



Quarantine protects Falkland Islands (Malvinas) cats from feline coronavirus infection

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Abstract

Feline coronavirus (FCoV) causes feline infectious peritonitis (FIP). Since 2002, when 20 cats on the Falkland Islands were found to be FCoV seronegative, only seronegative cats could be imported. Between 2005–2007, 95 pet and 10 feral cats tested negative by indirect immunofluorescence antibody (IFA) analysis using two strains of type II FCoV, two transmissible gastroenteritis virus assays, an enzyme-linked immunosorbent assay and rapid immunomigration test. Twenty-four samples (23%) showed non-specific fluorescence, mostly attributable to anti-nuclear antibodies (ANA). The reason for ANA was unclear: reactive samples were negative for *Ehrlichia canis* antibodies; seven were feline immunodeficiency virus positive, but 15 were negative. It was not possible to determine retrospectively whether the cats had autoimmune disease, hyperthyroidism treatment, or recent vaccination which may also cause ANA. The FCoV/ FIP-free status of the Falkland Islands cats should be maintained by FCoV testing incoming cats. However, ANA can complicate interpretation of IFA tests.

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The Falkland Islands (Malvinas) are a small archipelago of Islands in the South Atlantic Ocean. The human population is around 3000. The cat population is around 4000. Feral cat density is estimated at 8–10 adult cats per km²¹ and the cat population of Stanley alone is estimated at around 2000² with 1000 each of pet and feral cats. (Stanley, sometimes called Port Stanley, is the capital, and only true city on the islands.) Feral cats on the islands eat introduced mammals (house mice *Mus musculus*, ship rats *Rattus rattus* and rabbits *Sylvilagus* species) and the seabird, the thin-billed prion *Pachyptila belcheri*.²

There is only one veterinary practice in the Falklands: the Falkland Islands Government Veterinary Service which is part of the Department of Agriculture. The department is responsible for all import/export protocols. It is sometimes possible to prevent introduction of, or to eradicate, a disease within an island population of animals, so for that reason it was of interest to establish whether feline coronavirus (FCoV), the cause of feline infectious peritonitis (FIP), was already present within the cat population.

In a previous pilot study, all of 20 cats tested were negative for FCoV antibodies.² From that time on, in order to keep Falkland cats free of FIP, it became a

requirement to obtain a feline import permit that the cat be FCoV seronegative. Given the encouraging results of the pilot study, and that no cases of FIP were reported by the Islands veterinary surgeons, the study presented here was conducted to verify that the Falkland Islands are FCoV free and to justify the requirement of testing for FCoV antibodies in the feline import permit.

Previous studies on populations of pet cats with outdoor access have found a FCoV seroprevalence of 14–34%.^{3–5} Given a population of 4000 and assuming a

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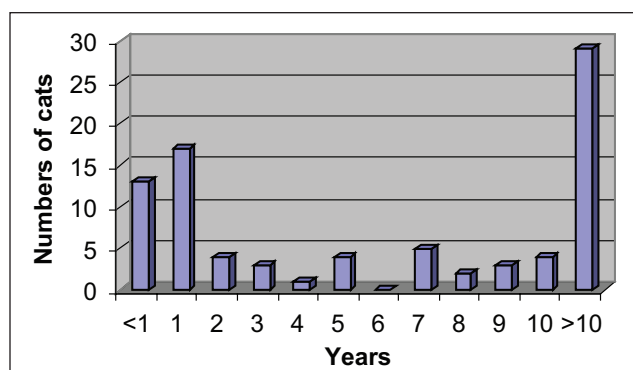


Figure 1 Age distribution of cats sampled. The ages of 20 (19%) cats were unknown, 30 cats (28.6%) were a year of age or under, 28 (26.7%) were over 10 years old

prevalence of 24% (confidence interval ± 10), a test of 70 cats would give a confidence level of 95% using the formula:

$$n = \frac{Z^2 p(1-p)}{e^2}$$

where n is the number of samples required
 Z is the Z value for the corresponding confidence level
 p is the estimated prevalence of 24% (± 10)
 e is the margin of error expressed as a decimal

Blood samples were collected from any cat brought through the veterinary practice between 2005 and 2007 (105 in total): 27 cats were brought to be neutered; 30 for euthanasia; others for various treatments or vaccination. Records stated that 17 cats were from Stanley and that 14 were from little farms or settlements in the countryside outside of Stanley, but the precise location of most cats was unrecorded. All 105 cats had outdoor access. Only two samples were from pedigree cats, the others were all domestic cats. Most of the cats were pet cats, 10 cats were described as 'wild' (meaning feral) and one as 'semi-wild'. Six feral cats were brought to be euthanased, one was to have an eye removed and one was trapped, neutered and returned. The ages of the cats are presented in Figure 1: the age of 20 (19%) cats was unknown, 30 cats (28.6%) were a year of age or under, 28 (26.7%) were over 10 years old. Forty-seven cats were female, 53 were male and the sex of five was unknown.

