGYIGATV	3874-3885	0	0	12,141
24480	VARRLLGLQTQT	5474-5475	54	69	21,406
24481	ARRLLGLQTQTV	5475-5486	13	0	14,830
25438	RCNLYNYGAQVR	6432-6443	92	2,648	0
28424	MVIGLLRKGKIL	6680-6691	4	284	18,403

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

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[2]	88.89 (79.95-94.79)%	80.00 (44.39-97.48)%
16433 (all cats)[1]	0.123[3]	56.67 (37.43-74.54)%	91.94 (82.17-97.33)%
16433 (Immunocomb +ve cats)[1]	0.123[3]	56.67 (37.43-74.54)%	90.38 (78.97-96.80)%

[1] 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.

[2] The average corrected OD_{450} value + 2SD obtained with samples from cats that were negative for feline coronavirus antibody by ImmunoComb. One outlier (#42) was excluded.

[3] The average corrected OD_{450} value + 2SD obtained with samples from all non-FIP cats, irrespective of their antibody status by ImmunoComb. Outtliers (#34 and #56) were excluded.

References

Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K., Hosie, M.J., Lloret, A., Lutz, H., Marsilio, F., Pennisi, M.G., Radford, A.D., Thiry, E., Truyen, U., Horzinek, M.C., 2009. Feline infectious peritonitis. ABCD guidelines on prevention and management. J Feline Med Surg 11, 594-604.

Addie, D.D., le Poder, S., Burr, P., Decaro, N., Graham, E., Hofmann-Lehmann, R., Jarrett, O., McDonald, M., Meli, M.L., 2015. Utility of feline coronavirus antibody tests. J Feline Med Surg 17, 152-162.

Balint, A., Farsang, A., Szeredi, L., Zadori, Z., Belak, S., 2014. Recombinant feline coronaviruses as vaccine candidates confer protection in SPF but not in conventional cats. Vet. Microbiol. 169, 154-162.

Brown, E., Lawson, S., Welbon, C., Gnanandarajah, J., Li, J., Murtaugh, M.P., Nelson, E.A., Molina, R.M., Zimmerman, J.J., Rowland, R.R., Fang, Y., 2009. Antibody response to porcine reproductive and respiratory syndrome virus (PRRSV) nonstructural proteins and implications for diagnostic detection and differentiation of PRRSV types I and II. Clin Vaccine Immunol 16, 628-635.

Duan, J., Yan, X., Guo, X., Cao, W., Han, W., Qi, C., Feng, J., Yang, D., Gao, G., Jin, G., 2005. A human SARS-CoV neutralizing antibody against epitope on S2 protein. Biochem. Biophys. Res. Commun. 333, 186-193.

Felten, S., Leutenegger, C.M., Balzer, H.J., Pantchev, N., Matiasek, K., Wess, G., Egberink, H., Hartmann, K., 2017. Sensitivity and specificity of a real-time reverse transcriptase polymerase chain reaction detecting feline coronavirus mutations in effusion and serum/plasma of cats to diagnose feline infectious peritonitis. BMC Vet Res 13, 228.

Go, Y.Y., Snijder, E.J., Timoney, P.J., Balasuriya, U.B., 2011. Characterization of equine humoral antibody response to the nonstructural proteins of equine arteritis virus. Clin Vaccine Immunol 18, 268-279.

Hagemeijer, M.C., Rottier, P.J., de Haan, C.A., 2012. Biogenesis and dynamics of the coronavirus replicative structures. Viruses 4, 3245-3269.

Johnson, C.R., Yu, W., Murtaugh, M.P., 2007. Cross-reactive antibody responses to nsp1 and nsp2 of Porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 88, 1184-1195.

King, A.M.Q., Adams, M.J., Carstens, E.B., Lefkowitz, E.J., 2012. Virus taxonomy: classification and nomenclature of viruses : ninth report of the International Committee on Taxonomy of Viruses, in: Division., I.U.o.M.S.V. (Ed.). Elsevier/Academic Press, Amsterdam, pp. x, 1327 p.

Kipar, A., Meli, M.L., 2014. Feline infectious peritonitis: still an enigma? Vet. Pathol. 51, 505-526.

