1 **Title: Feline leukemia virus in owned cats in Southeast Asia and Taiwan**

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17 **Abstract**
18 Feline leukemia virus (FeLV) is a retrovirus associated with fatal disease in cats with infection in its
19 progressive form. Although there are numerous reports on the occurrence of FeLV in the feline
20 population worldwide, there is a paucity of data on the associated infection in Asia. In this study,
21 we diagnosed FeLV infection by an ELISA-based test and a nested PCR assay in cats from different
22 countries in Southeast Asia (i.e., Thailand, Malaysia, Singapore, Philippines, Indonesia and Vi-
23 etnam) and Taiwan during 2017-2018. Overall 47 (7.7%) samples tested positive for FeLV with
24 prevalences ranging from 0 (Indonesia) to 22.7% (Thailand). A statistically significant association
25 ($p < 0.05$) was recorded between age, habitat variables, feline immunodeficiency virus serological
26 status, oral mucosa alterations, and FeLV antigenic and/or molecular positive results. A poor
27 agreement (K= 0.40; 95% CI, 0.20-0.59) between the ELISA test and nested PCR was found for the
28 diagnosis of FeLV infection. In-depth studies are needed in other countries in Southeast Asia to elu-
29 cidate the mosaic of knowledge about FeLV epidemiology, worldwide.

30

31 **Keywords**: cat; feline leukemia virus; blood; Asia

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Introduction

Feline leukaemia virus (FeLV) is an enveloped single-stranded RNA virus belonging to the family *Retroviridae*, genus *Gammaretrovirus*, occurring worldwide in felids, including domestic cats. Integration of a provirus DNA copy of the viral RNA in the cat's genome represents the molecular basis of virus persistence in its host (Sellon, 2012). Persistently viremic cats are a source of infection for other cats and being FeLV shed in saliva, nasal secretions, urine and faeces, transmission occurs via the oronasal route. The virus is mainly transmitted in feline communities though biting, mutual grooming, and sharing food bowls and litter boxes (Fromont, 1997; Pontier, 2009; Sellon, 2012) as well as through the placenta or lactation from the queen to kittens (Hartmann, 2012). Risk factors for FeLV infection include male gender, age, aggressive behavior and outdoor access (S. Gleich, 2009; Hoover, 1991; Sellon, 2012). FeLV infection may result in impaired bone marrow function with the development of cyto-proliferative (tumors) and cyto-suppressive (immunodeficiency, anemia) diseases with poor prognosis. However, the outcome of infection varies markedly on the basis of complex virus and host interactions, some of which needs to be elucidated (Hartmann, 2012). An unknown percentage of infected cats develops a strong immune response which prevents the spread of virus to target tissues (Hartmann, 2012). This "abortive infection" is likely to occur in healthy cats exposed to low doses of FeLV which is cleared without appearance of a viremia and antigenemia (Hartmann, 2012; Major, 2010). Alternatively, a partially effective immune response can control the infection after a primary viremia of a few weeks, but it does not prevent integration of proviral DNA in the host genome ("regressive infection"). Although latently-infected cats can terminate viremia, FeLV reactivation may occur (Hartmann, 2012; Sellon, 2012).

In the "progressive infection" cats are persistently viremic for a failure in the control of the infection, These cats are infectious for other cats and will develop FeLV associated neoplastic or non-neoplastic diseases (Hartmann, 2012; Sellon, 2012). "Focal infections" or atypical infections are characterized by a persistent local viral replication (e.g., in mammary glands, bladder, eyes). This replication can lead to intermittent or low-grade production of viral antigens (Hartmann, 2012; Major, 2010; Sellon, 2012), causing discordant results between the rapid antigen test and the molecular test results (Krecic, 2018; Lutz, 2009; Sellon, 2012; Westman, 2017; Westman, 2019).

