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Antibodies against Gag are diagnostic markers for feline foamy virus infections while Env and Bet reactivity is undetectable in a substantial fraction of infected cats

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Abstract

Spumaretroviruses or foamy viruses constitute a distinct subfamily of retroviruses. The biology of foamy viruses within the authentic host, their mode of transmission, and disease potential in the authentic host or after zoonotic transmission into human or other species are almost unknown. Using feline foamy virus (FFV) as model system, we established modular enzyme-linked immunosorbent assays (ELISA) suited to determine feline IgG and IgM antibody responses against structural and non-structural FFV proteins. We validated the ELISAs with standard reference sera. In 99 cats admitted to a Swiss veterinary hospital, overall FFV Gag antibody prevalence was 36%, reactivity against Env and the non-structural protein Bet each was about 25%, and 19% of the sera were directed against all three FFV antigens. With one exception, all Bet- and/or Env-positive sera were also positive for Gag. In this small epidemiological pilot study, FFV antibodies were not significantly associated with clinical disease.
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Keywords: Foamy virus; ELISA; Humoral immunity; Diagnostic marker

Introduction

Spumaretro- or foamy viruses (FVs) are a distinct group of retroviruses that gained increasing interest as novel viral vectors for gene delivery and vaccination (Linial, 1999; Rethwilm, 2003). FVs have a complex genomic organization with *gag*, *pol*, and *env* genes, the regulatory *bel 1/tas* transactivator gene, and *bet* (Rethwilm, 2003). Bet counteracts cellular APOBEC3-mediated restriction, is involved in particle release, and may have a role in establishing viral persistence (Alke et al., 2001; Löchelt et al., 2005; Meiering and Linial, 2002; Russell et al., 2005; Saib et al., 1995). Presently, insufficient data on FV replication in the infected individual, the sites of FV replication, the authentic target cell(s), and the extent of virus replication during life-long persistence are available.

The zoonotic potential of primate FVs is well established: Simian FV (SFV) types from chimpanzee, African green monkeys, and baboons have been detected in humans (Heneine et al., 2003). FV zoonoses were either traced back to simian caretakers who had been bitten by these primates (Heneine et al., 1998) or to Africans who were exposed to simian organs and body fluids by bush meat hunting and preparation (Wolfe et al., 2004). It is generally accepted that the prototypic human FV isolate HFV is actually a primate FV (PFV). In the few zoonotic cases known so far, no disease was associated with SFV infections and no further transmission to other humans occurred (Heneine et al., 2003). However, HFV/

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PFV may bear a disease potential since severe neurological disease consistently occurs in HFV-transgenic mice (Aguzzi et al., 1996).

In small-number studies of naturally infected cats or analysis of cats experimentally infected with wt or cloned FFV and FFV-derived vaccine vectors, we did not find evidence for a defined disease associated with FFV-infection (Alke et al., 2000; Schwantes et al., 2002, 2003). However, these studies confirmed active gene expression, replication, and progeny virus shedding in persistently infected cats clearly challenging the view that FV persistence is characterized by viral latency.

In order to study whether FFV infection is correlated with rare disease in cat, whether it may have a co-factorial role in other pathologies (Bandecchi et al., 1992; Glaus et al., 1997) and to analyze whether markers of FFV infections are detectable in man (Butera et al., 2000; Winkler et al., 1997b), sensitive and specific antibody assay systems are required. Besides virus reisolation and PCR genome detection, immunoblot reactivity against FV Gag and Bet proteins and immunofluorescence techniques are considered to be the most reliable methods to detect reactivity against FVs (Alke et al., 2000; Hussain et al., 2003; Khan et al., 1999; Winkler et al., 1997b; 1998, 1999). The only FFV Gag ELISA available at present showed considerable background reactivity (Winkler et al., 1997b). Using these diverse techniques, FFV prevalence ranged from 33% to more than 70% depending on the study and geographic region analyzed (Bandecchi et al., 1992; Daniels et al., 1999; Glaus et al., 1997; Winkler et al., 1998, 1999).

Here, we show with a novel ELISA technique that recombinant full-length FFV Gag is the diagnostic antigen of choice to identify experimental and natural FFV infections.

