Serological recognition of feline infectious peritonitis virus spike gene regions expressed as synthetic peptides and *E. coli* fusion protein

B. T. Suiter[1,3], N. E. Pfeiffer[1], E. V. Jones[2], A. P. Reed[2],
S. R. Klepfer[2], T. J. Miller[2], and S. Srikumaran[3]

[1]Biological Research and Development, SmithKline Beecham Animal Health,
Lincoln, Nebraska, [2]Molecular Genetics, SmithKline Beecham Animal Health,
King of Prussia, Pennsylvania, [3]Department of Veterinary Science,
University of Nebraska, Lincoln, Nebraska, U.S.A.

Summary. Cats exposed to feline infectious peritonitis virus (FIPV) or feline enteric coronavirus (FECV) cannot be differentiated by serological analysis. Three synthetic peptides and an *E. coli* recombinant fusion protein generated from FIPV 79-1146 spike gene sequence were produced. Coronavirus positive cat sera reacted to peptide aa 950–990 but were non-reactive to aa137–151 and aa 150–180 peptides as demonstrated by ELISA. Amino acid sequence 97–222 expressed as a galk fusion protein in *E. coli* was tested against coronavirus positive cat sera by western blot analysis. Only sera from cats exposed to the FIPV type-II strains DF-2 or 79–1146 that were exhibiting signs of FIP recognized the fusion protein. Sera from FECV exposed cats did not recognize the 97–222 fusion protein in western blot analysis.

Introduction

Feline infectious peritonitis virus (FIPV) is the causative agent of feline infectious peritonitis (FIP), a complex and fatal disease of cats. In contrast, feline enteric coronavirus (FECV) causes enteritis only. Both viruses are members of the *Coronavirus* family, a group of enveloped viruses that contain single-stranded positive sense messenger RNA.

Feline enteric coronavirus and FIPV can be distinguished based on virulence but cannot be separated antigenically or serologically. Based on cat challenge studies, the FIPV and FECV isolates have been designated in terms of increasing virulence as follows: FECV strain 1683, FIPV-UCD-2, FIPV-UCD-4, FIPV-UCD-3, FIPV-UCD-1, FIPV TN406, FIPV DF-2 and FIPV 79-1146 [26].

Several of the current in vitro assays, such as enzyme linked immuno-absobent assay (ELISA) or indirect fluorescent antibody assay (IFA), can identify coronavirus seropositive cats, but due to the high degree of cross reaction between FECV and FIPV, these tests cannot be used to distinguish one infection from the other [25]. In addition, there is very little correlation between ELISA or virus neutralization titer and clinical manifestation of disease.

Of the three FIPV structural proteins, the S glycoprotein is likely to be the most suitable target for diagnostic purposes. The S protein of coronavirus is known to bind to host-cell receptor glycoproteins and induce cell fusion [33]. The S protein induces a strong antibody response as well, and this response has been associated with FIPV disease enhancement [33]. In the case of other coronaviruses such as transmissible gastroenteritis virus (TGEV), infectious bronchitis virus (IBV), and mouse hepatitis virus (MHV), the S protein appears to be the major inducer of protective immunity.

The M and N proteins of FIPV and FECV contain group-specific epitopes whereas the S proteins contain type-specific epitopes [8]. Although the aa sequence for the S protein of FECV isolates is not available, heterogeneity among the S protein of several coronaviruses is located at a particular region. The S protein aa sequence heterology between FIPV strain 79–1146 and TGEV is approximately 61% for aa 1–274 and 7% among residues 275–1447 [11, 13]. Heterogeneity among other coronaviruses including IBV and MHV is located in the same region. It is likely that heterogeneity between FIPV and FECV is in the same area. The purpose of this study was to investigate if the predicted heterogeneity between FIPV and FECV could be exploited to generate reagents that could help differentiate between virulent and avirulent coronaviruses or to develop a test that would correlate with clinical disease.

Materials and methods

Peptide synthesis

Three peptides corresponding to FIPV WSU strain 79–1146 spike aa sequences 137–151, 150–180, and 950–990 were synthesized on a p-alkoxybenzyl alcohol resin [22]. Completed peptides were cleaved off the resin under acid conditions and purified by reverse phase HPLC. Purified peptides were conjugated via glutaraldehyde to ovalbumin (OVA) at a ratio of 50:500:1 [20]. Purified peptides were also conjugated via sulfo-MBS to human serum albumin (HSA) at a ratio of 10:40:1 [19].

Immunization of rabbits with conjugates

The three OVA conjugates were injected into three rabbits each designated 1-A, 2-A, 3-A, 1-B, 2-B, 3-B, 1-C, 2-C, and 3-C. Each rabbit received a priming dose equivalent to 125 µg of peptide in Complete Freund's Adjuvant intramuscularly (IM). Four weeks post immunization rabbits received a booster dose equivalent to 75 µg of peptide in Incomplete Freund's Adjuvant IM. Ten days after the 2nd immunization rabbits were bled. Reactivity of rabbit antisera to whole virus S protein was tested by western blot analysis and by ELISA. Rabbit antisera were shown to be peptide specific by ELISA (data not shown).

Cloning in bacterial expression vector, pOTSKF33

Cloning procedures were similar to those described by Maniatis [23]. The bacterial expression vector pOTSKF33 was developed by Dr. C. Debouck of SKF Labs, Swedeland, PA. POTSKF33 is designed to regulate the expression of cloned genes as galk fusion proteins in specific strains of E. coli. Expression of foreign genes is controlled by the lambda bacteriophage PL promoter. Transcription of galk is inhibited in the presence of the lambda c1 + repressor; when the repressor is removed, expression is induced. In POTSKF33, unique restriction sites have been introduced after the first 52 aa of the bacterial enzyme, galactokinase, coding sequence. Expression is induced in AR58 cultures upon heating to 42 °C where the repressor is thermolabile.