Legendre, A.M., Kuritz, T., Galyon, G., Baylor, V.M., Heidel, R.E., 2017. Polyprenyl Immunostimulant Treatment of Cats with Presumptive Non-Effusive Feline Infectious Peritonitis In a Field Study. Frontiers in veterinary science 4, 7.

Lei, J., Kusov, Y., Hilgenfeld, R., 2018. Nsp3 of coronaviruses: Structures and functions of a large multi-domain protein. Antiviral Res. 149, 58-74.

Oleksiewicz, M.B., Botner, A., Normann, P., 2001a. Semen from boars infected with porcine reproductive and respiratory syndrome virus (PRRSV) contains antibodies against structural as well as nonstructural viral proteins. Vet. Microbiol. 81, 109-125.

Oleksiewicz, M.B., Botner, A., Toft, P., Normann, P., Storgaard, T., 2001b. Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. J. Virol. 75, 3277-3290.

Pedersen, N.C., 2009. A review of feline infectious peritonitis virus infection: 1963-2008. J Feline Med Surg 11, 225-258.

Pedersen, N.C., 2014. An update on feline infectious peritonitis: virology and immunopathogenesis. Vet. J. 201, 123-132.

Pedersen, N.C., Eckstrand, C., Liu, H., Leutenegger, C., Murphy, B., 2015. Levels of feline infectious peritonitis virus in blood, effusions, and various tissues and the role of lymphopenia in disease outcome following experimental infection. Vet. Microbiol. 175, 157-166.

Perlman, S., Netland, J., 2009. Coronaviruses post-SARS: update on replication and pathogenesis. Nat Rev Microbiol 7, 439-450.

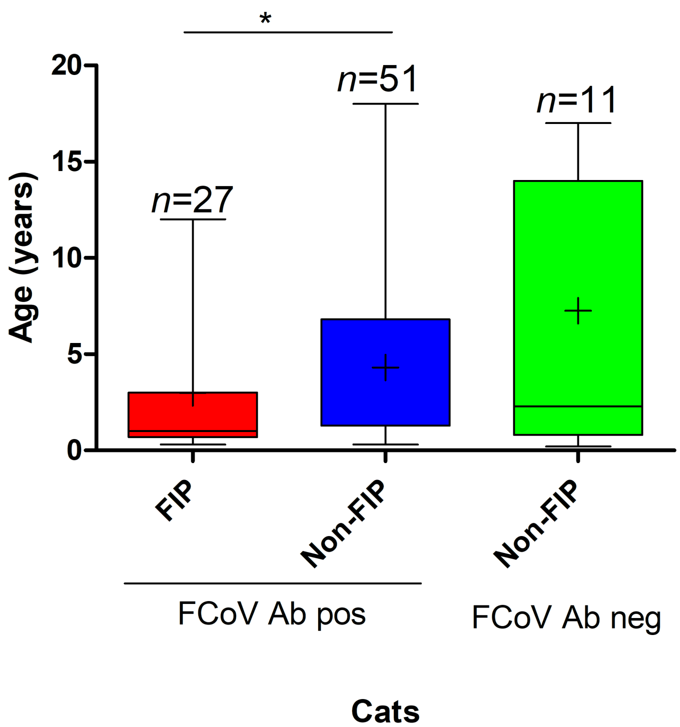
Riemer, F., Kuehner, K.A., Ritz, S., Sauter-Louis, C., Hartmann, K., 2016. Clinical and laboratory features of cats with feline infectious peritonitis--a retrospective study of 231 confirmed cases (2000-2010). J Feline Med Surg 18, 348-356.

