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Seroprevalence of feline foamy virus in domestic cats in Poland

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Abstract

Introduction: Feline foamy virus (FFVfca) is widespread and its prevalence in naturally infected domestic cats ranges between 30% and 80% worldwide. The infection is persistent, with a sustained antibody response in FFVfca-positive cats; however to date, no defined disease or clinical symptoms have been proved to be associated with it. The goal of the presented study was to determine the prevalence of FFVfca infection in domestic cats in Poland. **Material and Methods:** A total of 223 serum samples collected from domestic cats were tested with a glutathione S-transferase capture ELISA test to detect antibodies specific to capsid (Gag), accessory (Bet) and envelope (Env) FFVfca antigens. A Western blot test was used to confirm the ELISA results. **Results:** The cut-off value for the Gag antigen was established by calculation and evaluation with the immunoblotting assay. The cut-off values for Bet and Env were calculated from the reactivity of Gag-negative samples. The sera of 99 cats (44%) showed reactivity to Gag, those of 80 did so (35.9 %) to Bet, while only 56 samples (25%) were reactive to Env. Only 51 (22.9%) sera were positive for all antigens. The main diagnostic antigen was selected to be Gag. A statistically significant association was found between FFVfca status and the age of the cat. **Conclusions:** This study proved the high seroprevalence of FFVfca in domestic cats in Poland for the first time and confirmed that adult cats are at higher FFVfca infection risk than preadult cats. Its results correspond to those reported from other countries.

Keywords: feline foamy virus, domestic cats, ELISA, seroprevalence.

Introduction

Foamy viruses (FVs), also known as spumaviruses, belong to the *Retroviridae* family. Unique features in their replication cycle and a complex genomic organisation classify them into the *Spumaretrovirinae* subfamily (16, 21). Foamy viruses infect monkeys and other primate species, cattle, horses and cats (15). Feline foamy virus (FFVfca) was first isolated in 1969 (32) and since then it has been found to be broadly distributed worldwide in cat populations. Different studies from Europe, Australia, North and South America and Asia reported FFVfca prevalence ranging from approximately 30% to even 100% in domestic cats depending on sex, age and geographic region analysed (2, 3, 4, 5, 8, 13, 25, 30, 39). FFV-like viruses were also found in wild feline species and were detected in 35% of one such endemic to the Japanese island of Iriomote (*Felis iriomotensis*) and in one leopard cat species (*Felis bengalensis*) from Vietnam (24, 29). Recent studies using FFVfca antigens confirmed nearly 80% FFV seroprevalence in free-ranging pumas (*Puma concolor*) (14) from different geographic regions of the US. Many attempts have been made to find any link between FFVfca prevalence and demographic variables which could help to determine the main infection risk factors. While no consistent association with gender has been proven, greater prevalence was linked with advancing age (3, 25, 388), which is consistent with the studies on other FVs. Similarly to simian foamy viruses, FFVfca is mainly transmitted through close social or aggressive interactions among animals, such as grooming, licking and biting (38), which is supported by the oral mucosa having been confirmed as the main site of active FFVfca replication in domestic cats (4). The infection is persistent in cats and infected individuals have a sustained

antibody response (38). Several studies of naturally infected cats or individuals experimentally infected with FFVfca attempted to find a correlation between FFVfca infection and any pathology (1, 35, 36), or an association with some rare cat diseases such as feline polyarthropathy (28) or uncharacterised renal symptoms (20, 36, 38), but all of them have found the virus to be apathogenic in domestic cats. Furthermore, a putative co-factorial role of FFVfca in other viral infections of cats such as feline immunodeficiency virus (FIV) or feline leukemia virus (FeLV) has not been confirmed yet (2, 8, 388, 42).

In many hosts where FVs are prevalent, serum antibodies as well as neutralising antibodies against viral proteins have been associated with FV infection (10, 11); therefore, the diagnosis is mainly based on serological tests, while molecular methods are used rather for phylogenetic studies, especially those on interspecies transmission of simian foamy viruses to different primate species and humans (4, 31, 34). Serological methods like neutralising tests and immunoblots were explored in FFVfca research in the past, while currently the ELISA method described by Romen *et al.* (33) in 2006 remains the assay of choice not only in cats but also in wild feline species such as pumas (6, 13, 14).