To test the infection status of cats, point-of-care ELISA tests are widely used, which detect the p27 capsid protein in the blood (antigenemia). In order to confirm the ELISA results, or in case of false/non-interpretable results, antigenic assay should be followed by a confirmatory PCR test (Lutz, 2009; Sellon, 2012; Westman, 2017; Westman, 2019). Although overly sensitive and specif-

ic, the FeLV PCR test may give false negative results caused by mutations in the target region of the virus as it allows detection of regressive FeLV infections (Westman, 2019).
Reports of FeLV prevalence in cat population around the world are numerous (Burling, 2017; Chhetri, 2013; Hofmann-Lehmann, 2018; Levy, 2006; Little, 2009; Studer, 2019; Ueland, 1992; Westman, 2016) though no substantial data are available on the prevalence of FeLV in Asia. A recent study has highlighted the presence of high viral circulation in China, with prevalence up to 59.6% of the examined cats (Liu, 2020). On the other hand, there are no recent studies on the circulation of FeLV in Southeast Asia, with the exception of a study carried out on a limited number of stray cats in South Korea, where a prevalence of 12.1% was recorded (Hwang, 2016). A similar prevalence (12.2%) was reported in Malaysia (Bande, 2012), while a higher circulation (prevalence of 24.5%) in Thailand (Sukhumavasi, 2012). While in a Chinese study FeLV proviral DNA was searched for by molecular techniques, all other studies used ELISA detection of p27 (Bande, 2012; Liu, 2020; Sukhumavasi, 2012).
The aims of the present study were to estimate the proportion of FeLV-infected cats from different countries in Southeast Asia in 2017-2018 and the risk factors associated to FeLV infection by also assessing the diagnostic agreement between ELISA and nested PCR tests.

2. Materials and methods

2.1 Sample collection

A total of 609 blood samples were collected from a population of client-owned domestic cats during a multicenter study aiming to assess the diversity of endo- and ectoparasites in East and Southeast Asia carried out in 2017-2018 (Colella, 2020). Samples were collected in Thailand (n = 119), Malaysia (n = 46), Taiwan (n = 51), Singapore (n = 129), Philippines (n = 106), Indonesia (n = 43) and Vietnam (n = 115). Data regarding age, sex, reproductive status, breed, behavioral attitude, location of domicile and lifestyle were collected. Health status was evaluated from general physical examination, reporting abnormalities in rectal temperature, overall physical condition, body condition, nasal discharge, eyes, superficial lymph nodes, respiratory system (breathing), oral mucosa, skin/haircoat, and fecal consistency), while behavior was assessed based on cat demeanor as determined by the veterinarian. Feline blood samples (~2 ml) were collected in a tube with anticoagulant (e.g. EDTA, Sodium Heparin, Green-Top), stored and subsequently analyzed. After the bleeding, an aliquot of blood was used to detect FeLV antigen and feline immunodeficiency virus (FIV) antibodies by means of SNAP Combo FIV/FeLV (Idexx Laboratories, USA). Two spots of blood (125 µl each, 250 µl total blood per animal) were blotted onto the Whatman® FTA® cards (Sigma-Aldrich Corp. MO, USA), stored overnight (at least 6 h) at room temperature for blood to

dry, put in the zip-locked plastic bag and sent to laboratories of the Department of Veterinary Medicine, University of Bari and used for DNA extraction as described by Colella et al., 2020.

2.2 Molecular assays

2.2.1 Nested PCR

A nested PCR (nPCR) protocol previously described (Stiles, 1999), was used to detect FeLV proviral DNA. The 25-μl first-round PCR mixture was arranged using AccuPrime™ SuperMix II (Life Technologies) and primers 118 (5′-TTACTCAAGTATGTTCCCATG-3′) and 119 (5′-CTGGGGAGCCTGGAGACTGCT-3′) in order to amplify a 166-bp fragment of long-terminal repeat (LTR). The thermal protocol included a first step at 94° C × 2 min followed by 40 cycles of 94° C × 1 min, 50° C × 1' and 68° C × 1min, followed by a final extension of 68°C × 10 min. One microliter of a 1:100 dilution of the PCR product was used in the second-round PCR using the same mix and primers 120 (5′-GGTTAAGCACCTGGGCCCCTG-3′) and 121 (5′-GCAGCGGCCTTGAAACTTCTG-3′), which amplify an 85-bp internal fragment. The thermal protocol used was the same as for the first-round PCR except for the PCR cycles that were reduced to 30.