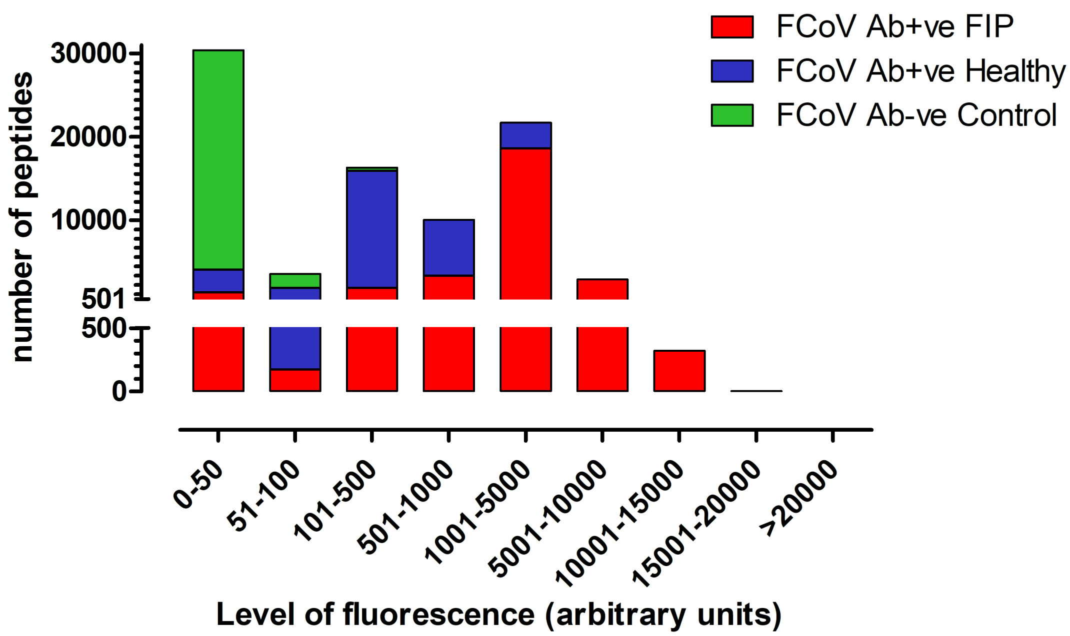
Satoh, R., Furukawa, T., Kotake, M., Takano, T., Motokawa, K., Gemma, T., Watanabe, R., Arai, S., Hohdatsu, T., 2011. Screening and identification of T helper 1 and linear immunodominant antibody-binding epitopes in the spike 2 domain and the nucleocapsid protein of feline infectious peritonitis virus. Vaccine 29, 1791-1800.

Takano, T., Morioka, H., Gomi, K., Tomizawa, K., Doki, T., Hohdatsu, T., 2014. Screening and identification of T helper 1 and linear immunodominant antibody-binding epitopes in spike 1 domain and membrane protein of feline infectious peritonitis virus. Vaccine 32, 1834-1840.

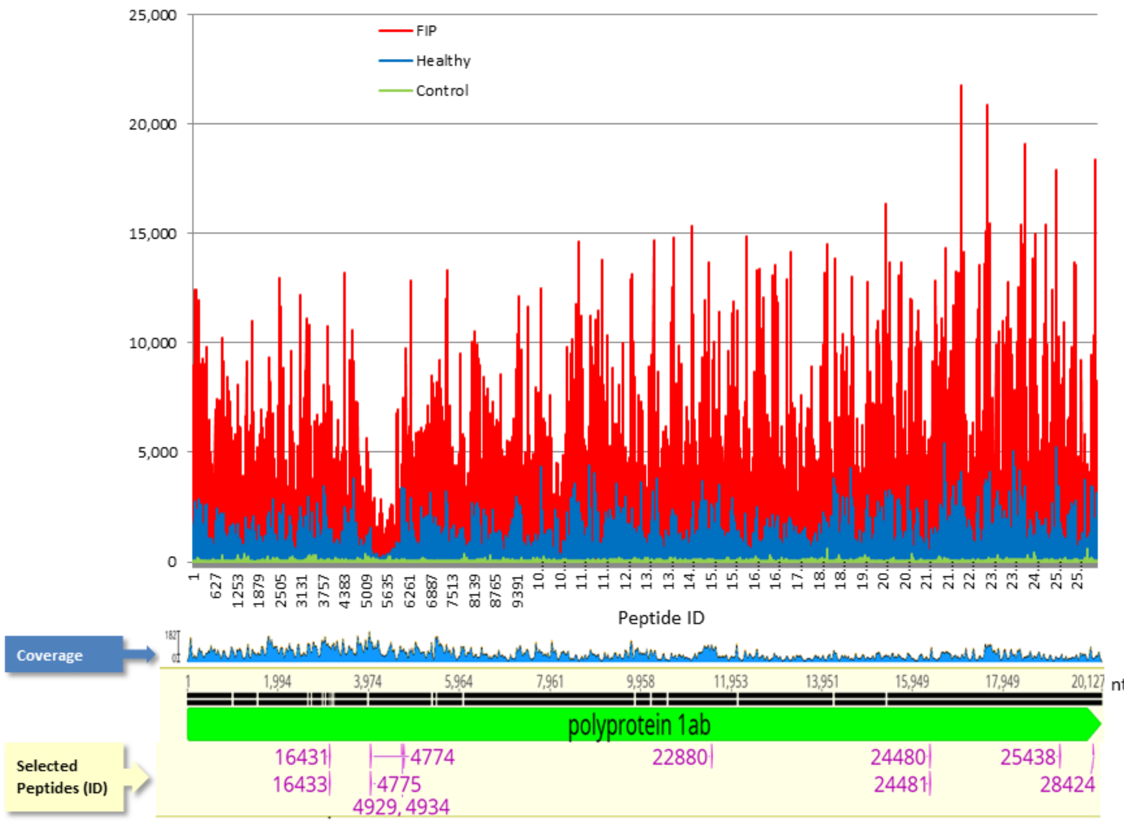
Tasker, S., 2018. Diagnosis of feline infectious peritonitis: Update on evidence supporting available tests. J Feline Med Surg 20, 228-243.