PCR amplification

The templates for PCR reactions were double stranded cDNA from the Ts-DF-2 variant of FIPV prepared from RNA extracted from infected cells. PCR standards specified by Cetus corporation were followed and incubated in a Perkin Elmer Cetus thermocycler for 30 cycles. The completed PCR reaction products were analyzed on a 1% agarose gel to confirm amplification of the predicted DNA fragment.

Cloning of AR58-3

The DNA from the PCR reaction mixture was digested with XmaI and StuI. The digested DNA was extracted with phenol followed by phenol/chloroform (1:1) extraction and ethanol precipitated at − 20°C. The XmaI/StuI digested DNAs were incubated in a ligation mix containing pOTSKF33 vector DNA digested with XmaI/StuI and dephosphorylated. Competent HB101 cells were transformed and insert-bearing clones identified by restriction digest of small scale DNA preparations. These DNAs from confirmed clones were used to transform the heat-inducible AR58 strain of E. coli. Sequence analysis of the gene encoding the AR58-3 recombinant spike fusion protein confirmed the predicted sequence [3].

Solubilization of FIPV recombinant spike fusion protein

The clone AR58-3 fusion protein contains 52 aa of the E. coli galk protein fused to aa 97–222 of the Ts-DF-2 FIPV spike protein. Clone AR58-3 cells were pelleted and treated with Lysozyme/EDTA (final concentration of 10 mM) at room temperature (RT) for 1 h. The mixture was sonicated for 6 min on ice and inactivated by the addition of 0.01% Thimerosal. Ten ml of AR58-3 inactivated extract was centrifuged at 27 000 × g for 30 min. The pellet was resuspended by vortexing for 10 min in 10 ml buffer A (50 mM Tris HCl pH 8.5, 5 mM EDTA, 1 mM DTT, 5% (v.v) Glycerol). This buffer also contained 0.2% (w/v) sodium deoxycholic acid and 1% (v/v) Triton X-100. The extract was centrifuged at 27 000 × g for 30 min. The pellet was resuspended in buffer A containing 1% Triton X-100 and 0.5M potassium chloride. The extract was centrifuged and the pellet was resuspended as before in 2 ml buffer A containing 8 M Urea. The extract was centrifuged again and the supernatant diluted 1:10 by the addition of 2 ml 10 mM PBS, pH 7.4. This material was applied to anti-galk affinity columns.

Purification of FIPV recombinant spike fusion protein with anti-galactokinase (Anti-galk) affinity column

Anti-galk monoclonal antibodies (MAbs) were obtained as mouse ascites fluid from SmithKline Beecham Department of Molecular Genetics. Anti-galk MAbs were semi-

purified by twice precipitating with saturated ammonium sulfate (45% and 40%), respectively. Ten milligrams of anti-galk MAbs were immobilized to each Pierce Immunopure TM Ag/Ab columns. Columns were equilibrated with 10 mM PBS, pH 7.4. Columns received 20 ml AR58-3 and were washed with 0.8 M urea and equilibrated with PBS. The AR58-3 protein was eluted with 0.1 M glycine-HCl, pH 3.0. Five 1 ml fractions were collected and each neutralized with 1 M Tris-HCl, pH 9.5. The second and third fractions contained AR58-3 proteins as shown by direct ELISA using anti-galk MABs.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in slab gels under reducing conditions. The separation gel and the stacking gel contained 12% and 3% polyacrylamide respectively. The sample buffer contained, 10% (v/v) beta-mercaptoethanol. The affinity-purified AR58-3 recombinant spike fusion protein at a concentration of approximately 70 μg/gel was mixed with sample buffer and boiled for 5 min. Electrophoresis was performed at RT for 6 h at a constant current of 20 mA [18].

Western blot analysis

The affinity purified AR58-3 recombinant spike fusion protein (70 μg) separated by SDS-PAGE was transferred to nitrocellulose sheets of 0.22 μm pore size. The transfer was carried out electrophoretically by the method of Towbin et al. [32]. The nitrocellulose sheets were blocked with buffer B (50 mM Tris, 150 mM sodium chloride, pH 7.4, and 5% (w/v) nonfat dried milk and incubated overnight at RT. Nitrocellulose strips were cut and placed into individual chambers and incubated 1 h at RT with cat serum diluted 1:30 in buffer B. Each strip contained approximately 2.3 μg of fusion protein (aa 97–223). The strips were washed once with buffer C [buffer B with 0.2% (v/v) Triton X-100] and then washed twice with buffer B. After washing was complete strips were incubated at RT for 1 hour with horseradish peroxidase-conjugated goat antibody against cat or mouse IgG (Kirkegaard & Perry Laboratories Gaithersburg, MD) diluted 1:1000 in buffer B. The strips were washed and incubated 15 min with BCIP/NBT phosphatase substrate system Kirkegaard & Perry.

Serology

The presence of antibodies to coronavirus in cat sera was determined by a direct antigen binding ELISA and virus neutralization against FIPV DF-2 [11].

Vaccination and challenge of cats

Specific pathogen-free (SPF) cats, 4 to 10 months of age, and free of serum anti-coronavirus antibodies were obtained from Liberty Labs, Liberty, NJ, USA. Some of these cats served as non-vaccinated controls, while others were vaccinated twice intranasally on days 0 and 21 with Primucell-FIP, a temperature sensitive modified five vaccine, (SmithKline Beecham Animal Health, West Chester, PA). Virulent FIPV (strains DF-2, 79–1146 or TN406) were titrated to $10^{4.5}$–$10^{4.9}$ TCID$_{50}$ per ml and one ml was given per cat orally 14 days post second vaccination [11]. In addition, cats with anti-coronavirus ELISA titers, but with negative DF-2 FIPV neutralization titers were received from Mason City and SmithKline Beecham Animal Health. Both sources of cats were part of colonies void of FIPV clinical symptoms. Naive SPF cats received three exposures of FECV strain 1683 at three week intervals.