2.2.2 Real-time PCR

Nested PCR positive samples were submitted to real-time PCR for quantification of the proviral DNA loads (Tandon, 2005). Primer pair FeLVU3-exo-f (5’- AACAGCAGAAGTTTCAAGGCC-3’) and FeLVU3-exo-r (5’-TTATAGCAGAAAGCGCGCG-3’) and probe FeLVU3probe (FAM-CCAGCAGTCTCCAGGCTCCCCA-BHQ1) were used, which target the LTR U3 region. Ten microliters of DNA were added to 15 μl of mix, prepared with iTaq™ Universal Probes Supermix (Bio-Rad Laboratories Srl, Milan, Italy), 400 nM of each primer and 200 nM of probe, for a total volume of 25 μl. The thermal protocol for FeLV proviral DNA included a first step at 95° C × 3 min, followed by 45 cycles of 95° C × 5 s and 60° C × 30 s. For absolute FeLV proviral DNA quantification a plasmid was used, which was prepared by cloning the LTR U3 region with the TOPO TA cloning kit (Life Technologies) following the manufacturer's instructions. Ten-fold dilutions of this plasmid, representing 10^0-10^9 copies of DNA/10 μl of template, were used to generate the standard curve for absolute quantification.

2.3 Data analysis

Statistical analysis of the variables was performed using the software R version 4.0.2 (R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/).

135 Categorical data were summarized as count and percentage. Fisher exact test or chi-square test,
136 when appropriate, were used to analyze the categorical variables. Univariate analysis was
137 performed to identify which categorical variables, country of animal origin, age (young [⩽ 12
138 months] vs. adult [> 12 months]), sex, neutering status, breed (mixed breed vs. pedigree),
139 behavioral attitude (usual vs. unusual behavior), location of domicile (urban area vs. countryside),
140 lifestyle (indoor vs. outdoor), rectal temperatures (normal [36.7° C ⩽ rectal temperature ⩽ 38.9°
141 C] vs. altered [rectal temperature < 36.7° C and rectal temperature > 38.9° C]), overall physical
142 condition, and evaluation of respiratory system, nasal mucosa, eyes, oral mucosa, superficial lymph
143 nodes, skin and stool, were significantly associated with FeLV infection. The magnitude of the
144 association between the variables and seropositivity is expressed as an odds ratio (OR) with 95%
145 confidence intervals (95% CI). A *P*-value < 0.05 has been considered as statistically significant.
146 The agreement between SNAP Combo FIV/FeLV (Idexx Laboratories, USA) and nested PCR was
147 calculated the Cohen's Kappa coefficient (k) according to Landis & Koch (1977).
148
149 **3. Results**
150 The analyzed samples (n = 609) were collected from different countries of Southeast Asia: 21.2%
151 (129/609) came from Singapore, 19.5 % (119/609) from Thailand, 18.9% (115/609) from Vietnam,
152 17.4% (106/609) from Philippines, 7.5% (46/609) from Malaysia, 7% (43/609) from Indonesia and
153 8.4% from Taiwan. Owned cats object of the study (i.e., n = 306 males and n = 300 females) aged
154 from 1 month to 20 years (mean 2.6 years, median 1.5 years). Of these, 47.3%, (282/596) were old
155 equal or less than 12 months (juvenile cats) and 52.7% (314/596) were older than 12 months (adult
156 cats). Most of the animals were mixed breed (n = 45, 84%, 451/537), exhibited usual behavior (n =
157 423, 77.2%, 423/548), came from urban areas (n = 473, 77.8%, 473/608) and lived indoor without
158 any chance to go outside (n = 333, 54.8%, 333/608) (table 1).
159 Regarding the clinical signs of the tested cats, the rectal temperature reported was between 35.7°C
160 and 42°C (mean 38.4°C, median 38.5°C). Of these, 79.4 % (444/559) had a rectal temperature in
161 the range of 36.7–38.9°C and 27.7% (155/559) had a rectal temperature outside the reference
162 interval of rectal temperature for healthy adult cats (Levy, 2015) (Table 2). The other cohorts in
163 which the animals were grouped according to their clinical status are shown in Table 2.
164 Overall 47 (7.7%; 95% CI: 5.7%-10.1%) cats were positive to FeLV by SNAP Combo FIV/FeLV
165 test (28/609, 4.6%; 95% CI: 3.1%-6.6%) and/or by nPCR (32/609, 5.2%; 95% CI. 3,6%-7,3%),
166 with poor concordance between the techniques (K= 0.40 (95% CI: 0.20-0.59). Only for 13 animals
167 (2.4%; 95% CI: 1.1%-3.6%) out of these 47 positive cats there was an agreement between the
168 results of the antigenic and molecular tests, whereas for 34 samples (5.6%; 95% CI: 3.9%-7.7%)

