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# Immunological properties of the transmembrane envelope protein of the feline foamy virus and its use for serological screening

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## Abstract

*The transmembrane envelope (TM) proteins of retroviruses are used as antigen in diagnostic immunoassays and they represent a conserved target for neutralizing antibodies. To analyze the situation in infections with the feline foamy virus (FFV), its recombinant TM protein was produced and used for ELISA and Western blot analyses. Screening sera from 404 German cats showed that 39% reacted against the TM protein, the same infection rate was determined using the Gag protein. Epitope mapping showed antibodies against the membrane proximal external region (MPER) of the TM protein in the sera from infected cats, but attempts to induce neutralizing antibodies by immunization with the recombinant TM protein failed. This is the first report demonstrating that the TM protein of the FFV is highly immunogenic and valuable for serological screening. Similar to HIV-1, but in contrast to different gammaretroviruses, immunization with the TM protein of FFV did not induce neutralizing antibodies.*

## Introduction

The retroviral transmembrane envelope (TM) proteins play an essential role during infection and thus their general structure is highly conserved. They are composed of a fusion peptide at the N-terminal end, an N-terminal helical region (NHR), a C-terminal helical region (CHR) and the membrane-spanning domain (MSD) (Chan et al., 1997). After insertion of the fusion peptide into the cellular membrane and due to an interaction of both helical regions, the TM proteins trigger the fusion of the cellular and viral membranes and infection of the cells can occur (Melikyan, 2008). The TM proteins of orthoretroviruses, e.g., gammaretroviruses and lentiviruses, including the human immunodeficiency virus HIV-1, are well studied. They contain an immunodominant cystein–cystein (Cys–Cys) loop between NHR and CHR with three Cys in the case of gammaretroviruses and two Cys in the case of HIV-1. Since most of the infected individuals produce antibodies against the TM protein, especially against the Cys–Cys loop ( [Gnann et al., 1987] and [Denner et al., 1994]), these proteins are used for diagnostic purposes ( [Eberle et al., 1997], [Manocha et al., 2003] and [Greenwald et al., 2006]). The TM protein gp41 of HIV-1 and the TM protein p15E of different gammaretroviruses including the porcine endogenous retrovirus (PERV), the feline leukemia virus (FeLV) and the Koala retrovirus (KoRV) are also the target for broadly neutralizing antibodies which recognize epitopes located in the membrane proximal external region (MPER) of their TM protein ( [Muster et al., 1993], [Stiegler et al., 2001], [Fiebig et al., 2003], [Fiebig et al., 2006], [Langhammer et al., 2005] and [Langhammer et al., 2006]).

The TM proteins of the foamy viruses (FVs), which belong to the sub-family of *Spumaretrovirinae*, differ significantly from that of the orthoretroviruses (Lindemann and Goepfert, 2003). They are larger (gp48 in comparison with gp41 of HIV-1 and p15E of gammaretroviruses, the last is not glycosylated), contain seven to eight cysteines in case of the feline and primate FV (FFV and PFV) and have an unusually prolonged central domain between their NHR and CHR that distinguishes the TM of FVs from that of all other retroviruses (Wang and Mulligan, 1999). FFV and PFV persistently infect cats and primates including man, respectively, however in contrast to the HIV-1, to the feline immunodeficiency virus (FIV) and to FeLV, no pathological consequences of FV infections are currently known ( [Linial, 2000] and [Alke et al., 2000]). In previous reports, the prevalence of FFV in wild and household cats

ranged from 33% to 70% depending on the method used and the geographic region analyzed: first data were obtained in a small group of animals in Italy, 10 out of 11 cats infected with FIV, six out of nine animals infected with FeLV and three out of 11 uninfected cats were positive for FFV (Bandedchi et al., 1992). FFV was found in 33% of 30 wild cats trapped in Scotland (Daniels et al., 1999), in 53% of 201 Australian cats (Winkler et al., 1997), in 57% of a larger group of Australian domestic cats (Winkler et al., 1999) and in 36% of domestic cats from Switzerland (Romen et al., 2006). The infection rate in German cats is still unknown. Although predominantly immunological assays were used to study the prevalence of FV infections, the immune response against the TM proteins was not yet investigated. In this report we describe the expression, purification and immunological properties of the FFV TM protein and its usage in a newly established ELISA for screening for FFV infection in German cats. Using TM and Gag protein ELISA data, the prevalence of FFV infection in cats in Germany was determined for the first time. Furthermore, we screened for neutralizing antibodies in the sera from FFV positive cats. We show that epitopes targeted by cat sera are similarly located as conserved epitopes recognized by antibodies neutralizing gammaretroviruses or HIV-1. However, attempts to induce antibodies neutralizing FFV in immunization studies with the recombinant TM in a goat and rats were not successful. This is similar to the situation with HIV-1 but in contrast to the situation with gammaretroviruses, where neutralizing antibodies were easily induced.