Clinical evaluation of cats

Cats challenged with FIPV were grouped based on clinical evaluation using a clinical scoring protocol described previously [11]. Symptoms such as febrile response, lymphopenia, leukopenia, icterus and decreased packed cell volume were all evaluated. The total clinical score was the sum of each clinical score for the duration of the study [10]. Cats with a total clinical score of less than 10 were designated as non-symptomatic (NS). Cats with a total clinical score of 10 or more were designated as symptomatic (S). All cats were routinely bled at pre-vaccination, post first vaccination, post second vaccination, and two, four, six and eight weeks post-challenge.

Results

Synthetic peptides

The aa sequence of the FIPV 1146 spike protein [3] was surveyed for potential antigenic sites using a number of criteria. The hydrophilic profile [12], surface probability [9], chain flexibility [15], and antigenic index factors [14] along with secondary structure predictions [2, 7] were analyzed using the University of Wisconsin Genetics Group programs [6]. A graphic representation of these factors appears in Fig. 1. Peptides homologous to high antigenic index residues 137–151, 150–180, and 950–990 of the published FIPV 79–1146 spike protein were synthesized. The two N terminal regions, residues 137 to 151 and 150 to 180, were selected based upon the heterogeneity between FIPV and TGEV in this region [11, 13]. The peptide 950–990 was chosen as a region with homologous sequence. These synthetic spike peptides were used as solid phase antigens in an ELISA in an attempt to differentiate FIPV and FECV antibodies in exposed cat sera, and 2) to generate polyclonal rabbit anti-peptide sera.

Reactivity of cat sera against S protein peptides

Cat sera were tested for their ability to recognize the three synthetic peptide conjugates. All naive (SPF) cat sera were negative for antibodies specific to the three HSA-conjugated peptides aa 137–151, aa150–180, and aa950–990 by ELISA. Sera from cats exposed to either FIPV 79–1146 or FECV 1683 were found to react only to conjugate aa950–990 (Table 1). The aa sequence of peptides in conjugates aa137–151 and aa150–180, which were not recognized by antibodies from these cats, are located within the spike N-terminal region (residues 1-274) that demonstrates heterogeneity between FIPV 79–1146 and TGEV [13]. Since the synthetic peptides may be too short for immune recognition, a longer peptide containing these two regions was produced by recombinant DNA technology. The recombinant *E. coli* fusion protein designated AR58-3 that contained a larger spike protein sequence was used to further investigate serological cross-reactivity in this region. AR58-3 contained 52 residues from galk and 126 residues representing aa 97–222 of the Ts DF-2 FIPV spike protein, which has exact homology to FIPV 1146 in this region. The affinity-purified fusion protein preparation was approximately seventy

FIPV WSU - 1146 SPIKE PROTEIN
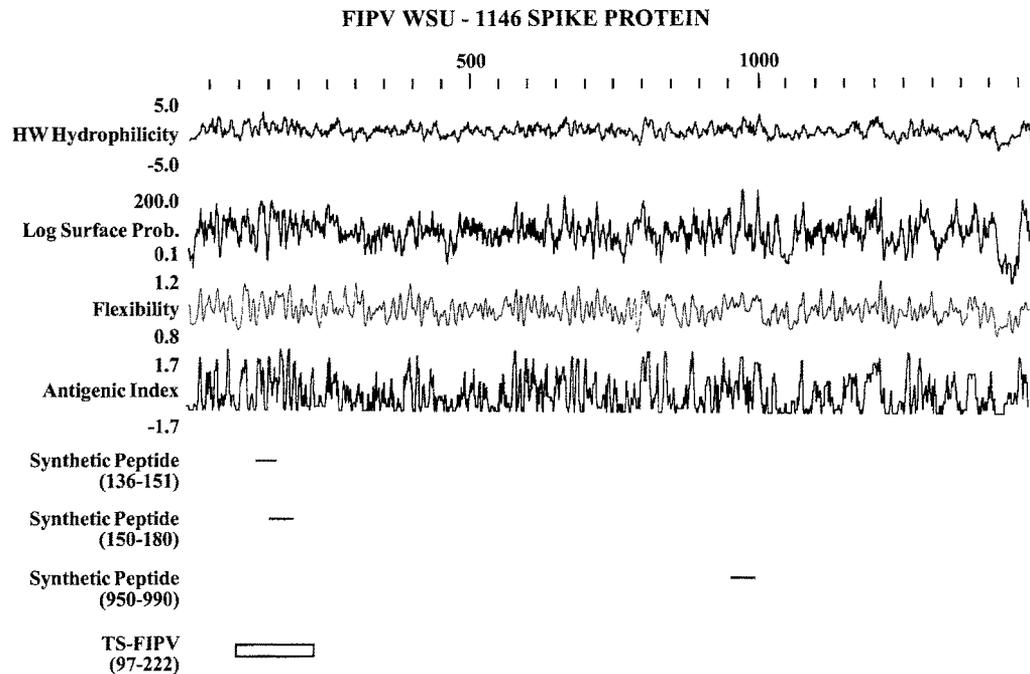
Fig. 1. A graphic representation of hydrophilicity, surface probability, flexibility or antigenic index as generated by Wisconsin Genetics Group programs are listed [6]. The location of the three synthetic peptides aa 136–151, aa 150–180, aa 950–990, and the spike fusion protein aa 97–222 are denoted respective to the spike protein

Table 1. Peptide specificity of antisera from cats exposed to FIPV strain 79-1146 or FECV by ELISA[a]

Exposure virus	Cat #	Conjugate #1	Conjugate #2	Conjugate #3
FIPV 79–1146	EU6	0.06	0.03	0.60
	FF2	0.06	0.04	2.00
	FH4	0.15	0.07	1.40
	EL5	0.07	0.07	2.00
	FN1	0.09	0.04	1.70
FECV	HC3	0.05	0.07	0.47
	21A	0.15	0.12	0.59
	21B	0.11	0.09	0.43
	21C	0.11	0.07	0.46
	22D	0.05	0.04	0.41