169 there was no agreement between the two tests. In particular, 15 samples (44.1%; 95% CI: 27.2%-
170 62.1%) tested positive by rapid antigen test and negative by nPCR, whereas 19 samples (55.9%;
171 95% CI: 37.9%-72.8%) tested positive by nPCR and negative by the SNAP Combo FIV/FeLV
172 assay. Real-time PCR was carried out on nPCR positive samples for quantification of the proviral
173 DNA loads (Tandon, 2005). The mean and median values of FeLV proviral DNA in the feline blood
174 samples were 2.3×10^{6} and 3.7×10^{3} proviral DNA copies per mL, respectively, with viral loads
175 ranging from 3×10^{0} to 5.9×10^{7} proviral DNA copies per mL.

176 The detection rates of FeLV infection by antigenic and/or molecular test in association with main
177 risk factors (country cat origin, age, sex, breed, neutering status, behavioral attitude, cat domicile
178 location and lifestyle) are reported in Table 1. The risk of FeLV infection in cats was significantly
179 associated with the country of sample origin (p-value = 0.2×10^{-8}). Cats living in Thailand and
180 Singapore were related to a higher risk of FeLV infection than those in Vietnam (OR = 33.12; 95%
181 IC: 5.25-1372.29 and OR = 9.51; 95% IC: 1.31-418.50 respectively). For the other countries, there
182 were no statistically significant differences between the country of cat origin and the positivity to
183 FeLV (Table 1).

184 A statistically significant association between age and FeLV infection was observed (p-value =
185 0.0006786). In particular, adults cat showed a higher risk than young cats (OR = 3.18; 95% IC:
186 1.54-7.08). Countryside and outdoor life, or indoor life with the possibility to go out in the garden,
187 was found to be protective factors for FeLV infection (OR = 0.30; 95% IC: 0.78-0.86, p-value =
188 0.01696 and OR = 0.34; 95% IC: 0.15-418.50, p-value = 0.002008, respectively). No association
189 was observed for the other risk factor usually related to FeLV infection (e.g., sex, reproductive
190 capacity, breed and behavioral attitude; $p > 0.05$) (Table 1). Overall, 44 cats were positive to FIV
191 (7.2%, 44/609), of which 18.2% (8/44) were also positive to FeLV. FeLV infection was
192 significantly associated to FIV infection with an OR = 2.98 (95% CI: 1.12–7.13, p = 0.0069).

193 The detection rates of FeLV infection by virologic and/or molecular test in association with clinical
194 signs are reported in Table 2. A statistically significant association between alteration of oral
195 mucosa and FeLV positivity was found (p-value = 0.009245). The cats with altered oral mucosa
196 present higher risk for FeLV than cats with normal oral mucosa (OR=3.11; 95% IC: 1.26-7.41). No
197 statistically significant association was observed between other clinical signs and FeLV positivity
198 (Table 2).