Results

Expression of recombinant FFV Gag, Env, and Bet fusion proteins and set-up of ELISAs

The FFV structural protein Gag, the FFV envelope-leader protein and SU ecto-domains (Wilk et al., 2001; Geiselhart et al., 2004; Lindemann and Goepfert, 2003), and the accessory Bet protein (Löchelt, 2003; Löchelt et al., 2005) were expressed as fusion proteins flanked by a N-terminal glutathione-*S*-transferase (GST) domain and by a C-terminal SV40-derived tag allowing immune detection by corresponding antisera (Sehr et al., 2001, 2002). The three FFV-fusion proteins were soluble and predominantly full-length and were purified from recombinant bacteria (data not shown).

ELISAs were based on a generic assay for Human Papillomavirus antibodies (Sehr et al., 2001, 2002). Recombinant FFV fusion proteins were directly adsorbed from cleared bacterial lysates to the ELISA plates as described (Sehr et al., 2001). Optimization of assay conditions was done using the FFV-positive cat reference serum 8014 and FFV-negative sera from specific pathogen-free (SPF) cats (Alke et al., 2000; Schwantes et al., 2003). Since background reactivity had been present in another FFV ELISA (Winkler et al., 1997b), the cat sera were pre-adsorbed with GST-tag-containing lysates decreasing background reactivity to 50 to 100 milli absorption units at 450 nm (mOD_{450}). The reproducibility of independently performed ELISAs was high (R^2 = 0.987).

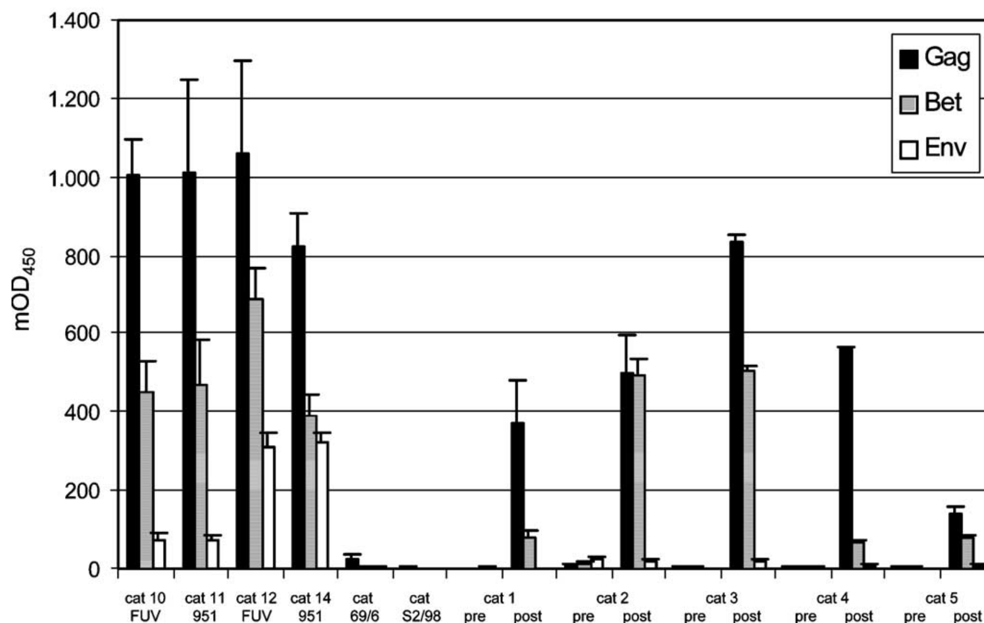

Fig. 1. Validation of FFV Gag, Env, and Bet ELISA by cat reference sera. Cats 10 and 12, and cats 11 and 14 were Australian cats naturally infected with FFV strains FUV and 951, respectively. FFV infection had been confirmed by serology, virus reisolation, and diagnostic PCR (Winkler et al., 1998). Cat 69/6 and S2/98 were FFV-negative SPF animals. Sera of SPF cats M1 to M5 were taken before (pre) and 10 weeks after (post) experimental FFV infection (Schwantes et al., 2003) and tested at 1:50 dilution. Results shown are antigen-specific absorption values (milli absorption units, mOD_{450}) with GST-tag background values subtracted. ELISAs were run against FFV Gag (black bars), Bet (grey bars), and Env (white bars) including error bars for standard deviation.