It is more difficult to establish a pathogen's absence from a population than its presence, and so a variety of different techniques were used to look for antibodies to FCoV, to detect exposure to the virus. Serology is more useful than detection of viral RNA in faeces as only about one seropositive cat in three sheds virus at any one time.⁶ To be effective, a screening of a cat population for FCoV depends on the sensitivity of the test employed. A panel of positive samples was also used on the tests

described in this paper in an ongoing study: the results of this separate study will be described elsewhere (Addie et al, manuscript in preparation) but suffice it to say that no test had a sensitivity of less than 80% and most were close to 100%.

A number of laboratories volunteered their services: samples were screened for FCoV antibodies by indirect immunofluorescence (IFA), using the Wellcome strain type II FCoV³ and an unidentified FCoV strain donated by the University of Liverpool, and transmissible gastroenteritis virus (TGEV)^{7,8} in two other laboratories. Samples were deemed negative if they gave no fluorescent signal at cut-off serum dilutions at 1:10, 1:8, 1:25 and 1:25, respectively. One-hundred-and-three samples were also screened by enzyme-linked immunosorbent assay (ELISA) (FCoV (FIP) Immunocomb; Biogal, Israel)^{5,9} and 83 by rapid immunomigration (RIM) (Speed F-Corona; BVT group, Virbac, France).

A summary of results is shown in Table 1: all samples were FCoV seronegative. Most of the Falkland cat samples were tested at least four times and many samples, especially those which had given non-specific results or results close to the test cut-off, were re-tested at the same and/or different laboratories by FCoV and TGEV IFA. However, due to limitations of sample size and funds, it was impossible for all of the samples to undergo all of the test systems involved in the study. When one particular test gave a positive result on a sample that the other tests found negative, the question arose whether that particular test was more sensitive than the others, or was giving a false positive result. One method of answering that question was to subject another aliquot of the same sample to that test again – if the second result was negative, then it was concluded likely that the first result was a false positive result. Thus it was that by rigorous screening, involving four different techniques across six different laboratories that these samples were found to be negative.

All techniques for FCoV antibody testing have a cut-off zone where interpretation is difficult. The ELISA (FCoV (FIP) Immunocomb; Biogal, Israel) gives grey spots which can be read in an ordinary photograph scanner, with software provided by the manufacturer.⁵ Results are given as 1–6 scores scaling, depending on intensity of greyness of the spots, which, in a previous study, correlated well with FCoV IFA titres.⁹ The absence of a spot gives a result of zero, correlating with a FCoV IFA titre of <1:10, deemed to be negative. In the previous study by Addie et al,⁹ the spots were read by eye, in the present study, they were read using a scanner (Epson 4000) which increased specificity to 100% in the present study. The tiny sample size (5 μ l) used in the ELISA test enabled testing of all but two of the samples; though one test attempted to use the dregs of the sample and possibly could not be counted. All the other 102 samples tested were negative: readings of 0 on 92 samples and 1

Table 1 Analysis of the various FCoV antibody tests used in this study

	FCoV IF† lab 1 37		FCoV IF‡ lab 2 63		TGEV IF lab 3 102		TGEV IF lab 4 16		ELISA§ 103		RIM¶ 83	
Total number of tests	Neg	nsf	Neg	nsf	Neg	nsf	Neg	nsf	Neg	Neg (10)	Neg	Neg/faint
FCoV lab 1 neg	30		17	*	5	23	5	4	24	4	23	3
FCoV lab 1 nsf		7	5	*	0	7	2	1	5	2	6	0
FCoV lab 2 neg	17	5	63	*	4	58	5	3	53	10	46	13
FCoV lab 2 nsf	*	*	*	*	*	*	*	*	*	*	*	*
TGEV lab 3 neg	5	0	4	*	26		3	0	25	1	15	0
TGEV lab 3 nsf	23	7	58	*		76	7	6	65	9	50	13
TGEV lab 4 neg	5	2	5	*	3	7	10		8	1	10	0
TGEV lab 4 nsf	4	1	3	*	0	6		6	5	1	6	0
ELISA neg	24	5	53	*	25	65	8	5	93		60	12
ELISA 10	4	2	10	*	1	9	1	1		10	7	3
RIM neg	23	6	46	*	15	50	10	6	60	7	69	
RIM neg faint line	3	0	13	*	0	13	0	0	12	3		14

nsf = non-specific fluorescence

*Although this laboratory did detect nsf, they recognised it as non-specific and reported the result simply as negative (<1:10). Unfortunately, they didn't keep a record of which samples gave non-specific fluorescence. Laboratories 1 and 3 also recognised and reported nsf results