HSA Human serum albumin
Conjugate #1 = (aa137–151-HSA)
Conjugate #2 = (aa150–180-HSA)
Conjugate #3 = (aa950–990-HSA)
[a]ELISA O.D. (405 nm) with HSA-conjugates as binding antigens
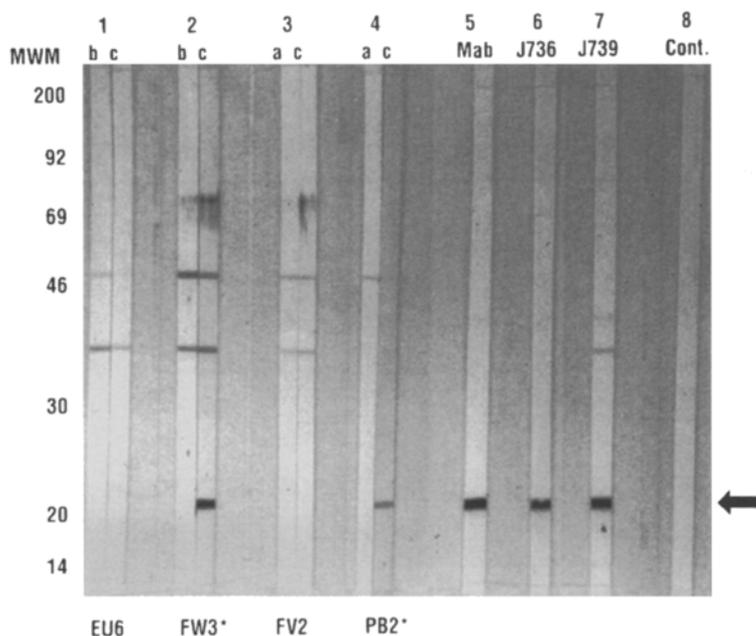
Fig. 2. Western blot analysis of purified galk fusion protein containing FIPV aa 97–222 sequence. *1 b* 3 weeks post 2nd Ts-FIPV vaccinated serum and *c* four weeks post FIPV strain 79–1146 challenged serum, both from the surviving cat #EU6; *2 b* 3 weeks post 2nd Ts-FIPV vaccinated serum and *c* four weeks post FIPV strain 79–1146 challenged serum, both from non-surviving cat #FW3; *3 a* non-vaccinated pre-challenge serum, *c* four weeks post FIPV strain 79–1146 challenge serum, both from surviving cat #FV2; *4 a* non-vaccinated pre-challenge serum, *c* four weeks post FIPV strain 79–1146 challenge serum, both from non-surviving cat #PB2; *5* (mab) anti-galactokinase monoclonal antibody; *6* (J736) serum from rabbit (1-A) which was immunized with peptide conjugate ovalbumin-glut-137aa-151aa; *7* (J739) serum from rabbit (4-B) which was immunized with peptide conjugate ovalbumin-glut-150aa-180aa; *8* (*cont.*) received only 2nd conjugated goat anti-cat-IgG-phosphatase antibody. *MWM* High molecular weight markers. Arrow demonstrates position of the recombinant galk fusion protein containing FIPV aa 97–222 sequence (21KD)

percent pure, and it was used in western blot analysis without further purification.

In western blot analysis, the 21 kD AR58-3 fusion protein was clearly identified by the reactivity of its galk portion with the anti-galk MAbs and by its FIPV spike protein portion with the rabbit antisera to the peptides aa 137–151 and aa 150–180 (Fig. 2).

Correlation between disease state and western blot reactivity

Initial western blot analysis of sera from eight cats demonstrated that only post-challenge sera from cats which did not survive FIP-V challenge contained antibodies that recognized the AR58-3 fusion protein. In contrast, antibodies from post-challenge sera of cats which survived FIPV challenge were non-
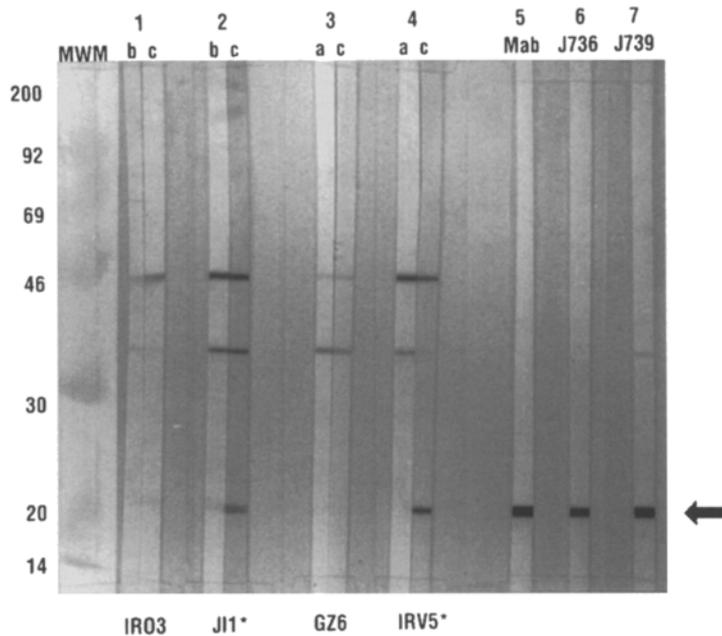
Fig. 3. Western blot analysis of purified galk fusion protein containing FIPV aa 97–222 sequence. *1 b* 3 weeks post 2nd Ts-FIPV vaccinated serum and *c* four weeks post FIPV strain DF-2 challenge serum, both from the surviving cat #IR03; *2 b* 3 weeks post 2nd Ts-FIPV vaccinated serum and *c* four weeks post FIPV strain DF-2 challenge serum, both from non-surviving cat #JI1; *3 a* non-vaccinated pre-challenge serum, *c* four weeks post FIPV strain DF-2 challenge serum, both from surviving cat #GZ6; *4 a* non-vaccinated pre-challenge serum, *c* four weeks post FIPV strain DF-2 challenge serum, both from non-surviving cat #IRV5; *5 Mab* anti-galactokinase monoclonal antibody; *6 J736* serum from rabbit (1-A) which was immunized with peptide conjugate ovalbumin-glut-137aa-151aa; *7 J739* serum from rabbit (4-B) which was immunized with peptide conjugate ovalbumin-glut-150aa-180aa. *MWM* Molecular weight markers. Arrow demonstrates position of the recombinant galk fusion protein containing FIPV aa 97–222 sequence (21KD)