199

200 **4. Discussion**

201 The present study represents the first large-scale epidemiological survey, performed across different
202 Southeast Asian countries, combining antigenic and molecular testing to assess the frequency of oc-
203 currence of FeLV infection in owned cats. The prevalence of FeLV recorded by antigen (4.6%) and
204 nPCR (5.2%) testing indicates that FeLV is widespread in the feline population of Southeast Asia.
205 The overall prevalence of 7.7% is lower than that observed in previous studies (Hwang, 2016; Liu,
206 2020). The large differences in the methods used for the identification of FeLV infection make di-
207 rect comparisons of the studies difficult. Indeed, in some studies, only FeLV-p27 antigen was de-
208 tected, but the genomic RNA or proviral DNA was not searched for. Interestingly, while for Thai-
209 land the prevalence observed using antigenic and/or molecular tests (22.7%) is almost similar to
210 that recorded in a study from more than 10 years ago, in which 24.5% of tested pet cats had circu-
211 lating FeLV antigen (Sukhumavasi, 2012), for Malaysia a much lower prevalence was found (4.3%)
212 compared to that reported in a previous study (12.2%) using a different antigenic test (SensPERT
213 FeLV Ag/FIV Ab kit) in owned and non-owned cats (Bande, 2012). As for the results obtained
214 from cats of Singapore, also in this case the prevalence was lower (7.7%, 10/119), albeit not as
215 much as previously observed (9.9%) in the domestic cat population (Chew-Lim, 1989). On the oth-
216 er hand, a greater prevalence was observed in Taiwan (5.9%) in comparison with the most recent
217 seroepidemiological survey (1.3%), which was conducted more than 20 years ago and was based on
218 detection of FeLV-p27 antigen in cats from veterinary hospitals, a breeding cattery and a homeless
219 shelter (Lin, 1995). Compared to other epidemiological studies carried out in Southeast Asia, we
220 observed a lower virus circulation (3.8%) in the Philippines with respect to a previous study
221 (11.2%) that had used antigen ELISA (Sukhumavasi, 2012). The presence of FeLV-p27 antigen,
222 even if in only one of the tested cats from Vietnam (0.9%) is in agreement with what reported in
223 previous studies suggesting that the absence of the FeLV circulation was associated with a particu-
224 lar management of domestic cats in this country, which did not allow the spread of FeLV infection
225 (Nakamura, 2000). Although no sample from Indonesia tested positive, it is noteworthy that for the
226 first time this country has been included in an antigenic and molecular survey for FeLV
227 (Sukhumavasi et al., 2012). Interestingly, a statistically significant association between the country
228 of cat origin and FeLV infection was found. Cats living in Thailand have an approximately 33-fold
229 higher risk of FeLV infection than those from Vietnam, while cats from Singapore have an approx-
230 imately 9-fold higher risk of FeLV infection than those from Vietnam.
231 In the present study, age, habitat variables (cat domicile location and lifestyle), FIV serological sta-
232 tus and oral mucosa alterations, were recognized as risk factors for FeLV infection.
233 Adult cats showed an approximately 3-fold higher risk than young cats. Although it is known that
234 cat susceptibility to FeLV is age-dependent and adulthood is recognized as a risk factor for the

FeLV infection (S. Gleich, 2009; Hoover, 1991; Sellon, 2012), there are conflicting data on the role of age as a risk factor for FeLV infection (Bande, 2012; Levy, 2006; Sellon, 2012; Studer, 2019; Westman, 2016).

Living in rural areas and outdoors, or indoors with the possibility to go out in the garden, seemed to be protective factors for FeLV infection, which was unexpected because it is well known that one of the main risk factors for FeLV infection is the possibility for the cat to have outdoor access, and that cats living in the countryside have a greater chance of getting out (Hartmann, 2012; Sellon, 2012; Studer, 2019). It should be noted that FeLV, like other feline viruses, is transmitted very efficiently in feline colonies due to the close proximity and high social contact rates between individuals and the common breeding of kittens by females (Fromont, 1997; Pontier, 2009; Sellon, 2012). Therefore, a higher prevalence of cats that did not have access to outside could be linked to a greater promiscuity of cats that may be more subject to mutual grooming and sharing food bowls and litter boxes. Moreover, the possibility that these results are due to the high variability between the outcome proportions in the analyzed cohorts cannot be excluded (urban area vs. countryside and indoor vs. outdoor life).

The significant statistically association between the two viral infection in 18.2% of the sampled cat population has been previously suggested (S. E. Gleich, 2009; Hartmann, 2012; Moraillon, 1990; Sellon, 2012) as an effect of the similar mode of transmission routes of FeLV not only through saliva but also bite wounds as for FIV (Pontier, 2009). Moreover, the coinfection with FeLV and FIV could lead to more negative health outcomes, compared to a single infection with either virus (Hartmann, 2012; Pedersen, 1990; Sellon, 2012).