## **Results**

### **Expression and purification of recombinant TM protein**

In order to express the TM protein of the FFV-FUV7 isolate, an expression plasmid was generated fusing the codon-optimized sequence of the ectodomain of the FFV TM protein to a glutathion S-transferase (GST) and a C-terminal His tag (Fig. 1A) in the prokaryotic expression vector pGEX-KG (Guan and Dixon, 1991). Testing several other vector systems and tags, this was the only construct to stably express the full-length protein. Since initial expression levels were low, a systematic optimization of the expression conditions using a 96-deep well expression screen was performed. Most suitable bacterial strains, media, IPTG concentrations and temperatures were selected (Mühle et al., in preparation). Since even under mildest expression conditions most of the protein was found expressed in inclusion bodies, resolubilization and binding to the NiNTA-affinity matrix was performed under denaturing conditions followed by a gradual replacement of the denaturant during the washing steps and subsequent elution. The purified protein had a purity of about 95% and was recognized by anti-His antibodies (Fig. 1B) and anti-GST antibodies (not shown). The produced protein was used in ELISAs, Western blot assays and for the immunization of a goat and rats described in this study.

### **ELISA setup and screening of cat sera for antibodies against the TM protein**

To investigate whether naturally infected cats produce antibodies specific for FFV TM, an ELISA was set up using the purified TM protein and optimal conditions including blocking reagents and antigen concentrations. Low background reactivity was achieved blocking with PBS containing 1% dried skim milk and 0.05% Tween 20; 200 ng TM protein per well was selected as standard antigen concentration and a serum dilution of 1:100 was chosen for all assays. In an initial screen, 261 cat sera with unknown infection status were investigated (Fig. 2A). As positive control, serum from goat 348 immunized with the FFV TM protein, as negative control the corresponding preimmune serum were used. 107 (41%) of the sera reacted positive in the ELISA with the TM protein. The ELISA results were confirmed in a Western blot assay using the same recombinant TM protein (Fig. 2B).

### **Comparison of FFV-TM ELISA results with results of established Gag and Bet ELISA**

To evaluate the TM protein based assay, the same sera were tested in well-established ELISA based on the Gag and the Bet proteins of FFV as described previously (Romen et al., 2006). 39% of the sera were positive for Gag and 33% for Bet (Bleiholder et al., submitted). Comparison of all three assays showed a clear correlation between the sera positive for antibodies against the TM protein and the sera positive for antibodies against the Gag and Bet proteins. ELISA results were confirmed in Western blot assays using FFV-infected CRFK cells as antigen (Bleiholder et al., submitted). The cut off in the ELISA using the FFV TM protein was calculated using twice the mean value plus three

standard deviations of 100 Gag-negative sera. Using this threshold, nine sera were positive for TM only. In addition, two sera showed ELISA reactivity against Gag (Fig. 3A) and Bet (not shown) but not the TM protein (Fig. 3A). Four of the nine sera positive for TM only were confirmed to be positive by Western blot analyses using recombinant TM protein, but none of these sera showed the characteristic 52 and 48 kDa Gag pattern in the Western blot assay using antigens from the FFV-infected cells (Fig. 3). A Western blot analysis of one of these four sera (serum 34713) is shown in Fig. 3B. Since the ELISA results of the five remaining sera were not confirmed by Western blot testing, these cats were considered not to be infected. Of the two sera positive only for Gag and Bet, both were positive in Western blots using FFV-infected cells, but not for the TM protein in a Western blot analysis (Fig. 3). Therefore, the prevalence of the FFV infection in the German cats was calculated to be 39%. In a second, smaller screen, another 143 sera were tested with the TM protein based ELISA and consistent with the previous finding, 41% of them were found to be positive for antibodies against the TM protein.

### **Epidemiology of FFV infection in German cats**

Analyzing age, sex and castration status of the animals, a strong correlation of the infection status with the age of the infected animal was found (Fig. 4). Significant differences in the infection rate were found when very young and older cats were compared. Whereas only 12.5% of the animals in the age of 0–1 year were FFV positive, already 42% were infected in the group of 1–2 years (P-value 0.001). The prevalence increased up to 75% for cats older than 12 years (Fig. 4A). Comparison between male and female cats and their castration status did not reveal any significant differences (P = 0.2, P = 0.3 and P = 0.2, respectively, Fig. 4B) but some influence of the castration status itself was measurable as an increase of FFV infections in desexed animals (47% compared to 34% in non-desexed animals, P-value 0.05, Fig. 4C).