reactive to the AR58-3 fusion protein (Figs. 2, 3). The reactivity of sera from non-surviving cats was independent of challenge strain. Of the four non-surviving cats two were challenged with FIPV 79–1146 (Fig. 2) and the other two with strain DF-2 (Fig. 3).

Table 2. Serological response against FIPV antigens in cats exposed to FIPV

Challenge virus	Disease state	# of cats	Western blot analysis		ELISA[a]
			positive	negative	range
FIPV type-II	symptomatic	50	82%	18%	100–204800
	non-symptomatic	22	14%	86%	100–102400

[a] Solid phase antigen used in the ELISA was partially purified FIPV DF-2. Antigen used in western blot analysis was affinity purified AR 58-3 fusion protein at 2.3 μg per lane
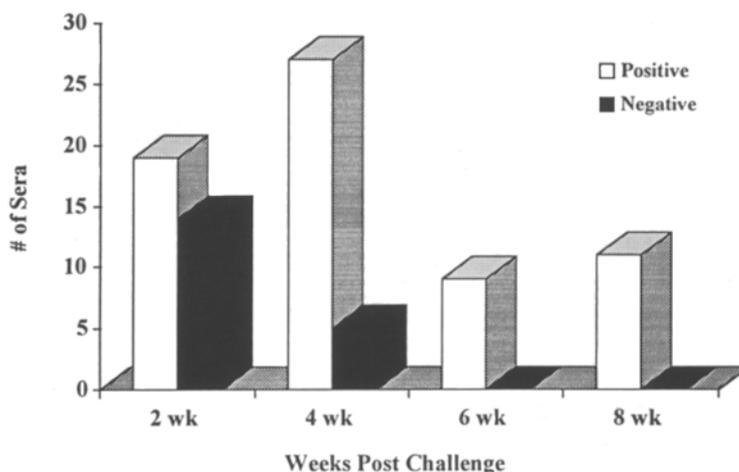
Fig. 4. Serological response against AR58-3 fusion protein in symptomatic cats exposed to virulent Type-II FIPV by western blot analysis

Further analysis of antibody reactivity was performed taking into consideration disease severity. Sera from additional cats challenged with either DF-2 FIPV or WSU 79-1146 FIPV were further grouped as symptomatic and non-symtomatic based on the clinical scoring system described earlier. Sera from these two groups of cats were tested for the ability to recognize the AR58-3 spike fusion protein by western blot analysis. As shown in Table 2, this antibody response correlated with the disease state of the cat. Eighty-two percent of the symptomatic cats contained antibodies that reacted to AR58-3. In contrast, eighty-six percent of the non-symptomatic cats were negative to this spike fusion protein.

All symptomatic cats which were negative to AR58-3 died between two and four weeks post challenge, therefore only two and four week post challenge sera were available for testing. Figure 4 shows the results of western blot analysis of all sera from symptomatic cats tested against the spike fusion protein. This figure shows that the majority of the negative reactivity in symptomatic cats occurred in two weeks post challenge sera, whereas all six and eight weeks post

Table 3. Serological response against FIPV antigens in non-symptomatic cats exposed to FECV

| Source of coronavirus | # of cats | Western blot analysis | | ELISA[a] |
		positive	negative	range
FECV 1683	3	0%	100%	6400–12 800
Mason city	8	0%	100%	3200–12 800
Norden enteric	5	0%	100%	12 800–51 200

[a] Solid phase antigen used in the ELISA was partially purified FIPV DF-2. Antigen used in western blot analysis was affinity purified AR58-3 fusion protein at 2.3 μg per lane
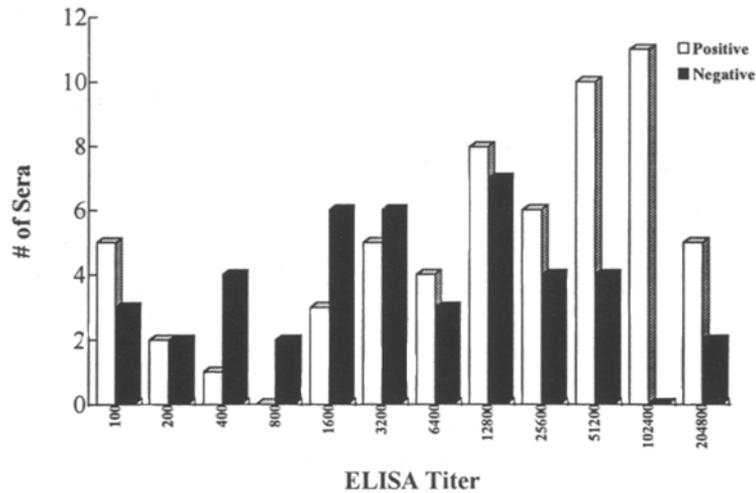
Fig. 5. Cat serum antibody ELISA titer vs reactivity to AR58-3 by western blot analysis
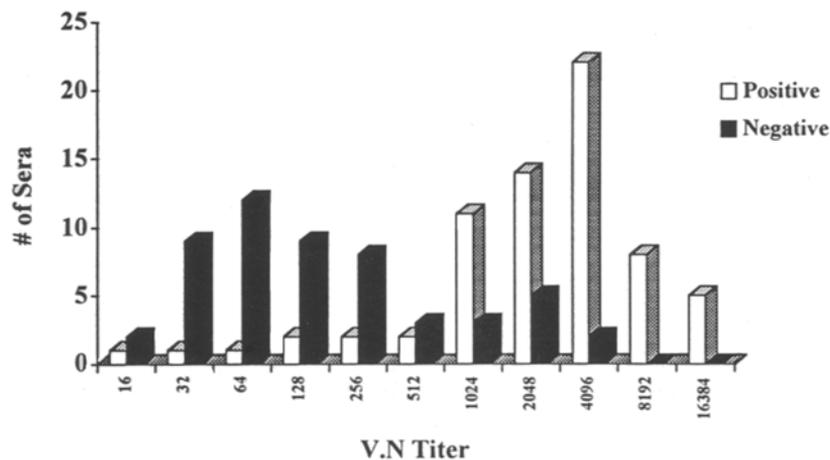
Fig. 6. Cat serum antibody virus neutralization vs reactivity to AR58-3 by western blot analysis