Yu, H., Jiang, L.F., Fang, D.Y., Yan, H.J., Zhou, J.J., Zhou, J.M., Liang, Y., Gao, Y., Zhao, W., Long, B.G., 2007. Selection of SARS-coronavirus-specific B cell epitopes by phage peptide library screening and evaluation of the immunological effect of epitope-based peptides on mice. Virology 359, 264-274.


*
Age (years)
20
15
10
5
0
n=27
n=51
n=11
FIP
Non-FIP
Non-FIP
FCoV Ab pos
FCoV Ab neg
Cats



FCoV Ab+ve FIP
FCoV Ab+ve Healthy
FCoV Ab-ve Control
number of peptides
30000
20000
10000
501
500
0
0-50
51-100
101-500
501-1000
1001-5000
5001-10000
10001-15000
15001-20000
>20000
Level of fluorescence (arbitrary units)


25,000
20,000
15,000
10,000
5,000
0
FIP
Healthy
Control
Peptide ID
Coverage
polyprotein 1ab
nt
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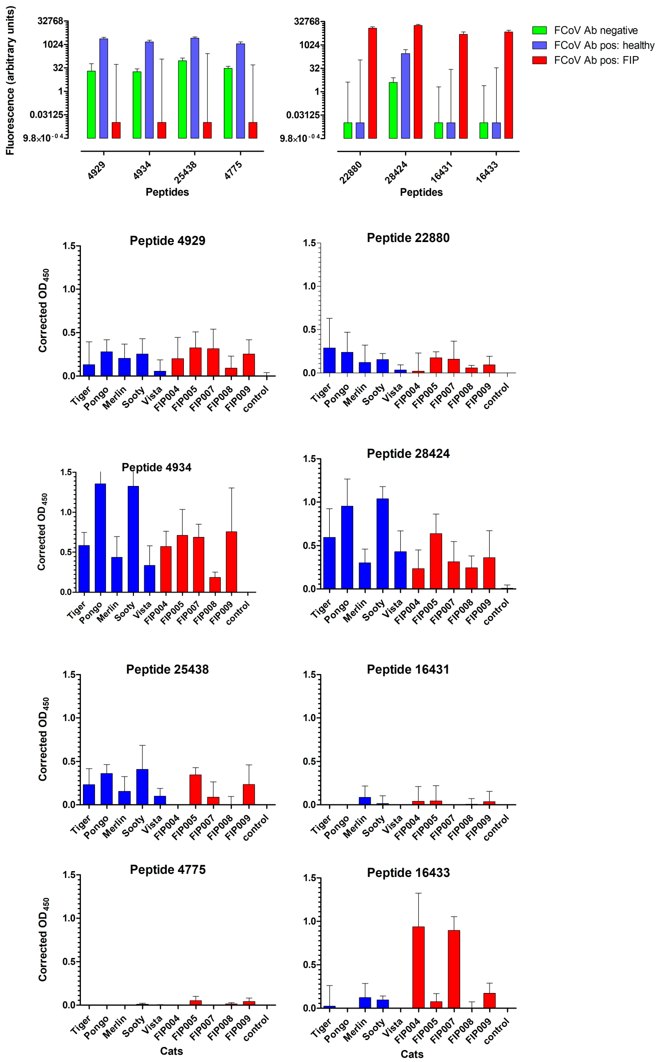
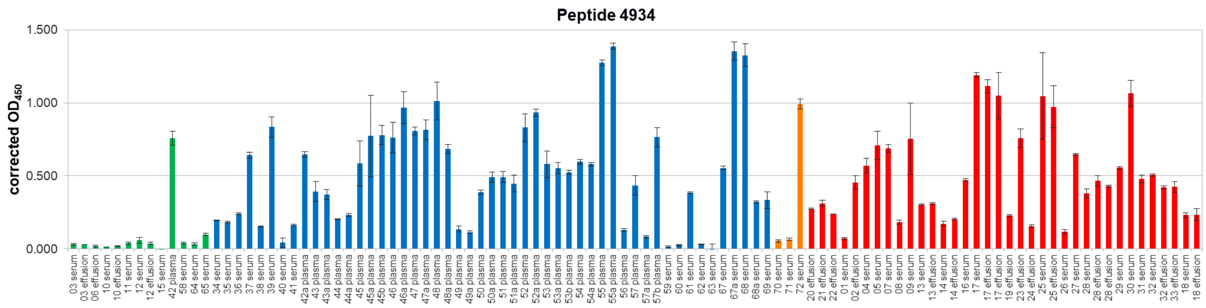
24480
24481
25438
28424