Our study aimed to investigate the seroreactivity of domestic cats from Poland for FFVfca antigens using sera collected from three distinct regions of the country. Additionally, we tried to investigate the association of FFVfca seroreactivity with selected demographic variables.

Material and Methods

Animal samples. Blood samples were collected from 223 cats in four veterinary clinics located in three agglomerations (Warsaw, Gdańsk and Kraków) and included 110 samples from Warsaw, 78 from Gdańsk and 35 from Kraków. Serum samples were obtained through centrifugation of blood samples, aliquoted and frozen at −20°C. Health status and demographic information was available only for the 113 cats from Gdańsk and Kraków and included age, gender, breed and the outdoor activity of the animals in the context of contacts with other cats. Most of the cats were healthy, of domestic short-haired cat breed, and had unknown status for FeLV and FIV; therefore, these variables were not considered in further analysis.

Serological study. GST capture ELISAs were performed as previously described (33). Briefly, 96-well plates were coated with glutathione casein, pre-adsorbed with blocking buffer (0.2% (w/v) casein in phosphate buffered saline, 0.05% (v/v) Tween-20) and then 100 µL of cleared *E. coli* BL21 lysates containing the GST-tag or GST-X-tag fusion proteins (X = capsid (Gag), accessory protein (Bet) or envelope (Env) corresponding to the surface unit with envelope leader peptide (ElpSU) of the FUV-7 serotype) (33) were added for 0.25 µg total protein in blocking buffer. Cat sera were pre-incubated in blocking buffer containing 2 µg/µL total protein from GST-tag-expressing *E. coli* BL21 at a dilution of 1:50. Pre-adsorbed sera were incubated for 1 h at room temperature in the coated plates, washed and incubated for 1 h at room temperature with Protein A-peroxidase conjugate (Sigma-Aldrich, Saint Louis, USA). Substrate reaction and quantification were carried out following a previously described procedure (33). All incubations were performed with a volume of 100 µL per well. For each serum sample, absorbance of the GST-tag was determined and subtracted from the absorbance with the GST-X-tag protein to calculate the specific reactivity to the FFVfca antigens. Optical density (OD) was measured with a 450 nm filter in duplicate and antibody levels were expressed as average netOD values. Sera from experimentally FFVfca infected domestic cats (positive samples nos 8013 and 8017)) or uninfected domestic cat (negative sample no. 8016) (1) were used as controls at dilution 1:50.

Cut-off values were calculated from the $netOD_{450}$ Gag values of all 223 samples as $2 \times (mean_{Gag} + 3\ SD)$. Positive outliers were excluded and the procedure was repeated until the calculated cut-off value no longer changed after their exclusion. The Bet and Env cut-off was calculated from the group of Gag-negative sera as $2 \times (mean_{BetorEnv} + 3\ SD)$ (33).

Immunoblotting. Total cell lysates of Crandell-Rees feline kidney cells (CRFK) infected with FFVfca and uninfected control cells of the same line were prepared as 10 µg aliquots and separated by SDS-PAGE; these served as the antigen for Western blotting analyses (3). Cat sera were used at 1:100 dilutions (v/v in 0.1% bovine albumin, 0.01% Tween 20, Tris-buffered saline) and Protein A-peroxidase conjugate at 1:5,000 dilution. A pool of serum samples from FFVfca-infected cats was used as a positive control at dilution 1:100. The chromogenic peroxidase substrate 4-chloro-1-naphthol (Sigma-Aldrich, Saint Louis, USA) was used for the detection of specifically bound antibodies.

Statistical analysis. Scatter plot analyses were performed to calculate linear correlations of the $netOD_{450}$ values obtained for the Gag, Bet and Env antigens in ELISA tests. The data were analysed statistically by chi-squared test for detection of any association between FFVfca seroreactivity and age, gender and contact variables. In statistical tests, P values of less than 0.05 were considered to represent a significant association. Calculations were made and graphs generated using MedCalc Statistical Software version 20.014 (MedCalc Software, Ostend, Belgium).