### **Analysis of binding and neutralizing antibodies in the sera from infected and immunized animals**

To estimate the titers of TM binding antibodies in infected cats, ELISAs using recombinant TM protein and serial sera dilutions were performed. In average, titers of TM binding antibodies ranging from  $0.8 \times 10^2$  to  $2 \times 10^4$ , medium  $3 \times 10^3$ , were measured (Table 1). To detect neutralizing antibodies, ELISA positive sera were analyzed in neutralization assays using FeFAB indicator cells and the FFV–FUV7 isolate and an improved 96 well protocol (see Material and methods). In a panel of 90 sera tested, 13 were found to efficiently neutralize at serum dilutions of 1/200 and higher. To confirm the specificity of the antibodies neutralizing FFV, an HIV-1 specific neutralization assay using TZM-bl reporter cells and HIV-1 strain pNL-4.3 was performed and no neutralization observed. When the sera from goat 348 and rats immunized with the recombinant FFV TM protein were investigated, all were strongly positive in ELISA and Western blot assays using recombinant TM protein with and without the GST fusion part with titers in the range of  $10^5$  to  $10^6$ , indicating that the antibodies are directed against the TM protein. When the sera from immunized animals were analyzed for the presence of FFV-neutralizing antibodies using the FeFAB indicator cell assay and FFV–FUV7, none of the sera could prevent the infection.

### **Characterization of the antibody response by epitope mapping**

In order to identify the epitopes recognized by the sera and purified IgG from naturally FFV-infected cats and to compare them with those recognized by the sera from goat 348 and the rats immunized with the TM protein, overlapping peptides corresponding to the ectodomain of the FFV TM protein were used (Figs. 5A and B). This method identified several epitopes (between three and seven), with the most obvious difference that in the case of the sera from infected cats epitopes were missing in the unusually prolonged central domain between NHR and CHR, which contains eight cysteine residues (Fig. 5C) and is predicted to be highly glycosylated (Wang and Mulligan, 1999). In contrast, the sera from goat 348 and from rats immunized with the recombinant FFV TM protein recognized epitopes mainly in this central region, independent from the immunized species. Whereas all the sera from the FFV infected cats recognized epitopes in the MPER and the fusion peptide proximal region (FPPR), the sera from the immunized did not recognize the epitopes in this region.

## **Discussion**

Here we show that cats infected with FFV build up a strong immune response against the viral TM protein and that this antigen can be used to detect FFV infections in cats. In comparison with the results of previously established assays using the group specific antigen Gag and Bet, similar results were obtained when a large population of German cats was screened for FFV infection. Approximately 39% of the animals were found infected. This prevalence is in line with findings of other European areas ([Bandecchi et al., 1992], [Daniels et al., 1999], [Winkler et al., 1997], [Winkler et al., 1999] and [Romen et al., 2006]). Epidemiological data showed that the percentage of infected animals increases with age, and that infection is predominantly acquired after the first year of birth. This time point correlates with sexual maturity, where cats start roaming and get in contact with other cats, increasing the probability of infection. Interestingly the number of animals infected with FFV seemed higher among the desexed animals when compared with the non-desexed. This may be explained by toleration of straying of desexed rather than non-desexed cats by the pet holder, or differences in social behavior caused by the castration itself. Winkler et al. (1999) also reported a higher number of positive animals among the desexed, but only among young animals (less than 5 years) and not among older. In contrast to what has been described for FIV, no differences in the infection rate between male and female cats was found, suggesting a different way of transmission such as prolonged intimate contact instead of aggressive behavior (Winkler et al., 1999).

In order to diagnose a retroviral infection besides virus isolation and PCR provirus detection, sensitive and specific antibody detection methods may be used, since infections with retroviruses are always accompanied by antibody production. Among the immunological detection assays such as Western blot analysis, immunofluorescence and others, ELISAs have numerous advantages: the assay is quick, sensitive, reproducible, cheap and easy to use for screening large quantities of sera. Therefore an ELISA using the TM protein as antigen was established. This assay gave similar results compared with a well-characterized ELISA based on the Gag protein (Romen et al., 2006). Upon screening 261 sera in both assays, there were 11 sera with unclear results, nine detected only the TM protein, two only the Gag protein. As in the case of the HIV diagnostics, Western blot assays were performed to confirm the results, using either the recombinant TM protein or FFV-infected CRFK cells expressing predominantly the Gag. Both assays showed a good correlation with the ELISA results with exception of the nine sera described (Fig. 3). Due to the lack of appropriate additional material (e.g., blood or tissue samples) non-serological analyses such as PCR or virus reisolation could not be performed. Additional investigations should clarify such results. From our point of view, it should be evaluated whether a combined assay using both the Gag and the TM protein may be used for future screening. Since serological testing for HIV-1 infection gave some false-positive responses to HIV-Gag, strong criteria had been developed requesting confirmatory Western blot assays that show antibodies against one of the envelope proteins on one hand and the Gag on the other hand (Sayre et al., 1996).