Validation of the FFV Gag, Env, and Bet ELISAs using feline reference sera

To validate reactivity against the three FFV proteins, we used reference sera 10, 11, 12, and 14 from naturally FFV-infected Australian cats (Winkler et al., 1998), pre- and post-exposition sera M1 to M5 from cats infected with empty replication-competent FFV vectors (Schwantes et al., 2003), and sera from FFV-negative SPF cats (Fig. 1). At 1:50 dilutions, FFV-negative cats 69/6 and S2/98 and pre-exposition sera were antibody-negative since the reactivity towards all three FFV antigens was always below 23 mOD_{450}. In contrast, all sera taken about 10 weeks after FFV infection showed clear reactivity (more than 142 mOD_{450}) against Gag, two animals reacted strongly and three weakly with Bet, and none showed clear reactivity towards Env (all below 30 mOD_{450}). Sera from naturally FFV-infected cats (Winkler et al., 1998) showed stronger reactivity. Gag was recognized best, Env reactivity was low in two and strong in two other cats, and Bet was consistently recognized at intermediate levels. Env reactivity was not correlated with the serotype of the infecting FFV isolate: the FFV FUV-derived diagnostic antigen was similarly recognized by FFV FUV-infected cats 10 and 12 or by FFV 951-infected cats 11 and 14 (Winkler et al., 1998). In summary, the observed ELISA reactivity of the reference animals correlated well with their infection status.