Results

The distribution of the individual reactivities showed that no clear distinction of FFV Gag-positive and negative reactivity was apparent (Fig. 1), as was also previously reported (3). Therefore the cut-off value for

Gag was newly calculated as described in the previous section. To confirm the calculated cut-off values for Gag ($netOD_{450}$ = 0.199), selected sera were used for immunoblot analysis. In total, 22 cat sera were tested by immunoblotting with cellular antigen of CRFK cells infected with FFVfca. The tested samples were those displaying Gag reactivity around and above the statistically calculated Gag cut-off value as well as clearly FFVfca-positive and -negative sera as determined by ELISA. Uninfected CRFK cell lysate was used as a control. Cat sera clearly positive in ELISA were also positive in immunoblot, since the FFVfca Gag proteins (52 kDa precursor and 48 kDa processed Gag) were detected (Fig. 2); similarly ELISA-negative sera did not react with FFVfca antigen in immunoblot.

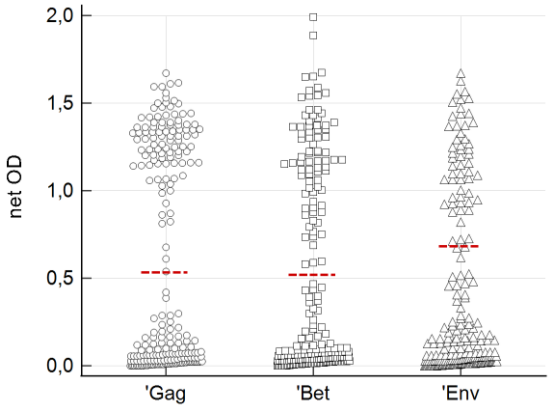

Fig. 1. Distribution of feline foamy virus Gag, Bet and Env antigens seroreactivity in 223 domestic cats from Poland. Dashed red lines indicate determined cut-off values

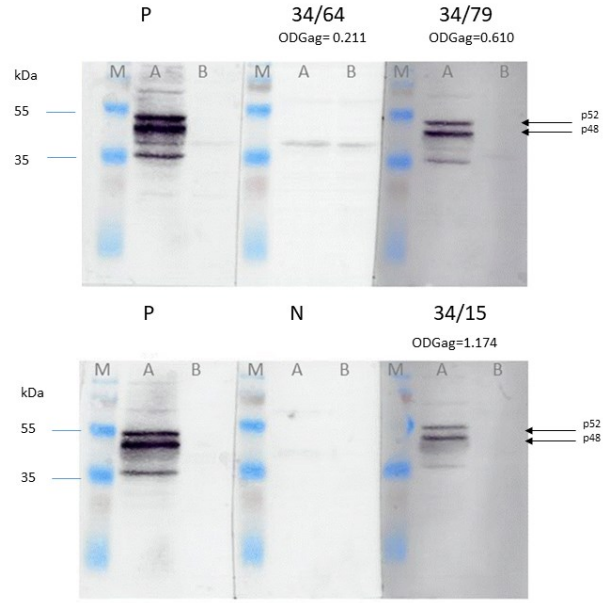

Fig. 2. Detection of FFVfca-specific antibodies by immunoblotting assays with a cellular antigen in representative feline serum samples. M – prestained protein ladder; A – lane with Crandell-Rees feline kidney cells (CRFK)/FFVfca cells lysate as antigen; B – lane with uninfected CRFK cells lysate as antigen; P – FFVfca positive control serum; N – FFVfca negative control serum; 34/64, 34/79, 34/15 – representative samples with their ELISA reactivity to FFVfca Gag antigen (OD Gag)

However, there were 10 serum samples which had been positive in ELISA (net OD_{450} from 0.211 to 0.421) but which showed no reactivity with FFVfca Gag proteins in immunoblot. Sera with $netOD_{450}$ equal or higher than 0.539 were also positive in immunoblot assay. Such re-evaluation resulted in a Gag cut-off value of $netOD_{450}$ = 0.539 with sera at or above this value being Gag positive and sera below this value being scored Gag negative (Fig. 1). This new cut-off clearly distinguished a group of low-level Gag-reactive sera that were negative in immunoblotting from those that were Gag positive in both tests (Fig. 1). Previous reports confirmed that Bet is not consistently detectable in cat sera by immunoblot assays (1, 3), and therefore we could not use the same procedure as for Gag antigen to experimentally determine the Bet cut-off value. Thus, we calculated the cut-off for Bet from Gag-negative sera as $netOD_{450}$ = 0.517. The cut-off for Env antigen was calculated in the same way and was established as $netOD_{450}$ = 0.654 (Fig. 1).