Previous reports showed that antibodies against the surface envelope protein of the foamyvirus FFV are rare in infected cats (Romen et al., 2006). This is in contrast to the situation in animals infected with orthoretroviruses, which produce high amounts of antibodies against their surface envelope protein, e.g., gp120 in HIV-1 infected individuals. The reasons for that discrepancy are still unclear, one explanation may be the presence of non-infectious, capsidless subviral Env-particles or the release of Env-Bet or Env-Bel2 fusion proteins that lack the membrane-spanning domain of TM in animals infected with FFV ([Bodem et al., 1998], [Pietschmann et al., 2000] and [Shaw et al., 2003]). These subviral particles or soluble Env derivatives may act as decoys adsorbing antibodies specific for the surface envelope protein, but not the antibodies against the TM protein, which is hidden under the surface protein. In contrast to foamy viruses, orthoretroviruses are unable to produce Env-Particles, but they produce particles composed of Gag without Env (Luttge and Freed, 2010).

Different epitopes were identified when the sera obtained by immunization with the ectodomain of the TM protein of FFV and the sera from infected cats were analyzed (Fig. 5). The sera from immunized animals (independent whether goat or rats) recognized epitopes in the region characterized by potential glycosylation sites ([Wang and Mulligan, 1999] and [Luftenegger et al., 2005]). This may be explained by the fact that the administered antigen was the non-glycosylated TM protein produced in bacteria. In contrast, the sera from infected cats do not recognize these epitopes, but mainly the epitopes outside the glycosylated region (Fig. 5C). These data indicate that in the infected animals the epitopes may be hidden by glycosylation ([Lindemann and Goepfert, 2003] and [Luftenegger et al., 2005]) and antibodies against the protein backbone were not produced. Since for the epitope mapping

synthetic peptides and for ELISA and Western blot analyses recombinant proteins produced in bacteria were used, we cannot rule out the possibility that antibodies against the glycosylated protein are generated. The sequences of the epitopes recognized by the sera from infected animals are highly conserved when different isolates were compared, suggesting that there is no variability due to escape mutations.

The sera from goat 348 and the four rats immunized with the TM protein did not inhibit FFV infection, indicating that the antigen in the applied form did not induce neutralizing antibodies. We have previously demonstrated that neutralizing antibodies were induced by immunization with the recombinant ectodomain of the TM protein p15E of the porcine endogenous retrovirus (PERV) (Fiebig et al., 2003), the feline leukemia virus (FeLV) ([Langhammer et al., 2005], [Langhammer et al., 2006] and [Langhammer et al., 2010]) and the Koala retrovirus (KoRV) (Fiebig et al., 2006) and that main epitopes recognized were located in the FPPR and in the MPER of the corresponding TM proteins. The epitopes located in the MPER of the TM protein of these gammaretroviruses (FeLV, PERV, and KoRV) resembled epitopes located in the MPER of the TM protein gp41 of HIV-1 which are targeted by broadly neutralizing antibodies such as 2F5 and 4E10. These antibodies had been isolated from HIV-1 infected patients and they prevent infection of up to 95% of primary HIV-1 isolates. Furthermore, despite the evolutionary divergence between HIV-1 and the gammaretroviruses, three identical amino acids were found in the epitope of the antibody 4E10 neutralizing HIV-1 and the epitopes recognized by the sera neutralizing PERV, FeLV and KoRV ([Fiebig et al., 2003], [Langhammer et al., 2005] and [Langhammer et al., 2006], unpublished data). However, immunizations with the ectodomain of gp41 of HIV-1 did not result in neutralizing antibodies and no epitopes located in the MPER were found (unpublished data). Results similar to the findings obtained with HIV-1 were described here: the sera from goat 348 and the rats immunized with the TM protein of FFV were not neutralizing and did not recognize the epitopes close to the membrane spanning domain. In contrast, the sera from cats naturally infected with FFV were neutralizing and recognized the epitopes in the MPER. Obviously the large size, the high complexity and the strong glycosylation of the TM proteins of HIV-1 and FFV do not support the conformations able to induce neutralizing antibodies when immunizing with the entire ectodomain. Since the influence of oligomerization, glycosylation and conformation on the ability of the FFV TM protein to induce neutralizing antibodies is unknown, we first used the recombinant protein produced in the bacteria for reasons of cheapness and ease of expression. It remains the subject of further studies to clarify whether immunization with a glycosylated TM protein produced in eukaryotic cells will result in the production of neutralizing antibodies.