In agreement with other investigations (Bandecchi, 2006) (Danner, 2007), no statistically significant association was found between sex or reproductive capacity and FeLV infection (Table 1). However, other authors consider male intact cats to be more exposed to FeLV infection (Bande, 2012; Hartmann, 2012; Major, 2010; Sellon, 2012; Studer, 2019). On the other hand, aggressive behavior and mixed breed were no risk factors for FeLV infection , which was unexpected on the basis of previous studies (Sellon, 2012; Studer, 2019).

As previously reported (Hartmann, 2012; Kornya, 2014; Sellon, 2012) a statistically significant association between alteration of oral mucosa and FeLV positivity was found. Cats with altered oral mucosa had approximately 3-fold higher risk to be FeLV positive than cats without oral lesions. Our findings suggest that cats presenting evident alterations of the oral mucosa at the clinical examination should be investigated on their retroviral status. Unexpectedly, no statistically significant association was observed between other clinical signs and FeLV positivity. These results might be related to differences in the type of feline populations being studied and the sample size of

269 the different courts understudy, as well as to the lack of complete signalment for several cats that
270 were tested.

271 Finally, in the present study the agreement between the results obtained with the rapid test and the
272 nPCR was evaluated. Nineteen samples that tested negative on the SNAP Combo FIV/FeLV assay
273 were positive by nPCR, confirming that molecular tests are generally higher sensitive, because they
274 detect FeLV proviral DNA, which is also present in cats with regressive infection (that have passed
275 the phase of transient viremia). In contrast, ELISA tests look at the free p27 protein in the blood (or
276 saliva), which is the expression of an active viral replication observable in the transient and
277 persistent viremia phases, but not in the regressive phase (Westman, 2017; Westman, 2019).
278 Interestingly, 15 samples tested positive for FeLV antigen but negative by the molecular assay,
279 which could be attributed to false-negative results obtained by nPCR, possibly caused by
280 mismatches between primers and target proviral DNA, or degradation of this DNA as a
281 consequence of the long-term storage of DNA extracts. Our study showed a poor agreement
282 between SNAP Combo FIV/FeLV and nPCR, in contrast to what previously reported. Indeed,
283 different studies have generally shown a good agreement between the ELISA and molecular test
284 results (Krecic, 2018; Lutz, 2009; Westman, 2017; Westman, 2019). Detection of FeLV p27 antigen
285 in EDTA blood using in-clinic assays is considered the most suitable protocol for routine diagnosis
286 of FeLV infection since it is less expensive and gives rapid results that facilitate clinical decision-
287 making, pending definitive confirmatory tests at a PCR facility (Lutz, 2009; Westman, 2017).
288 Indeed, though PCR could have limits (Westman, 2019), it remains the most sensitive test for the
289 diagnosis of FeLV infection (Lutz, 2009).
290 Epidemiological surveillance studies, particularly in the countries of Southeast Asia, which are of-
291 ten poorly studied, are increasingly needed to complete the mosaic of knowledge of the worldwide
292 spread of FeLV. The lower prevalence or absence of FeLV infection in some countries as Vietnam
293 and Indonesia could be linked to factors not strictly related to the virus biology or the host, such as
294 particular management of domestic cats, which should be further investigated in order to highlight
295 other potential risk factors related to FeLV infection, which presently may be unknown or underes-
296 timated.
297 Further studies are needed that correlate the clinical signs, not only with the presence of the FeLV-
298 p27 antigen, but also with the proviral DNA load in the cat blood and with viral RNA in oral swabs,
299 in order to provide to clinicians the tools to implement early preventive measures against the infec-
300 tion spread and treatment strategies.
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302

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311
312
313 **Ethical Approval**
314 The protocol of this study was approved by the Ethics Committee of the Department of Veterinary
315 Medicine, University of Bari (protocol no. 13/17). At partner institutions, animal owners read, ap-
316 proved, and signed an owner informed consent, which contained information about study proce-
317 dures.
318
319 **Conflict of Interest Statement**
320 Dr. Thibault, Tan, Tronel, Halos and Beugnet are Boehringer Ingelheim Animal Health employees.
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