Fluorescence (arbitrary units)
32768
1024
32
1
0.03125
9.8x10-04
4929
4934
25438
4775
22880
28424
16431
16433
Peptides
FCoV Ab negative
FCoV Ab pos: healthy
FCoV Ab pos: FIP
Peptide 4929
Peptide 22880
Peptide 4934
Peptide 28424
Peptide 25438
Peptide 16431
Peptide 4775
Peptide 16433
Corrected OD450
1.5
1.0
0.5
0.0
Tiger
Pongo
Merlin
Sooty
Vista
FIP004
FIP005
FIP007
FIP008
FIP009
control
Cats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corrected OD_{450}
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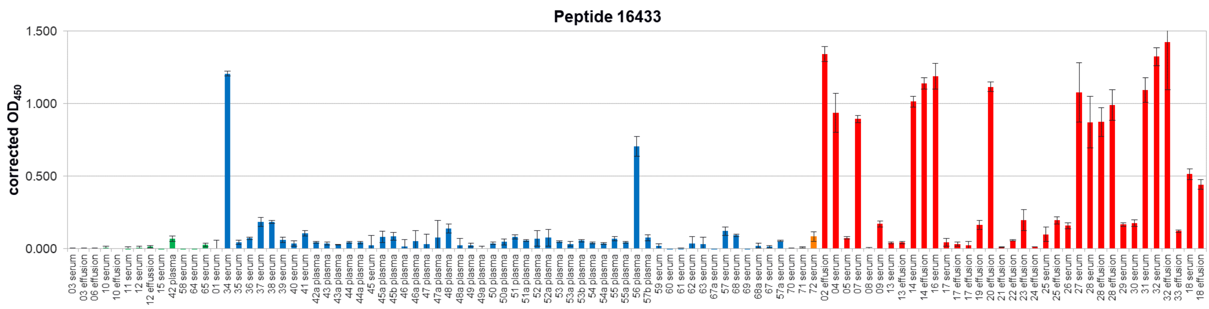
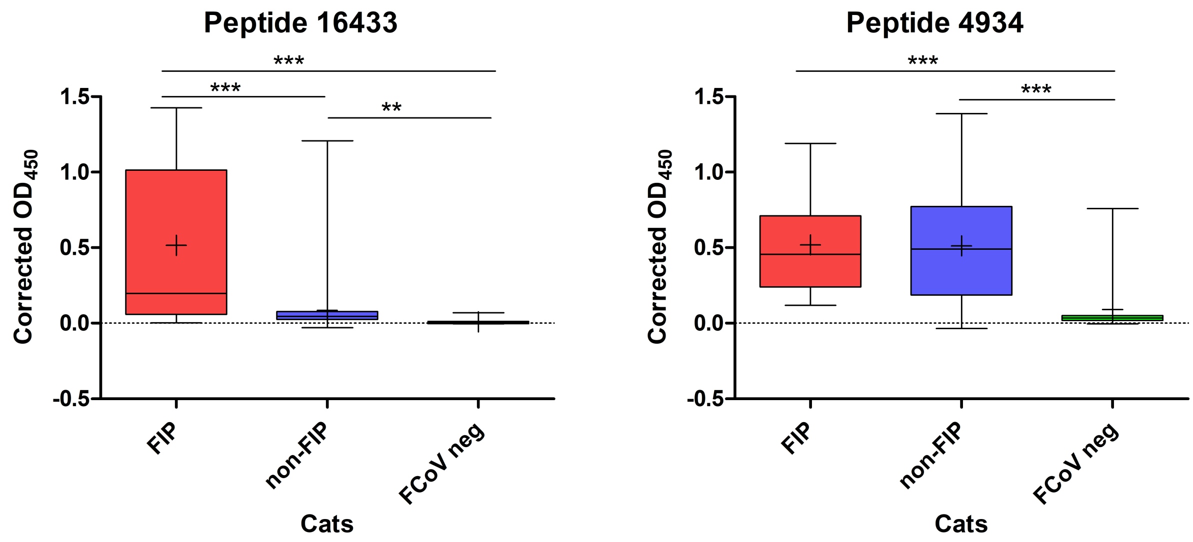
1.000
0.500
0.000