challenge serum antibodies from symptomatic cats were positive to AR58-3. Therefore, it is clear that an even higher percent of symptomatic cats could be identified if sera collected at several time points were tested.

All sera tested by western blot from cats challenged with FIPV demonstrated anti-coronavirus antibodies when analyzed by ELISA and virus neutralization analysis as shown in Fig. 5 and Fig. 6, respectively. This demonstrates that all cats responded serologically to FIPV post challenge. The virus neutralization titers showed better correlation than ELISA titer to AR58-3 reactivity by western blot analysis. Serum antibodies of cats exposed up to three times with FECV strain 1683 did not react to the AR58-3 spike fusion protein by western blot analysis. However, serum from all sixteen cats did show anti-coronavirus antibody titers by ELISA, see Table 3.

Table 4. Serological response against FIPV antigens in cats exposed to FIPV

Challenge virus	Disease state	# of cats	Western blot analysis		ELISA[a]
			positive	negative	range
FIPV type-I	symptomatic	8	0%	100%	3200–102 400
	non-symptomatic	8	0%	100%	3200–102 400

[a] Solid phase antigen used in the ELISA was partially purified FIPV DF-2. Antigen used in western blot analysis was affinity purified AR 58-3 fusion protein at 2.3 µg per lane

Sera from either symptomatic or non-symptomatic cats challenged with the FIPV strain TN406 did not recognize AR58-3. Serum from all sixteen cats tested showed high anti-coronavirus antibody titers by ELISA, Table 4. Thus the AR58-3 fusion protein was not useful in identifying cats exposed to FIPV strain TN406.

Discussion

Feline infectious peritonitis virus and FECV are natural pathogens of cats, causing strikingly different diseases, yet sharing extensive cross-reactivity. At the present time it is not possible to differentiate one infection from the other by serological means. However, the S gene of these viruses is a likely target for variablity. The S gene from FIPV 79-1146 has been sequenced and shown that when compared to the related coronavirus TGEV, the heterogeneity is confined to the N-terminal 274 aa [9, 10]. Feline enteric coronavirus and FIPV may also differ in their N-termini. At the time of this study, FECV sequence was not available. Synthetic peptides and *E. coli* recombinants were used to define regions of possible heterogeneity within the FIPV Ts DF2 spike protein. The N-terminal aa 97–222 fragment of Ts DF-2 FIPV expressed by *E. coli* recombinant clone, AR58-3, may contain epitopes unique to FIPV strains 79–1146 and DF-2 but not present in FECV.

Three peptide regions from the S protein of FIPV were selected based on antigenic profiles and their location within the spike protein. The two N-terminal peptides were designed to look for regions of heterogenicity, while the C-terminal peptide was expected to be recognized by antibodies from cats infected by any coronavirus. These peptides had excellent antigenic profiles based on computer analysis of the primary aa sequence which predicted these regions to be surface-exposed. This approach was used by Talbot et al. [31] to produce a synthetic peptide homologous to residues 993–1002 of murine hepatitis virus (MHV) strain JHM. This MHV spike peptide was conjugated to keyhole limpet hemocyanin and found to elicit high levels of neutralizing antibodies which protected mice against lethal MHV challenge [31]. Three groups of rabbits were immunized with the OVA conjugate of either aa 137–151, aa 150–180, or aa 950–990 homologous to the S protein of FIPV strain 79–1146. Sera from these rabbits contained antibodies that did not react to viral antigen

of FIPV strains DF-2 or 79–1146 by ELISA or western blot analysis (data not shown).

Although the anti-peptide antibodies raised in rabbits did not recognize the native FIPV spike protein, at least one of the peptides was recognized by antisera from cats that had been exposed to FIPV. The synthetic peptide conjugates which contained either N-terminal or C-terminal FIPV strain 79-1146 spike protein aa were also screened against sera from cats exposed to FIPV or FECV by ELISA. Only the C-terminal synthetic peptide was recognized by the cat sera from either FIPV or FECV exposed cats. Therefore, antibodies to this peptide could not differentiate between FIPV and FECV. The C-terminal peptide contained residues 950–990 which is larger than the N-terminal peptides of 137–151 and 150–180. This increased size may have allowed the C-terminal peptide to fold and therefore mimic conformational epitopes found in native FIPV or FECV spike proteins [4]. However, other investigators have identified linear neutralizing epitopes in MHV strain A59 aa 848-aa 856 and MHV strain JHM aa 993-aa 1002 that are at similar locations as this residue sequence (aa 950-aa 990, [16, 29]).