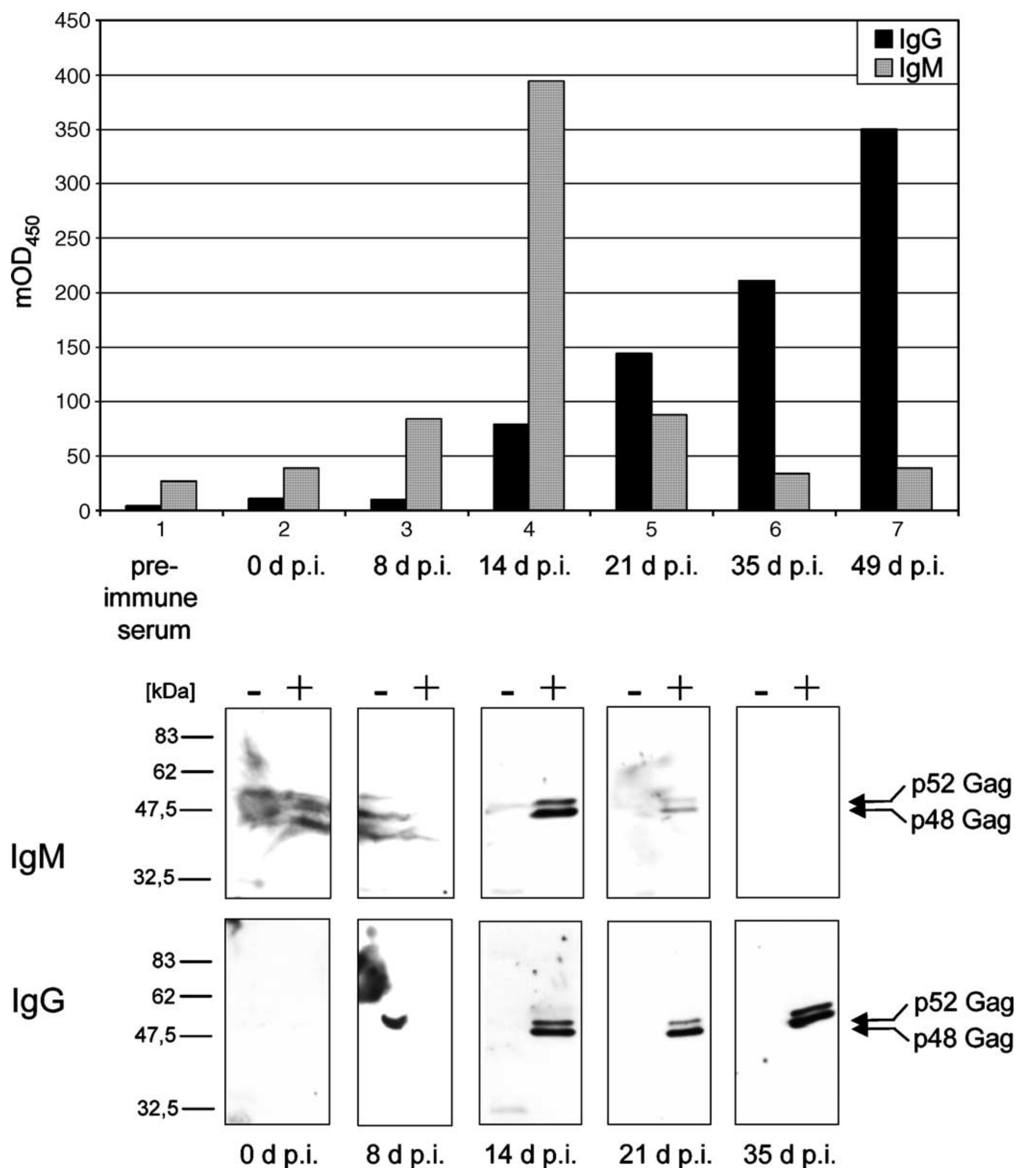

Fig. 2. Kinetics of FFV Gag-specific IgG and IgM as determined by GST-ELISA (top, single measurements) and FFV immunoblot (bottom). Sera from experimentally FFV FUV-infected cat M2 (Schwantes et al., 2003) taken before (pre) and 0, 8, 14, 21, 35, 49 days after infection (d.p.i.) were assayed for Gag reactivity using peroxidase conjugates of protein A (IgG) and goat anti-feline IgM (IgM), respectively. For immunoblots with the sera taken between 0 and 35 d.p.i., enriched FFV particle preparations (+) and mock preparations (−), respectively, were blotted after protein gel separation and the same secondary antibodies were used as above. Besides some unspecific reactivity, two forms of Gag proteins, p52 and p48, were detected.

Kinetics of anti FFV IgG and IgM in experimentally infected cats

To assess whether FFV-specific IgG and IgM antibodies can be discriminated, ELISAs were run in parallel with a protein A conjugate detecting cat IgG antibodies (Yamamoto et al., 1985) and a peroxidase-coupled antiserum specific for feline IgM. Due to the differential temporal appearance of IgG and IgM antibodies, serially taken blood samples from six cats infected with empty FFV vectors were analyzed (Schwantes et al., 2003). The sera were also subjected to IgG- and IgM-specific immunoblotting using the same secondary reagents. As shown for cat M2 (Schwantes et al., 2003), IgM reactivity peaked 14 d p.i. and then dropped rapidly whereas IgG reactivity increased steadily from day 14 (Fig. 2). The IgM-specific background reactivity was slightly larger than that for IgG but still always