Peptide 16433
corrected OD_{450}
1.500
1.000
0.500
0.000


Peptide 16433
***
***
**
1.5
1.0
0.5
0.0
-0.5
Corrected OD_{450}
FIP
non-FIP
FCoV neg
Cats
Peptide 4934
***
***
1.5
1.0
0.5
0.0
-0.5
Corrected OD_{450}
FIP
non-FIP
FCoV neg
Cats

Consensus (060 … 1,070 … 1,080 … 1,090): PDEQLSSVEEKVEVSAKNDPWAAAVDEQEAEQI

Peptide	Sequence	FIP cats Mean	FIP cats SD	FIP cats p	Non-FIP cats Mean	Non-FIP cats SD	Non-FIP cats p
6566	DEKVEVSAKNDP	747	483	4.10E 02	81	245	7.00E 01
7553	EEEVDVSAKNDP	578	633	3.90E 01	26	70	5.30E 01
8532	EEKVEISAKNDP	989	380	1.20E 03	184	126	1.20E 01
9699	EEKVDVSAKNDP	199	267	5.50E 01	50	69	6.00E 01
12339	EEKVEVTAKNDP	1,220	413	4.10E 04	112	119	2.90E 01
14989	EENAEVSAKNDP	198	182	3.80E 01	160	161	3.60E 01
16428	EEKVETSAKNDP	3,840	1,682	2.00E 02	0	62	1.00E+00
18084	MTKAEVSAKNDP	263	369	3.50E 01	134	62	1.10E 01
20074	EKKDEVSAKNDP	544	433	1.50E 01	118	45	6.20E 02
1606	EKVEASAKNDPW	991	469	1.40E 01	23	34	9.60E 01
7554	EEVDVSAKNDPW	3,163	1,277	1.40E 02	1	89	8.30E 01
8533	EKVEISAKNDPW	3,500	918	4.60E 05	537	349	1.10E 01
9700	EKVDVSAKNDPW	1,497	463	1.60E 03	884	361	1.60E 02
11058	EKVEVSAKNDPW	1,182	471	6.10E 02	244	119	6.10E 02
12340	EKVEVTAKNDPW	2,246	984	1.20E 02	739	295	1.10E 02
14990	ENAEVSAKNDPW	1,248	509	1.70E 02	211	86	2.20E 02
16429	EKVETSAKNDPW	6,782	1,888	2.80E 04	176	101	1.60E 01
17453	EIVEVSAKNDPW	2,735	638	2.30E 05	288	214	1.50E 01
18085	TKAEVSAKNDPW	1,423	736	4.10E 02	214	166	2.80E 01
20075	KKDEVSAKNDPW	1,871	632	2.00E 03	477	194	2.20E 02
1607	KVEASAKNDPWA	438	187	4.50E 01	0	18	1.00E+00
7555	EVDVSAKNDPWA	2,682	1,129	1.90E 02	11	90	7.50E 01
8534	KVEISAKNDPWA	2,050	617	2.10E 04	557	278	4.00E 02
9306	NAEVSAKNDPWX	362	584	5.80E 01	71	65	3.70E 01
9701	KVDVSAKNDPWA	709	520	2.00E 01	398	161	1.70E 02
11059	KVEVSAKNDPWA	483	318	5.60E 01	65	48	3.60E 01
12341	KVEVTAKNDPWA	1,096	531	1.20E 02	324	168	4.50E 02
14991	NAEVSAKNDPWA	1,011	484	4.40E 02	223	179	2.40E 01