Using the determined cut-off values, 100 out of the 223 domestic cat sera showed the presence of FFVfca antibodies, of which 99 reacted to Gag and 80 to Bet antigens, while only 56 did so with Env. Moreover, 80% of Gag-positive samples also reacted to Bet antigen, while only 55.5% with Env antigen. The scatter plot analysis confirmed a stronger correlation between the reactivities to Gag and Bet than between those to Gag and Env antigens (Fig. 3). Interestingly, only 51 out of 223 tested samples reacted to all three antigens. Only one serum sample showed reactivity exclusively to the Env antigen. These results seem to be concordant with those of previously reported studies, which showed that Gag was the antigen of choice for serological surveys of FV infections.

In summary, 44% of the examined domestic cat sera from Poland were positive for FFVfca. Among them, 35% represented cats from the Warsaw, 45% from the Gdańsk and 74% from the Kraków agglomerations (Fig. 4).

Since demographic information was available for 113 cats from Gdańsk and Kraków (Table 1, Fig. 5) a chi-squared test was used to analyse the association between FFVfca infection and variables such as age, gender and contact with other cats. A statistically significant association between the prevalence of FFVfca infection and the age of the cat was observed (χ^2 = 10.30, P = 0.0061). Only 30% of domestic cats under the age of 1 year tested positive for FFVfca. The seroprevalence of FFVfca was clearly higher in the two remaining age groups: among adult cats (1–10 years) 67% of individuals were assessed FFVfca positive and in the group of the oldest cats (over 10 years) 55% showed seroreactivity to FFVfca Gag. In domestic cats, no significant association was found between FFVfca infection and gender or between infection and contact with other cats (Table 1).

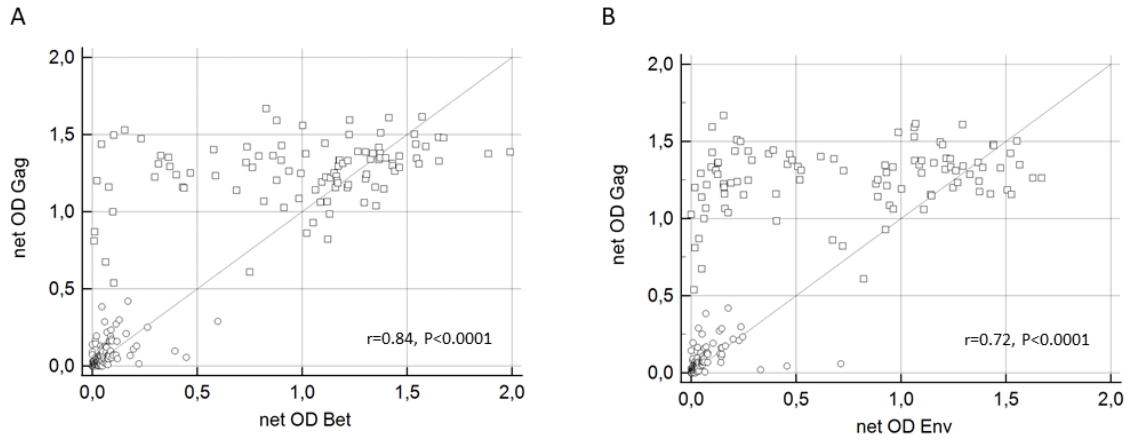

Fig 3. Scatter plot of ELISA results showing relationship between (A) feline foamy virus Gag and Bet and (B) Gag and Env antigens; correlation coefficients are indicated on both graphs. Circles indicate Gag ELISA negative and squares Gag ELISA positive samples

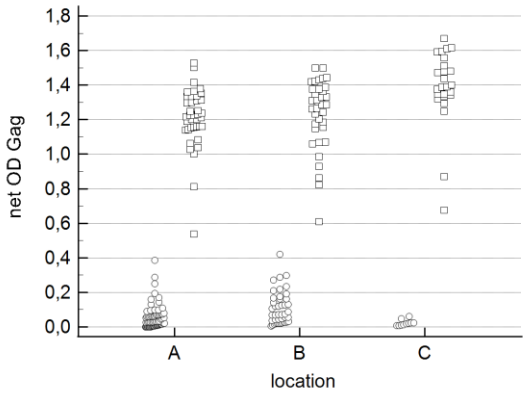

Fig. 4. Distribution of the cat serum samples' reactivity to feline foamy virus Gag antigen by animal origin. Black circles indicate Gag ELISA-negative and grey squares Gag ELISA-positive samples; A – Warsaw; B – Gdańsk; C – Kraków agglomerations