## **Materials and methods**

### **Protein production**

Expression vector pGEX-TMop-His containing the codon-optimized ectodomain of the feline foamy virus FUV7 strain (Uniprot Acc.-Nr. O56861, amino acids 563 - 944) was obtained by cloning of the XhoI digested Pfx-PCR fragment amplified from the expression plasmid pGEX-TMop using primers MM001 5'-aggatctggctctggatctggtatcgaggaagggatctgaacgatcagaaactg-3' and MM002 5'-atactcgagtagtgatggtgatggtgatggtgctgcggtgcaaaaattccacc-3' into SmaI/XhoI digested pGEX-KG. A C-terminal 8xHis tag was introduced using a corresponding reverse primer. The construct was transformed into *E. coli* BL21 and expression conditions were optimized. Induced cells were pelleted by centrifugation and frozen at -20° until needed. Purification was performed by resuspending pellets in Buffer A (6 M GuHCl, 100 mM NaCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM imidazol, 10 mM TrisHCl, Complete EDTA free protease inhibitor, and pH 7.0) and thoroughly sonification. After centrifugation (10.000 xg, 20 min) cleared lysate was loaded on a HisTrap FF Crude NiNTA-column integrated in an automatic chromatography system (ÄktaExplorer 10 S, GE Healthcare), the column was washed intensively with buffer B (8 M urea, 100 mM NaCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 80 mM imidazol, 10 mM TrisHCl, and pH 7.0) followed by a 20 column volume refolding gradient to Buffer C (1 M urea, 100 mM NaCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 80 mM imidazol, 10 mM TrisCL, 1 mM β-ME, and pH 7.0) and elution with buffer D (1 M urea, 100 mM NaCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 500 mM imidazol, 10 mM TrisHCl, and pH 7.0). Protein concentration was estimated by measuring absorption at 280 nm or SDS-PAGE using a BSA standard dilution series.

## **ELISA**

Purified recombinant protein (100 µl/well) diluted in water at a final concentration of 20 ng/µl was adsorbed overnight at 37 °C in 96-well plates. Plates were washed once with PBS-T (phosphate buffered saline, 0.05% Tween 20) and blocked with blocking buffer (1% non-fat dry milk in PBS-T). Sera (1:100 in blocking buffer) were added and incubated for one hour at 37 °C. After washing HRP-conjugated anti-cat IgG (Bethyl Laboratories, 1:3000) were added. Detection was performed as described (Denner et al., 1994). The GST capture ELISA to measure antibodies specific for Gag and Bet were performed as described (Romen et al., 2006).

## **Sera and immunoglobulins**

The sera from household and street cats were obtained from LABOKLIN GmbH (Hannover, Germany), a laboratory for clinical diagnostics. All sera were collected between July and August 2009. Goat #348 and four rats were immunized three times with 500 or 250 µg purified GST-TMop-His in oily adjuvans (1:1) i.m and s.c. in a three week interval following internationally recognized guidelines under approval of the Lageso, Berlin, reference numbers H0028/03 and H0201/02. IgGs were isolated from the sera using a Protein G based purification kit (Ab SpinTrap IgG Purification Kit, GE Healthcare) as recommended by the manufacturer.

## **Western blot analysis**

Western blot analyses using the TM protein (Tacke et al., 2001) or FFV infected CRFK cells (Winkler et al., 1998) were performed as described.

## **Neutralization assay**

An indicator cell line based assay (Zemba et al., 2000) was modified using 96 well plates and an ELISPOT reader. Briefly,  $2 \times 10^4$  FeFAB cells, were seeded into each well one day before infection and supplied with 50 µl fresh DMEM containing 0,5 µg/µl G418 on the day of the assay. 25 µl of each serum dilution (1:50, 1:100, 1:200 and 1:400) and 25 µl FFV containing supernatant ( $3 \times 10^4$  infectious particles/ml) were incubated for 30 min at 37 °C, added to the cells and incubated for two days. To test for antibodies neutralizing HIV-1,  $2 \times 10^4$  TZM-bl cells and 25 µl HIV-1 pNL-4.3 ( $0.63 \times 10^4$  infectious particles/ml) were used. To determine the number of infected cells, medium was removed; cells were washed twice with PBS, fixed for 10 min with 2% paraformaldehyde and after two additional washing steps staining was performed using X-gal containing staining solution. Blue colored cells were counted in an ELISPOT reader (AID, Strassberg, Germany). Neutralization titers were expressed as serum dilutions that showed more than 75% reduction of plaque forming units compared to the infected control. All measurements were performed in triplicates.

## **Epitope mapping**

Pepspot libraries comprising the ectodomain of the FFV TM protein as 15-mer peptides overlapping by 13 amino acids were used to identify epitopes in the described sera. Membranes were saturated for at least two hours in TBS-T (Tris-buffered saline, pH 7.5, 0.05% Tween 20) containing 5% dry milk powder and then incubated with Protein G purified IgGs (Ab Spin Trap, GE Healthcare) from goat (1:800), rat (1:800) and cat sera (1:100), washed three times for 10 min with TBS-T and then incubated for 2 h with an appropriate peroxidase-conjugated secondary antibody diluted 1:3,000. Binding was detected using a chemiluminescence detection solution (ECL, Amersham Pharmacia Biotech). Core epitopes were defined as the centrally shared amino acids of overlapping peptides recognized with identical signal intensity.