16430	KVETSAKNDPWA	4,449	1,114	4.80E 05	0	51	1.00E+00
17454	IVEVSAKNDPWA	1,005	397	1.40E 02	0	40	1.00E+00
18086	KAEVSAKNDPWA	538	518	2.30E 01	54	81	8.10E 01
20076	KDEVSAKNDPWA	1,551	426	1.30E 04	223	163	2.50E 01
27316	KVEGSAKDDPWA	1,647	891	7.00E 02	469	274	7.50E 02
1608	VEASAKNDPWAA	336	328	9.10E 01	0	353	1.00E+00
8535	VEISAKNDPWAA	1,845	605	5.80E 04	325	171	4.70E 02
9307	AEVSAKNDPWXA	0	261	1.00E+00	85	70	3.00E 01
9702	VDVSAKNDPWAA	681	282	2.30E 02	296	170	9.60E 02
11060	VEVSAKNDPWAA	306	284	9.80E 01	98	75	3.00E 01
12342	VEVTAKNDPWAA	949	899	1.90E 01	213	118	5.50E 02
14992	AEVSAKNDPWAA	477	288	1.30E 01	182	147	2.50E 01
16431	VETSAKNDPWAA	5,076	2,005	1.00E 02	0	28	1.00E+00
18087	AEVSAKNDPWAV	508	460	2.00E 01	14	74	1.00E+00
20077	DEVSAKNDPWAA	1,125	515	1.90E 02	98	61	2.90E 01
27317	VEGSAKDDPWAA	1,062	1,255	4.10E 01	183	99	4.20E 02
1609	EASAKNDPWAAA	0	153	1.00E+00	0	18	1.00E+00
8536	EISAKNDPWAAA	0	175	1.00E+00	287	192	1.20E 01
9308	EVSAKNDPWXAA	98	206	7.70E 01	168	91	8.80E 02
9703	DVSAKNDPWAAA	314	291	3.40E 01	322	294	2.90E 01
12343	EVTAKNDPWAAA	243	228	3.40E 02	176	124	1.20E 01
16432	ETSAKNDPWAAA	4,767	1,131	1.80E 05	0	15	1.00E+00
18088	EVSAKNDPWAVA	438	495	2.90E 01	84	63	4.30E 01
20078	EVSAKNDPWAAA	621	438	1.10E 01	0	34	1.00E+00
27318	EGSAKDDPWAAA	1,579	774	4.60E 02	325	288	2.30E 01
1610	ASAKNDPWAAAV	443	226	5.20E 01	86	48	1.80E 01
8537	ISAKNDPWAAAV	0	192	6.20E 01	748	490	1.20E 01
9309	VSAKNDPWXAAV	384	255	1.70E 01	206	96	4.40E 02
12344	VTAKNDPWAAAV	1,489	439	8.40E 05	282	277	2.90E 01
16433	TSAKNDPWAAAV	6,952	2,256	1.80E 03	0	35	1.00E+00
18089	VSAKNDPWAVAV	0	291	1.00E+00	0	40	1.00E+00
20079	VSAKNDPWAAAV	811	499	7.40E 02	99	86	4.50E 01
27319	GSAKDDPWAAAV	3,281	1,286	1.20E 02	0	8	1.00E+00
9310	SAKNDPWXAAVD	833	370	3.10E 02	411	167	1.70E 02
12345	TAKNDPWAAAVD	2,816	858	3.70E 04	217	125	6.50E 02
18090	SAKNDPWAVAVD	573	621	2.90E 01	114	204	6.90E 01
20080	SAKNDPWAAAVD	3,286	1,140	3.10E 03	261	104	2.90E 02