†Using the Wellcome type II strain of FCoV

‡Using an unspecified type II strain of FCoV kindly donated by the University of Liverpool

§FCoV (FIP) Immunocomb; Biogal, Israel

¶Rapid immunomigration (RIM) (Speed F-Corona; BVT group, Virbac, France)

Table 2 Signalment and FIV status of 24 cats whose samples gave non-specific fluorescence on FCoV/TGEV antibody tests

Cat reference	Age (years)	Sex	Breed	Origin	nsf titre (TGEV IFA)	FIV	Antibody titres		
							FCV	FHV	FPV
3	5	F (N)	DSH Tabby	SCH	1:100	Negative	256	16	320
15	8	M (N)	DSH Black	SCH Countryside	1:25	Negative	256	4	320
17	Elderly	F (N)	DSH Tabby	Wanderer town	1:25	No sample left			
24	16	F	DSH Tabby	Countryside	1:25	Negative	256	128	10
26	Unknown	M	DSH Black	Feral	1:25	Positive	256	64	40
28	<1	F	DSH Tabby	2-cat household	1:25	Positive	256	Neg	160
34	Unknown	F	DSH Tabby	Feral	1:25	No sample left			
35	7	F (N)	DSH Black	3-cat household	1: 5	Negative	>256	16	320
37	12	M (N)	DSH Tabby	2-cat household	1: 5	Positive	256	64	160
39	9	M (N)	DSH Tabby	Unknown	1:100	Negative	>256	64	320
40	2	M (N)	DSH Tabby	Unknown	1:100	Negative	256	16	320
51	8	M	DSH	At least 3-cat household	>100	Negative	>256	16	320
52	16	M	DSH	2-cat household	>100	Positive	>256	32	160
63	1	M	DSH	2-cat household	>100	Negative	128	32	Neg
64	Adult	F	DSH	2-cat household	>100	Negative	256	Neg	320
65	<1	M	DSH	SCH	>100	Positive	256	32	320
67	1997/13*	M	DSH	3-cat household	>100	Positive	256	32	160
70	Unknown	M	DSH	2-cat household	>100	Negative	256	4	320
72	Old	M	DSH	3-cat household	>100	Positive	128	128	80
87	Unknown	F	DSH	Feral	>100	Negative	128	32	320
88	14	F	DSH	2 cat household	>100	Negative	256	Neg	Neg
99	2	F (N)	DSH	SCH	>100	Negative	128	8	80
100	Unknown	M	DSH	Feral	>100	Negative	>256	Neg	>320
103	12	M	DSH	2-cat household	>100	Negative	>256	32	320

*Conflicting data given in submission – if born in 1997, the cat would only be 8 or 9 years old when tested

F = female, M = male, (N) = neutered

SCH = single cat household

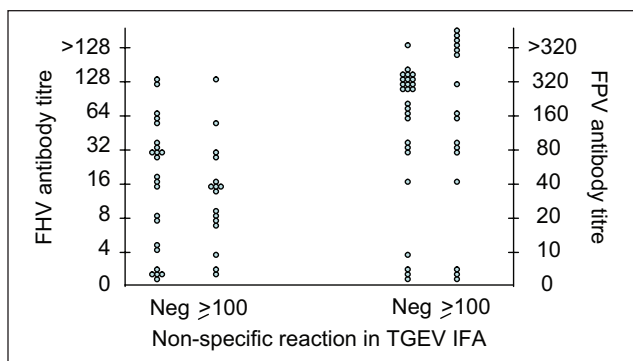


Figure 2 A comparison of antibody titres against FHV and FPV in samples without and with non-specific reactions. Comparison of FHV and FPV antibody titres in 16 samples which had a non-specific fluorescence result of 100 or more (shown as ≥ 100 on the graph) with 23 samples with no non-specific fluorescence (labelled Neg): mean FHV and FPV antibody titres were slightly lower in the non-specific fluorescence group than the Neg group, however, there was no significant difference ($P = 0.77$ for FHV; $P = 0.73$ for FPV). If recent vaccination was the cause of non-specific fluorescence in some of the samples, it was not discernable by looking at antibody titres. Neg = no non-specific immunofluorescence on TGEV IFA test. ≥ 100 = high non-specific immunofluorescence on TGEV IFA test

(or $<1:10$) in 10 cases (see Table 1). A score of 1 is less than the control spot and is deemed negative.^{5,9}

There was sufficient sample remaining to test 83 samples by RIM. The uncertain area for interpretation of RIM results is where a very faint or so-called 'ghost' line appears, which, at the time of testing, the manufacturers recommended as being interpreted as negative (ie, specificity for this test was 100%), but which in a later formulation of the kit is counted positive. As shown in Table 1, 69 samples were clearly negative and 14 (17%) showed ghost lines. These 14 samples were all negative by FCoV IFA and ELISA (though three had ELISA readings of 1).