### **Statistical analysis**

To evaluate the statistical significance between selected groups, Pearson's Chi-Square test was used. P values of 0.05 and less were considered significant.

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## Tables and Figures

**Table 1.** Sera characterized by epitope mapping.

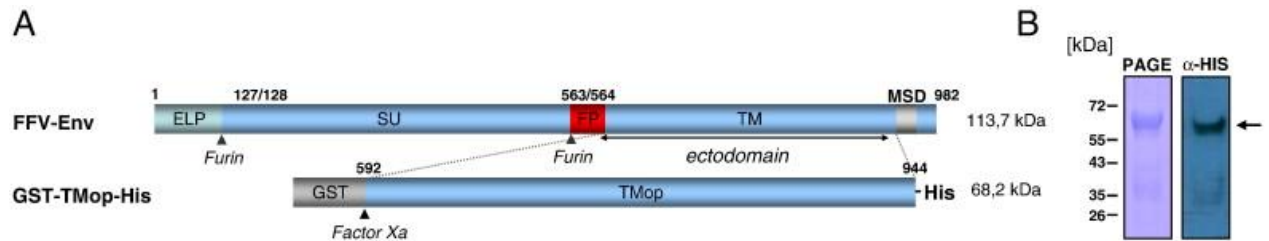
Serum	Sex of the animal	Age of the animal <sup>a</sup>	Antibody titre	
			ELISA	Neutralization
			FFV-TM <sup>b</sup>	FFV-FUV7 <sup>c</sup>
Goat 348	Female	n.a.	$1,0 \times 10^6$	<10
Rat 321-2	Female	n.a.	$5,0 \times 10^5$	<10
Rat 321-3	Female	n.a.	$1,4 \times 10^6$	<10
Cat 40202	Female	14	1280	<10
Cat 49499	Female	3	2560	<50
Cat 44093	Male	8	320	<50
Cat 56503	Male	6	1280	<50
Cat 30623	Female	13	20480	200
Cat 47831	Male	3	80	400
Cat 35051	Male	n.a.	2560	>400
Cat 41746	Male	14	640	>400

<sup>a</sup> Age of animal in years, n.a. — no information available.

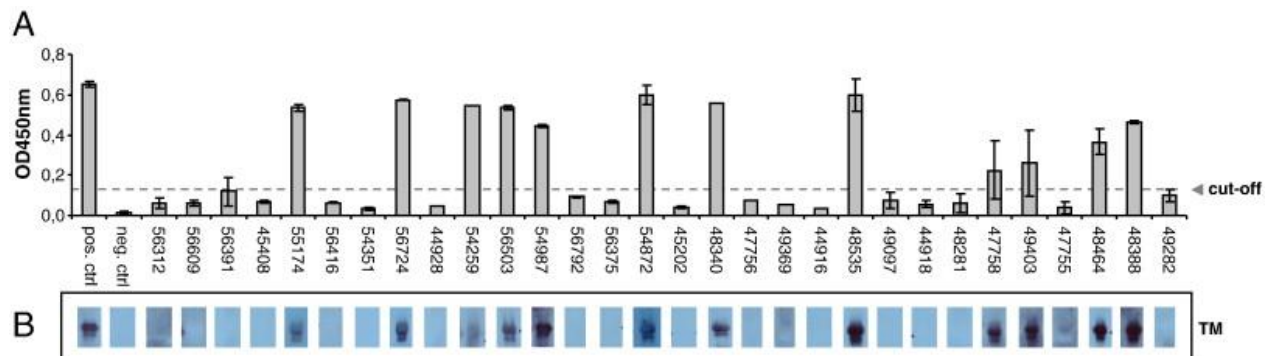
<sup>b</sup> Reciprocal serum dilution reacting positive (signal above  $2 \times (\text{OD}_{490 \text{ nm}} \text{ of median preimmune serum} + 3 \text{ standard deviations})$ ) in an ELISA using FFV-TM.

<sup>c</sup> Reciprocal serum dilution resulting in more than 75% reduction of X-Gal staining in a neutralization assay.