The main objective of this study was to differentiate between cats exposed to FIPV or FECV. However, as described previously, the two synthetic peptides based on N-terminal spike protein sequence were not recognized by the cat sera antibodies which were evaluated. The two N-terminal peptides may represent non-immunodominant regions or portions of a discontinuous epitope and were therefore not targets of natural FIPV or FECV induced humoral response [25]. Therefore, a larger spike protein fragment was produced that included sequences of the two smaller peptides. The recombinant E. coli clone AR58-3 expressed a galk/Ts-DF-2 S fusion protein which contains 56 aa of the host galactokinase protein and 126 aa of (Ts DF-2 FIPV) spike protein (homologous to residues 97–222 of Ts DF2 FIPV). The fusion protein AR58-3, expressed in E. coli forms aggregated structures in inclusion bodies. Strong denaturing conditions were required for inclusion body solubilization prior to affinity purification. In addition, AR58-3 was reduced before western blot analysis. Therefore, recombinant E. coli antigens are most suitable for identifying the same category of epitopes that are detected by the use of synthetic peptides, namely linear or sequential epitopes.

Western blot analysis of coronavirus positive cat sera showed that only sera from cats challenged with FIPV 79-1146 and DF-2 reacted with AR58-3 fusion protein. Furthermore, seventy-two cats that had been challenged with either FIPV 79-1146 or DF-2 could be separated into two groups based upon manifestation of FIPV clinical symptoms. Of the fifty symptomatic cats, eighty-two percent were positive and eighteen percent were negative to the recombinant AR58-3 fusion protein. Of the twenty-two non-symptomatic cats, eighty-six percent were non-reactive and fourteen percent recognized this aa 97–222 sequence. The symptomatic cats may have had difficulty in clearing the virus compared to non-symptomatic cats. Therefore, extensive viral replication may have occurred in symptomatic cats, giving their immune system

the opportunity to produce antibodies to epitopes not seen by the non-symptomatic animals. Thus, differentiation between diseased and healthy cats may be possible using AR 58-3 fusion protein by western blot analysis.

In order to demonstrate that reactivity to AR58-3 fusion protein was not just a function of antibody concentration, all post challenge sera were titrated by ELISA and virus neutralization analysis using FIPV DF-2. Although cat sera with very high ELISA titers usually showed positive reactivity to AR58-3 by western blot analysis, there were exceptions as shown in Fig. 5. Therefore, very little correlation could be established between reactivity against the aa 97–222 peptide and ELISA titer. However, a correlation was established between the reactive of cat serum antibodies to the fusion protein by western blot analysis and the virus neutralization titer (Fig. 6).

The protein concentration of AR58-3 fusion protein was constant in all western blot analyses and all cat sera were diluted thirty fold. However, the total concentration of anti-spike protein specific antibodies probably varied among some of the cat sera tested. The previously described ELISA results indicate that reactivity by western blot is not a function of high antibody titer alone and supports a theory that antibody response to AR58-3 fusion protein is related to the disease state of each cat.

Cats exposed to FECV did not recognize the recombinant AR58-3 fusion protein. This observation was encouraging because it suggested that it might be possible to differentiate between cats exposed to the virulent FIPV strains DF-2 and 79-1146 and the avirulent FECV. The DF-2 and FECV S gene have been recently sequenced [28]. When compared to the published 79-1146 S gene, some interesting results were shown in terms of aa homology. The DF-2 and 79-1146 show greater than 99% aa homology. Although the DF-2 and 79-1146 share greater than 93% aa homology with FECV, of their 50 and 52 aa differences respectively, all are within the N-terminal half of the protein. In addition, a very high aa divergence was observed between aa 133–159 (8 of 18 aa differ between FIPV and FECV) [28]. The ability of the fusion protein AR58-3 to differentiate between FIPV and FECV exposed cats may in part be due to the divergence observed in the region aa 97–222.

However, sera from cats exposed to the virulent FIPV strain TN 406 did not detect the DF-2 aa 97–222 AR58-3 fusion protein. The TN 406 strain of FIPV is classified as a virulent Type-I virus, whereas DF-2 and 79–1146 are virulent Type-II viruses, and FECV 1683 is an avirulent Type-II virus [26]. Based on the lack of reactivity to AR58-3 and inability of sera from cats exposed to TN 406 to cross neutralize, it would appear that the Type I FIPV strains may contain regions of considerable heterogeneity. Therefore, a diagnostic assay using the recombinant AR58-3 fusion protein as antigen substrate to differentiate cats exposed to virulent strains of FIPV from those exposed to avirulent strains of FIPV or FECV would not be possible. Spike protein gene sequence data is needed on the Type-I FIPV's, namely strains TN 406, UCD-1, UCD-3, and UCD-4. This information would help to locate possible conserved regions unique for all virulent strains of FIPV.

In conclusion, the recombinant *E. coli* fusion protein AR58-3 is recognized by antibodies from cats with clinical manifestations of FIP, if exposure is from virulent Type-II strains of FIPV. In contrast, antibodies from cats exposed to FECV 1683 an avirulent Type-II virus did not react to AR58-3 by western blot analysis. However, sera from cats infected with strain TN 406 FIPV, irrespective of whether the cats became sick or remained healthy, they did not recognize the fusion protein. Spike gene sequencing of other virulent and avirulent strains of FIPV are needed to identify regions containing type-specific epitopes. Furthermore, expression of additional recombinant spike protein fragments should be explored to target regions of biological importance.