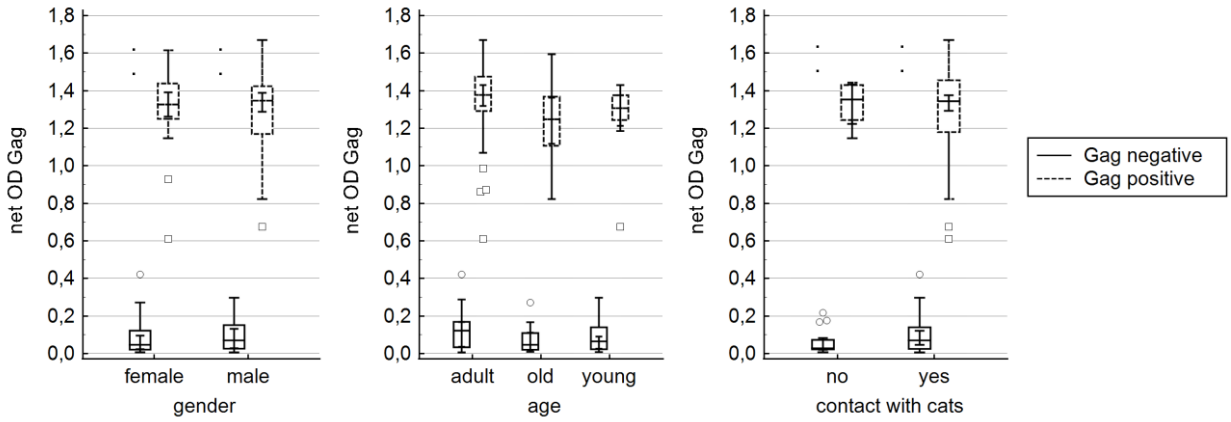

Fig. 5. Box-plot distribution of feline foamy virus Gag-seropositive and -seronegative cat samples from the Gdańsk and Kraków agglomerations analysed by variables

Table 1. Serological prevalence of FFVfca in domestic cat populations from the Gdańsk and Kraków agglomerations aligned with demographic variables

Variable		Number of cats	
		FFVfca seropositive	Total
Gender	Female	22	51
	Male	37	61
Age	Young (≤1 year)	10	22
	Adult (1–10 years)	33	49
	Old (>10years)	16	29
Contact with other cats	Yes	48	86
	No	10	23

Discussion

Serum samples from 223 domestic cats originating from three agglomerations were tested for the presence of FFVfca Gag-, Bet- and Env-specific antibodies using GST-capture ELISAs for the first time in Poland. In serological studies on FVs, Gag protein was acknowledged as the antigen of choice in the detection of infection with such viruses. Gag protein is known to be highly immunogenic and contains sequences which are conserved among different isolates of FFVfca, including two FFVfca serotypes identified due to differential neutralisation (7, 37). It has also been demonstrated that Gag protein elicited a strong and long-lasting immune response in all species infected by FVs even through interspecies transmission (1, 12, 177, 22, 377). Therefore, in our study FFVfca seroprevalence was determined based only on the seroreactivity of cat sera to Gag antigen. Such seroprevalence as determined, 44%, is in accordance with that found in previous studies in domestic cats in Australia, Vietnam, Germany, Switzerland and US showing 30–80% FFVfca-positive individuals depending on age, gender and geographic region (3, 8, 13, 24, 26, 33, 388). In a further similarity to previous studies (13), the rate of FFVfca-positive animals varied by location.