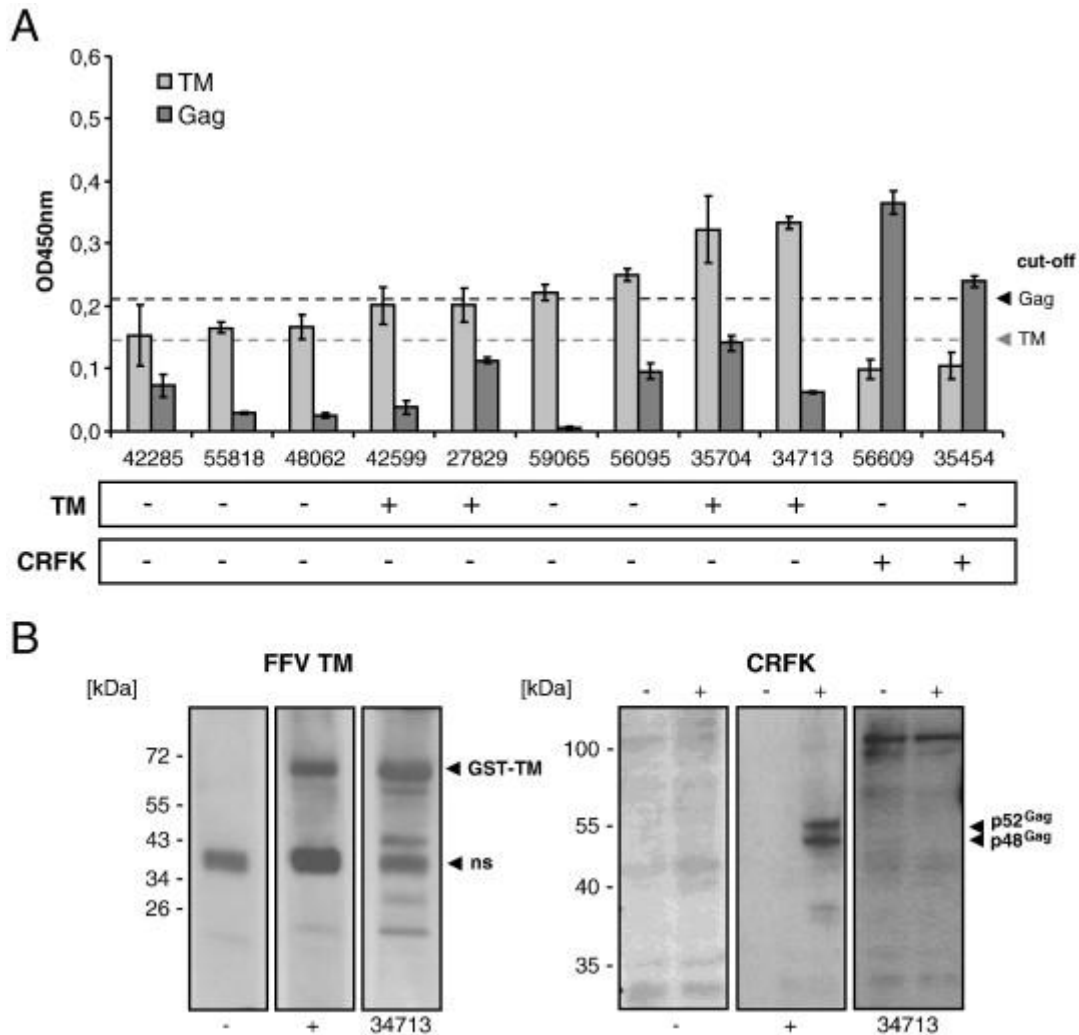
**Figure 1.** Expression, purification and characterization of the TM protein of FFV. (A) Schematic presentation of the envelope protein of FFV containing the envelope leader protein (Elp), the surface envelope protein (SU), the transmembrane envelope protein (TM) and furin cleavage sites indicated by arrows. The TM protein is further characterized by the fusion peptide (FP) and the membrane spanning domain (MSD). The ectodomain of the TM protein was cloned with a GST and His tag, the arrow indicates the factor Xa cleavage site used for the removal of the GST residue. (B) Characterization of the purified TM protein by SDS-PAGE and a Western blot assay using anti-His tag antibodies. The arrow indicates the TM protein with a predicted molecular weight of 68 kDa.