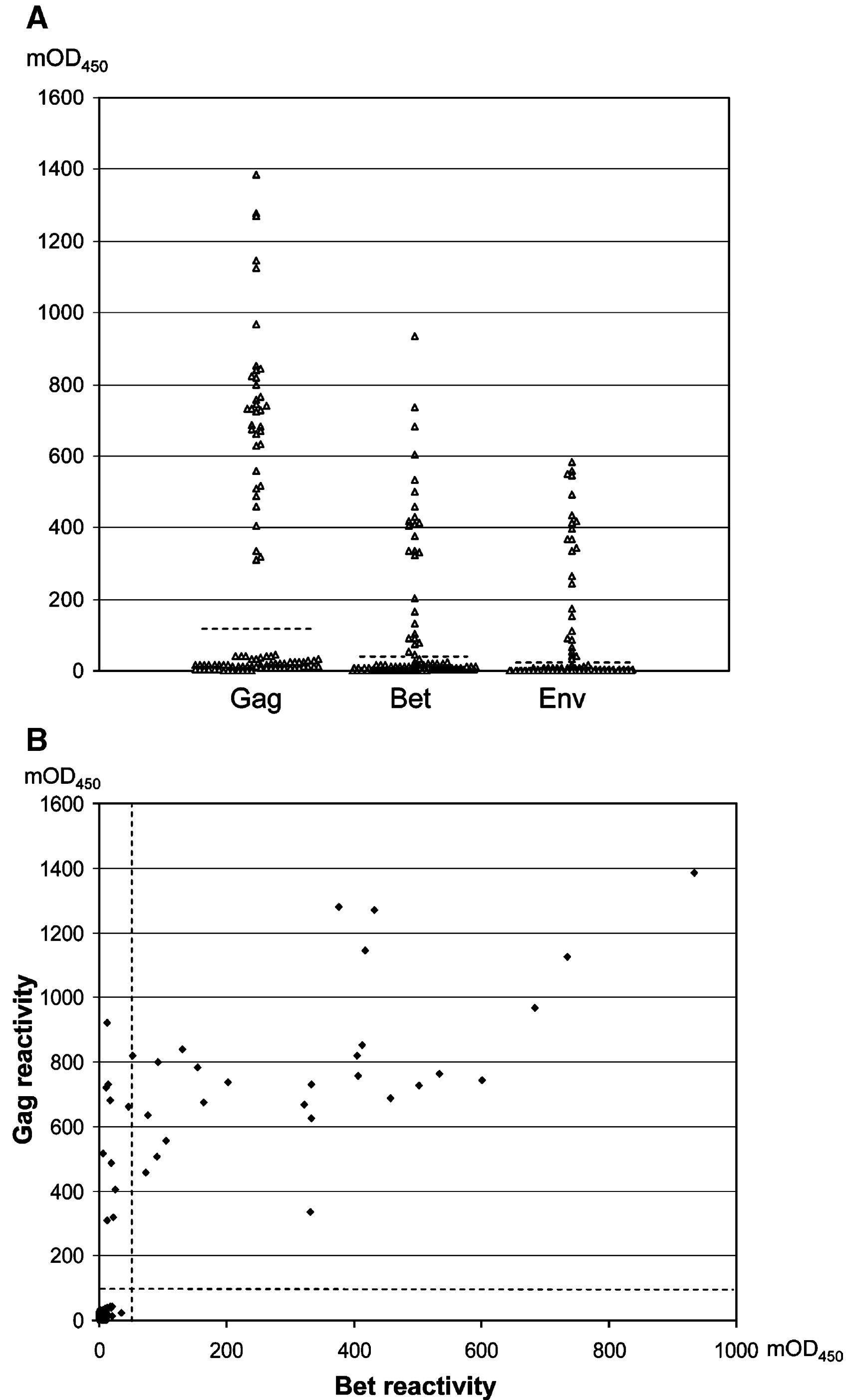

Fig. 3. Distribution of Gag, Bet, and Env antibody reactivity in 99 Swiss cat sera. In panel A, ELISA reactivity of all 99 sera towards FFV Gag, Bet, and Env is shown in dot-plot presentations. Dashed lines indicate the calculated cut-off values. In panel B, Gag (vertical axis) and Bet reactivity (horizontal axis) is presented in a two-dimensional plot. Each point represents a data pair of an individual serum. The upper right sector shows double-positive, the lower-left double-negative, the lower-right sector would display sera positive for Bet only, and the upper left sector represents sera positive for Gag only.

below 50 mOD_{450}. The ELISA results paralleled the immunoblot data and showed a sensitivity allowing IgM detection already at 8 d.p.i.

Pattern of FFV-specific IgG antibodies in sera of hospitalized cats

Sera of 99 Swiss domestic cats taken to hospital for diverse reasons were assayed for IgG antibodies against FFV Gag, Env, and Bet. Reactivity towards Gag was strongest and most frequent and showed a bimodal distribution (Fig. 3A). Gag reactivity of 63 sera was below 45 mOD_{450} and above 300 mOD_{450} for the remaining 36 sera. A stringent cut-off value of 112 mOD_{450} was calculated as 2 × (mean + 3 SD). Bet and Env antibodies showed not such a bimodal distribution of reactivity. Over 50% of these sera displayed very low values of less than 10 mOD_{450}, the remaining sera were evenly distributed among higher values. Bet and Env reactivities of Gag-negative sera were all very low (Fig. 3B and data not shown) suggesting that Gag negativity is correlated with negativity for Env and Bet. Thus, stringent cut-off values for Bet and Env were calculated from only the Gag-negative sera (excluding positive outliers) resulting in values of 46 and 21 mOD_{450} for Bet and Env, respectively.

ELISA reactivity of sera gradually declined upon serial dilution and remained above the cut-off at dilutions of 1:100 to 1:1.600 for Gag, 1:400 to 1:800 for Bet, and 1:100 to 1:800 for Env (not shown). Thus, 1:50 dilutions are required to detect all FFV-positive sera.

Of all 99 sera, 37 showed FFV antibodies, of which 36 reacted with Gag, 26 with Bet, and 25 with Env (Table 1). About half (19/37) of the antibody-positive sera reacted with all three antigens whereas only 6 displayed single reactivity against Gag or Env. The remaining sera contained Gag plus Bet or Gag plus Env antibodies whereas no Bet plus Env only serum was detected. The strengths of reactivity of double-positive sera were grossly correlated, with Env or Bet reactivities >200 mOD_{450} mainly coinciding with Gag reactivities >600 mOD_{450} (Fig. 3B and data not shown). The combined reactivity of cats against Gag and Bet (26/36) is similar to that seen for SFV-infected chimpanzees (Hahn et al., 1994). Only a single serum was slightly Env-positive but negative for Gag and no serum was Bet-positive in the absence of Gag antibodies. This antibody pattern makes Gag the diagnostic antigen of choice for the serological detection of FFV-infected cats.