Twenty-four samples showed non-specific fluorescence (nsf) likely attributable to anti-nuclear antibodies (ANA) (Tables 1 and 2). Non-specific fluorescence occurs when the uninfected negative control cells, as well as the infected cells, fluoresce. This phenomenon was observed more in the TGEV IFA plates than in the FCoV IFA plates and can be mistaken for a positive signal by an inexperienced technician.

ANA can be present because of concurrent infections with *Ehrlichia canis*;¹⁰ *Anaplasma (Ehrlichia) phagocytophilum*;¹¹ or feline immunodeficiency virus (FIV) (Maria Grazia Pennisi, personal communication); autoimmune disease;¹² certain treatments for hyperthyroidism (ie, felimazole, methimazole, propylthiouracil, thiamazole).^{12–14}

Ehrlichiosis was considered an unlikely cause of ANA because ticks were never observed on the Falkland Islands (Vic Epstein, personal communication) but to be

sure, the samples from the cats listed in Table 2 were tested using *E canis* slides (VMRD; Pullman, Washington, USA) – all were negative. FIV was reported in 8% of pet cats and 28.5% of feral cats in the Falklands.² As shown in Table 2, 22 samples which fluoresced non-specifically were tested for FIV antibodies by anti-transmembrane ELISA:¹⁵ seven cats were FIV positive and 15 were negative (there was insufficient material to test the remaining three cats). Therefore, FIV infection did not explain all the non-specific fluorescence observed. It was not possible to ascertain whether any of the cats were on treatment for hyperthyroidism, but given that six cats were older, this was certainly possible.

Cells used in one of the two TGEV IFAs used in the study were regularly screened for feline leukaemia virus, FIV, feline herpesvirus, feline parvovirus, feline calicivirus (though not mycoplasma contamination) by real-time polymerase chain reaction,⁸ thus eliminating the possibility that non-specific reactions in this test were due to antibodies to contaminants of the cell culture.

Non-specific reactions to FCoV antibody tests can also be induced by vaccination due to induction of antibodies against components in the vaccine which are also present in the test assay (for example, ruminant serum components which are used in cell culture).^{16–18} The post-vaccinal reaction lasts up to 15 weeks.¹⁶ Unfortunately, it was not possible to ascertain from the cats' records whether they had been vaccinated in the 15 weeks prior to blood sampling, although it was known that the feral cats had not been vaccinated (4/24 cats were feral). Hypothesising that recent vaccination would cause the presence of antibody titres to all three vaccine components, 22/24 non-specific fluorescence samples, and 37 clearly FCoV negative samples, were screened by ELISA to determine antibody titres to core vaccine components, feline calicivirus (FCV), feline herpesvirus (FHV) and feline parvovirus (FPV) (Immuno Comb Feline VacciCheck; Biogal, Israel). All 59 samples tested were seropositive for FCV (100%), 43 (73%) had antibodies to FHV, and 46 (78%) were seropositive for FPV. Of the 22 non-specific fluorescence samples screened (Table 2), five were negative for antibodies to at least one of the three core vaccine viruses, indicating that recent vaccination was unlikely. The Feline VacciCheck gives results as a number between 0 and 6, 0 corresponding with seronegativity and 6 with a high titre. For FHV the mean of the samples without nsf was 2.87, and the mean of samples with non-specific fluorescence to a dilution of 1:100 or more was 2.69 ($P = 0.77$, Fisher's exact test); for FPV the means were 4.43 and 4.19, respectively ($P = 0.73$) (see Figure 2). As a cause of at least a few non-specific reactions vaccination was not completely ruled out, but appears unlikely.

FCoV has been found in every country in which it has been looked for⁷ except the Galapagos Islands.¹⁹ Based

on the UK and Australian models, where the cats are predominantly granted outdoor access, we expected that, if FCoV were present, around 14–34% of Falkland Island cats would be seropositive.^{3–5} Thus a sample size of 105 should have been adequate to detect antibodies if the virus had been present. FCoV can only survive a matter of weeks outside the cat. It is likely that the outdoor lifestyle, where cats bury their faeces, enabled the virus, if it was ever present, to die out. The system of quarantine, preventing introduction of the infection to the islands, has probably protected the cats ever since.

Conclusion

Maintaining a population free of FCoV infection, and therefore FIP, is possible by screening incoming cats for FCoV antibodies. However, care must be taken with the reading of indirect immunofluorescence antibody tests, because of the occasional presence of non-specific antibodies in some cats. It is likely that non-specific fluorescence is caused by many possible factors: anti-nuclear antibodies, FIV infection and possibly recent vaccination. Samples where the result is questionable should be retested using a different technology.

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Conflict of interest The authors declare that there is no conflict of interest.

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