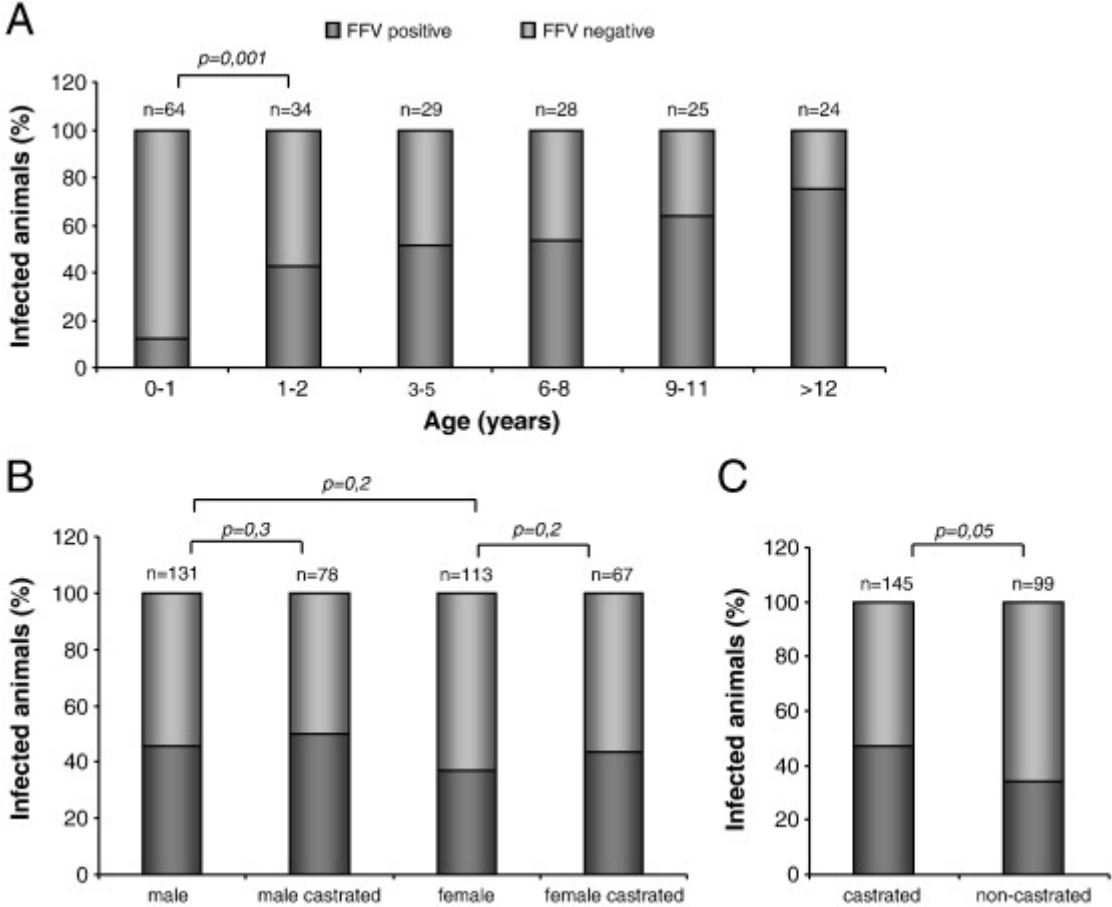
**Figure 2.** Screening of cat sera for antibodies against FFV. (A) Representative results of an ELISA using the TM protein and the sera from cats with unknown serological FFV infection status; the calculation of the cut off is described in the text. (B) Testing of the same sera in a Western blot assay using the same TM protein. The sera used in the ELISA were diluted 1:100, in the Western blot assay 1:1000.



**Figure 3.** Confirmatory tests of the sera recognizing only FFV TM protein or only FFV Gag. (A) In the top panel, ELISA results using the TM and Gag protein as antigens and the corresponding cut offs are indicated. Below, the results of the Western blot analyses of the same sera using the recombinant TM protein (TM) or FFV-infected CRFK cells (CRFK) as antigen are shown; (+) indicates a positive result, (-) a negative result in the Western blot analyses. (B) Western blot results of representative serum 34713, a positive serum from an infected cat (+) and a negative serum from an uninfected cat (-) using recombinant FFV TM protein (left figure) and infected CRFK cells as antigen (right figure). Arrows indicate the position of recombinant GST-TM protein, a commonly detected non-specific band (ns), and the characteristic 52 and 48 kDa Gag bands of FFV. In the right figure (-) indicates uninfected and (+) infected CRFK cells.



**Figure 4.** Prevalence of FFV antibodies in dependence on the age of the animals. Animals were stratified by age, sex, castration status and the percentage of the sera positive in a TM protein based ELISA, the total number of investigated animals and the P values are given.



**Figure 5.** Epitope mapping of the sera from animals immunized with the TM protein of FFV and from cats infected with FFV. (A) Representative epitope mapping of the goat serum 348. The central box shows a membrane spotted with overlapping peptides corresponding to the entire ectodomain of the TM protein after incubation with goat serum 348 and development with secondary antibody as described in Materials and methods. The sequences of the positive peptides were shown and the corresponding epitopes are framed. (B) The sequence of the TM protein of FFV (accession number 056861) and sequences of the epitopes recognized by all sera. (C) Schematic presentation of the TM protein of FFV showing the location of the fusion peptide (FP), the fusion peptide proximal region (FPPR), the membrane proximal external region (MPER) and the membrane spanning domain (MSD). The location of the predicted glycosylation sites (Y) and the cysteine residues (C) is indicated. In the lower part the localization of the epitopes recognized by the sera from immunized and infected animals is shown.