None of the 99 Swiss cat sera exhibited IgM reactivities against Gag (data not shown) indicating that the FFV infections observed here were not recent. Selected cat sera were also analyzed by immunoblotting using purified FFV particles as antigen. For Gag antibodies, data from ELISA and immunoblot were fully concordant while the Env ELISA was superior when compared to immunoblots (data not shown). In addition, Bet antibodies are known to poorly react in immunoblots (Alke et al., 2000) suggesting the Bet ELISA to be better suited for Bet antibody detection.

Table 1
FFV antibody patterns in hospitalized Swiss cats

Total number of sera	99
Number of triple-negative sera	62
Number of positive sera	
For any antigen	37
For Gag	36
For Bet	26
For Env	25
Number of single-positive sera	
For any antigen	6
For Gag	5
For Bet	0
For Env	1
Number of double-positive sera	
For any combination of two antigens	12
Gag + Bet	7
Gag + Env	5
Bet + Env	0
Number of triple-positive sera (Gag + Bet + Env)	19

Discussion

We describe novel ELISAs for the sensitive and specific detection of antibodies against both known FFV serotypes. The specificity of the ELISAs was confirmed using defined reference sera from naturally and experimentally infected cats (Winkler et al., 1998; Schwantes et al., 2003). Gag, Bet, and Env antigens are recognized with higher sensitivity in the novel ELISAs than in immunoblots. Gag was shown to be the diagnostic antigen of choice. The new FFV Gag ELISA is superior to a previous one which showed substantial background reactivity (Winkler et al., 1997b), a critical issue when analyzing sera with a low-level of reactivity e.g. from recently infected animals or from humans who might be zoonotically infected with FFV.

Utilization of multiple antigens and the capacity to discriminate early and transient IgM responses from long-lasting IgG reactivity allow detection of antibodies as marker for past or previous/current infections. In addition, the FFV ELISAs established here allow direct determination of antibody patterns in order to potentially associate individual pattern with disease, the kinetics of infection, and the host–virus interplay. Such sero-epidemiological studies for FFV will certainly extend our understanding of FV infection and replication in general. The direct comparison of feline reactivity towards Gag, Env, and Bet revealed that Gag reactivity is strongest. This clearly corresponds to the general observation that reactivity towards Gag, either in ELISA or immunoblots is diagnostic for FV infection in primates (including zoonotically infected human) and cats (Alke et al., 2000; Hussain et al., 2003; Khan et al., 1999; Winkler et al., 1998). Strong Gag reactivity obtained with all positive sera clearly differentiates Gag-negative from Gag-positive sera as shown by the bimodal distribution of Gag reactivity (Fig. 3A). This bimodal distribution allowed easy calculation of the Gag cut-off value. Since Bet and Env did not display such clear pattern of reactivity, their cut-off values were determined from the Gag-negative sera. A single Gag-negative serum slightly exceeded the calculated Env cut-off.

Since this serum was clearly negative in immunoblots (data not shown) and since reactivity against Bet and Env generally ranged from strong to very low, this animal is considered FFV-negative and the Env reactivity unspecific. As all Gag-specific reactivity was strong, background and specificity problems are not relevant for this diagnostic antigen thus making Gag the antigen of choice to diagnose FFV infection by serology.

The small-scale study performed did not reveal any significant association of a defined disease and FFV infection. Thus, larger epidemiological surveys are required including detailed information on the cats (age, sex, disease status, other infections etc.) to clarify whether FFV infection does induce disease or whether FFV infection is associated with feline immunodeficiency virus infections (Glaus et al., 1997; Bandecchi et al., 1992). In addition, such studies may reveal whether the pattern of sero-reactivity towards different antigens differs during infection. For instance, the Australian cats (Winkler et al., 1998) showed in general higher levels of reactivity than FFV vector-infected animals 60 days p.i. (Schwantes et al., 2003; Fig. 2) which may indicate that Bet and Env reactivity reaches high levels only after long-term infection or which may point to differences between the Australian field virus and the molecular cloned FFV used as vaccine vector.