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References

1. Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res 7: 1513
2. Chou PY, Fasman G (1978) Prediction of the secondary structure of proteins from their amino acid sequence. Annu Rev Immunol 2: 67–101
3. De Groot RJ, Maduro J, Lenstra JA, Horzinek MC, Van Der Zeijst BA, Spaan MJ (1987) cDNA cloning and sequence analysis of the gene encoding the peplomer protein of feline infectious peritonitis virus. J Gen Virol 68: 2639–2646
4. De Groot RJ, Luytjes W, Horzinek MC, Van der Zeijst BA, Spaan WJ, Lenstra JA (1987) Evidence for a coiled-coil structure in the spike proteins of coronaviruses. J Mol Biol 196: 963–966
5. Delmas B, Gelfi J, Laude H (1986) Antigenic structure of transmissible gastroenteritis girus. II. domains in the peplomer glycoprotein. J Gen Virol 67: 1405–1418
6. Devereux J, Haeberli P, Smithies O (1984) A comprehensive set of sequence analysis programs for the vax. Nucleic Acids Res 12: 387–395
7. Emini EA, Hughes JV, Perlow DS, Boger J (1983) Induction of hepatitis A virus-neutralizing antibody by a virus-specific synthetic peptide. J Virol 55: 836–83
8. Fiscus SA, Teramoto YA (1987) Antigenic comparison of feline coronavirus isolated: evidence for markedly different peplomer glycoproteins. J Virol 61: 2607–2613
9. Garnier J, Osguthorpe DJ, Robson B (1978) Analysis of the accuracy and implication of simple methods for predicting the secondary structure of globular protein. J Mol Biol 120: 97–120
10. Gerber JD, Pfeiffer NE, Ingersoll JD, Chistianson KK, Landon RM, Selzer NL, Beckenhauer WH (1990) Characterization of an attenuated temperature sensitive feline infectious peritonitis vaccine virus. In: Coronaviruses and their diseases. Plenum Press, New York, pp 481–489
11. Gerber JD, Ingersoll JD, Gast AM, Christianson KK, Selzer NL, Landon RM, Pfeiffer NE, Sharpee RL, Beckenhauer WH (1990) Protection against feline infectious peritonitis by intranasal inoculation of a temperature-sensitive FIPV vaccine. Vaccine 8: 536–542
12. Hopp TP, Woods KR (1981) Prediction of protein antigenic determinants from amino acid sequences. Proc Natl Acad Sci USA 78: 3824–3828

13. Jacobs L, Groot R, Van der Zeijst BA, Horzinek MC, Spaan W (1987) The nucleotide sequence of the peplomer gene of porcine transmissible gastroenteritis virus (TEGV): comparison with the sequence of the peplomer protein of feline infectious peritonitis virus (FIPV). Virus Res 8: 363–371

14. Jameson BA, Wolf H (1988) The antigenic index: a novel algorithm for predicting antigenic determinants. Comput Appl Biosci 4: 181–186

15. Karplus PA, Schulz GE (1985) Prediction of chain flexibility in proteins. Naturwissenschafter 72: 212–213

16. Kusters JG, Jager EJ, Lenstra JA, Koch G, Posthumus WP, Meloen RH, Van der Zeijst BA (1989) Analysis of an immunodominant region of infectious bronchitis virus. J Immunol 145: 2692–2698

17. Kusters JG, Niesters GM, Lenstra JA, Horzinek MC, Van der Zeijst BA (1989) Phylogeny of antigenic variants of avian coronavirus IBV. Virology 169: 217–221

18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

19. Lerner RA (1981) Chemically synthesized peptides from the nucleotide sequence of the hepatitus-B virus genome elicit antibodies reactive with the native envelope protein of dane particles Proc Natl Acad Sci USA 78: 3403–3407

20. Liu FT (1979) New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry 18: 690–693

21. Luytjes W, Geerts D, Posthumus W, Meloen R, Spaan W (1989) Amino acid sequence of a conserved neutralizing epitope of murine coronaviruses. J Virol 63: 1408–1412

22. Merrifield RB (1963) Solid phase peptide synthesis: the synthesis of a tetrapeptide. J Am Chem Soc 85: 2149–2154

23. Maniatis T, Sambrook J, Fritsch EF (1989) Molecular cloning, a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

24. Mitraki A, King J (1989) Protein folding intermediates and inclusion body formation. Biol Tech 7: 690–697

25. Pedersen NC, Boyle JF, Floyd Km Fudge A, Barker J (1980) An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. Am J Vet Res 42: 368–377

26. Pedersen NC, Floyd K (1985) Experimental studies with three new strains of feline infectious peritonitis virus: FIPV-UCD2, FIPV-UCD3, and FIPV-UCD4. In: Viral disease of small animals. 34th Annual Symposium, Continuing Education Article #5, Vol 7, No 12: pp 1001–1011

27. Posthumus WP, Lenstra JA, Schaaper WM, Nieuwstadt AP, Enjuanes L, Meloen RH (1990) Analysis and simulation of a neutralizing epitope of transmissible gastroenteritis virus. J Virol 64: 3304–3309

28. Reed P, Klepfer S, Jones EV, Pfeiffer NE, Suiter BT, Miller TJ (1991) Recombinant feline coronavirus S proteins. WO 93US365

29. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors Proc Natl Acad Sci USA 74: 5463–5467

30. Talbot PJ, Buchmeier MJ (1985) Antigenic variation among murine coronaviruses: evidence for polymorphism on the peplomer glycoprotein, E2. Virus Res 2: 317–328

31. Talbot PJ, Dionne G, Lacroix M (1988) Vaccination against lethal coronavirus-induced encephalitis with a synthetic decapeptide homologous to a domain in the predicted peplomer stalk. J Virol 62: 3032–3036

32. Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from

polyacrylamide gels to nitrocellulose: procedure and some applications. Proc Natl Acad Sci USA 76: 4350–4354

33. Vennema H, Groot RJ, Harbour DA, Dalderup M, Jones TG, Horzinek MC, Spaan WJ (1990) Early death after feline infectious peritonitis virus challenge due to recombinant vaccinia virus immunization. J Virol 64: 1407–1409

Authors' address: Dr. N. E. Pfeiffer, Biological Research and Development, Smith Kline Beechan Animal Health, Lincoln, NE 68521, U.S.A.