Similarly to the study reported by Bleiholder *et al.* (3), Gag reactivity of cat sera did not show apparent biphasic distribution to FFVfca-positive and -negative groups (Fig. 1), although such distribution was shown previously (33). Since we could not use the same cut-off (33), we re-calculated the FFVfca Gag cut-off using all 223 sera, but this approach failed to determine values allowing for reasonable distinction between positive and negative samples. As reported previously (3), the problems with clear cut-off determination may be due to differences in serum quality: the serum samples used in this study were collected and stored in different veterinary practices for some time before they were sent to the laboratory at the National Veterinary Research Institute, so some differences in their reactivity due to variant sampling procedures, storage conditions or shipping processes cannot be excluded. Nevertheless, to define the acceptable Gag cut-off value, we re-analysed 22 cat sera for their reactivity in an immunoblot assay with the cellular antigen of FFVfca. This analysis included the sera with reactivity in and above the range of the primary calculated cut-off of $netOD_{450}$ = 0.199, as well as sera evaluated as strongly positive or strongly negative in ELISA. This resulted in a newly defined cut-off of $netOD_{450}$ = 0.539. Although this cut-off clearly distinguished between Gag-positive and -negative sera, it was still over twice as high as the one defined previously by Bleiholder *et al.* (3). Therefore, we can suspect that there may be other factors influencing the reactivity of the sera in our ELISA assays. One possible explanation can be linked to the recent report from Moskaluk *et al.* (25) proving that sera from a population of domestic cats negative for FIV and FFVfca showed higher reactivity to the antigens specific to both viruses than the sera of virus-negative specific pathogen–free (SPF) cats. Additionally, sera from SPF cats experimentally infected with FIV or FFVfca showed some cross-reactivity with an antigen of the other virus. These findings suggest that antibodies produced during infection with off-target pathogens may non-specifically bind to diagnostic antigens in enzyme-linked immunosorbent assays, explaining the increased reactivity of some negative field samples. Indeed, 99% of the cats from our study were not tested for FIV and FeLV, so their serological status was unknown and it cannot be excluded that the antibodies specific to one of these or to some other cat pathogens influenced the ELISA reactivity.

Additionally to Gag, we also used Bet and Env antigens to test all cat sera. As previously observed (3), we also confirmed the stronger correlation between the reactivities to Gag and Bet than Gag and Env, however, both antigens reacted with clearly lower number of sera than the Gag antigen. Similar results were noted previously not only for FFVfca but also for other FVs (9, 23) and can be explained by the cessation of production of Bet protein after the productive phase of FVs infection and its diminution in the persistent one lessening its diagnostic value (33). The assay using the Env antigen was primarily developed in order to find an alternative to serotype-specific PCR and neutralisation assays (3, 377, 41) for distinguishing between FFVfca serotypes. Unfortunately, this antigen did not detect serotype-specific antibodies, probably due to its C-terminus fusion to the GST moiety, which alters the quaternary structure of the resulting fusion protein and limits the access of antibodies to the specific epitopes. This fact, of course, disqualifies Env fusion protein as the main diagnostic antigen but does not negate its supportive value, similarly to Bet antigen (18, 19).

In order to investigate the risk factors favouring FFVfca infection, we performed statistical analysis based on serological data and some demographic information available for most of the cats from the Gdańsk and Kraków agglomerations. Significant association of FFVfca infection with cat age was observed, which is consistent with the results of previous studies and supportive of the hypothesis that FFVfca infections are preferentially accumulated in cat populations through horizontal transmission (27, 38). In this context, the fact that no association was found between FFVfca infection and outdoor contact with other cats is quite surprising, especially since social contacts are considered to be the main route of FV transmission (15). However, it can be simply explained by the unequal size of the tested groups of cat sera having led to biased results. Additionally, in concurrence with previous reports from Australia, we also noted that FFVfca prevalence did not vary between genders (388). Interestingly, recent reports from the USA suggested that male cats are at higher risk of FFVfca infection;

however, since this observation was made in cat shelters in Colorado, regional variation or spurious association can be considered (13).

This study is the first report confirming FFVfca infections among cats in Poland. Using a GST-capture ELISA, we detected FFVfca-specific seroreactivity in 44% of cat serum samples. Additionally, we found a significant association between seroreactivity to FFVfca antigen and the age of cats, suggesting that adult animals have a higher probability of being infected with FFVfca. Since FFVfca has been associated with a higher risk of other retroviral infections, further epidemiological and clinical studies should be conducted to investigate the potential influence of infection with this virus on the health status of domestic cats.

Conflict of Interests Statement. The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement. The approval from ethics committee was not required according to national regulation (Act on the Protection of Animals Used for Scientific or Educational Purposes of 15 January 2015 published in the Journal of Laws of 2015, item 266). Informed approval was sought from the owners of the cats prior to commencement of sampling.

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