Materials and methods

Molecular cloning and recombinant proteins

The complete FFV FUV (Winkler et al., 1997a) *gag* ORF was amplified by PCR with primers Gag-s and Gag-as (Table 2) thereby introducing restriction sites for *BamH*I and *Sal*I. The FFV *bet* gene was amplified from a Bet expression plasmid (Alke et al., 2001) with primers Bet-s and Bet-as introducing sites for *Bgl*II at the 5′ and *Sal*I at the 3′ end. The ectodomain of the Elp-SU part of FFV Env (Wilk et al., 2001; Geiselhart et al., 2004) was amplified with primers Env-s and Env-as introducing *Eco*RI and *Sal*I sites. All PCRs were done using Herculase Hotstart proof reading DNA polymerase (Stratagene, Heidelberg, Germany) at 95 °C for 2 min plus 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 75 s. PCR products were digested with restriction enzymes cleaving at the introduced sites. Purified DNA fragments were fused in frame between the 5′ GST domain and the 3′ SV40 tag (KPPTPPPE-PET) of correspondingly digested pGEX4T3tag derivatives (Sehr et al., 2002). Clones were identified by restriction enzyme digestion and DNA sequencing.

For fusion protein expression, *E. coli* BL21 cells were transformed with pGEX-X-tag plasmids and recombinant proteins were purified as described (Sehr et al., 2001, 2002).

GST capture ELISA and cut-off definition

The ELISAs were performed essentially as described (Sehr et al., 2001, 2002). 96-well microtiter plates (Thermo Labsystems, Dreieich, Germany) were coated with glutathione casein, pre-adsorbed with blocking buffer (0.2% (w/v) casein in PBS, 0.05% (v/v) Tween 20), and then reacted with 100 µl cleared *E. coli* lysates containing the GST-tag or GST-X-tag fusion proteins (0.25 µg/µl total protein in blocking buffer).

Sera from naturally and experimentally infected cats (Alke et al., 2000; Schwantes et al., 2003) or from 99 cats with unknown immune status collected at a veterinarian hospital in Zurich, Switzerland, were pre-incubated in blocking buffer containing 2 µg/µl total protein from a GST-tag expressing *E. coli* BL21 (Sehr et al., 2001) at a dilution of 1:50 (for protein A peroxidase conjugate, Sigma, Munich, Germany, 1:5000 dilution), or 1:100 (for anti cat IgM peroxidase conjugate, Bethyl, Montgomery, USA; 1:10,000 dilution). Pre-absorbed sera were incubated for 1 h at RT in the coated ELISA plate wells, washed, and incubated for 1 h at RT with either conjugate. Substrate reaction and quantification were done as described (Sehr et al., 2001). Unless otherwise noted, all incubations were performed with a volume of 100 µl/well.

For each serum, the background absorbance with GST-tag was determined and subtracted from the absorbance with the GST-X-tag protein to calculate its specific reactivity against the FFV antigens. Measurements were done in duplicate on different plates and the mean value of the specific reactivity of the duplicate was taken as the readout.

Cut-off values were calculated from the group of Gag-negative sera as 2 × (mean + 3 SD) excluding positive outliers.

Immunoblot analyses of recombinant proteins and FFV particles

FFV particles from cell culture supernatants of FFV-infected CRFK cells were enriched by ultracentrifugation through a 20% (w/v) sucrose cushion (30,000 × *g*, 2 h, 4 °C), and served as antigen for immunoblot analyses (Wilk et al., 2001).

Table 2
PCR primers for generating FFV Gag, Bet, and Env GST fusion proteins

Primer	Sequence	Restriction site
Gag-s	5′-CGAGTCGGATCCATGGCTCGAGAATTAAATCCTCTCC	*Bam*HI
Gag-as	5′-GCATGAGTCGACATCTTTACCCCCTTTCTTTCCACCG	*Sal*I
Bet-s	5′-CGAGTCAGATCTATGGCTTCAAAATACCCGGAAGAAG	*Bgl*II
Bet-as	5′-GCATGAGTCGACTTCAGAGTCAGATGACTCAGATGTTG	*Sal*I
Env-s	5′-CGAGTCGAATTCTCAATGGAAAGAAGCAATAACACATCC	*Eco*RI
Env-as	5′-GCATGAGTCGACTTGTCTTCTACCTTTCTTTCTTTCACAAG	*Sal*I

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