

A study of multiple *Felis catus* papillomavirus types (1, 2, 3, 4) in cat skin lesions in Italy by quantitative PCR

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**A study of multiple *Felis catus* Papillomavirus types (FcaPV-1, 2, 3, 4) in cat skin lesion in Italy by q-PCR**

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Keywords:	feline, papillomavirus, skin, viral plaque, in situ Bowenoid carcinoma, squamous cell carcinoma, actinic keratosis, q-PCR
Abstract:	<p>Abstract</p> <p>Objective: Aim of the study is to investigate by q-PCR the presence of Papillomavirus in feline viral plaques (VP), Bowenoid in situ carcinoma (BISC), squamous cell carcinoma (SCC), and actinic keratosis (AK).</p> <p>Methods: Twenty-nine cases with previously established diagnosis of feline VP, BISC, invasive SCC, and AK were selected from a dermatopathological database. A critical re-evaluation of diagnosis was performed by defining clear criteria toward carcinomatous vs non carcinomatous, in situ vs invasive (if carcinomatous) and viral vs actinic. Cases were evaluated for p16 immunolocalisation. The presence of the target viral genes for FcaPV-1, 2, 3 and 4 was determined by q-PCR. Data generated Cq values, which represent a normalized measure of DNA viral quantity. Samples with a positive Cq values were submitted to sequence analysis.</p> <p>Results: 4 VPs, 19 BISCs 4 SCCs and 1 AK were included. By Cq analysis we found that all VPs were positive for FcaPV-1 or FcaPV-2; and 8 BISCs for FcaPV-1, 2, 4. FcaPV-2 was the most prevalent among the group of VPs and BISCs.</p> <p>Conclusion and relevance: Using Cq method we report the first evidence of FcaPV-1, 2 and 4 in Italy, FcaPV-2 was the most frequently detected. To a lesser extent also FcaPV-1 and FcaPV-4 has been detected in our examined samples while FcaPV-3 was never associated to viral induced lesions by Cq investigation. Compared to conventional PCR the Cq method has the advantage to establish a possible role of the virus in the outcome of infection.</p>



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2 **cat skin lesion in Italy by q-PCR.**

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17

18 **Key words:** feline papillomavirus, skin, viral plaque, *in situ* Bowenoid  
19 carcinoma, squamous cell carcinoma, actinic keratosis, q-PCR

## 20 **Abstract**

21 **Objective:** Aim of the study is to investigate by q-PCR the presence of  
22 Papillomavirus in feline viral plaques (VP), Bowenoid *in situ* carcinoma (BISC),  
23 squamous cell carcinoma (SCC), and actinic keratosis (AK).

24 **Methods:** Twenty-nine cases with previously established diagnosis of feline VP,  
25 BISC, invasive SCC, and AK were selected from a dermatopathological  
26 database. A critical re-evaluation of diagnosis was performed by defining clear  
27 criteria toward carcinomatous vs non carcinomatous, *in situ* vs invasive (if  
28 carcinomatous) and viral vs actinic. Cases were evaluated for p16  
29 immunolocalisation. The presence of the target viral genes for FcaPV-1, 2, 3  
30 and 4 was determined by q-PCR. Data generated  $\Delta\Delta Cq$  values, which  
31 represent a normalized measure of DNA viral quantity. Samples with a positive  
32  $\Delta\Delta Cq$  values were submitted to sequence analysis.

33 **Results:** 4 VPs, 19 BISCs 4 SCCs and 1 AK were included. By  $\Delta\Delta Cq$  analysis  
34 we found that all VPs were positive for FcaPV-1 or FcaPV-2; and 8 BISCs for  
35 FcaPV-1, 2, 4. FcaPV-2 was the most prevalent among the group of VPs and  
36 BISCs.

37 **Conclusion and relevance:** Using  $\Delta\Delta Cq$  method we report the first evidence of  
38 FcaPV-1, 2 and 4 in Italy, FcaPV-2 was the most frequently detected. To a  
39 lesser extent also FcaPV-1 and FcaPV-4 has been detected in our examined  
40 samples while FcaPV-3 was never associated to viral induced lesions by  $\Delta\Delta Cq$   
41 investigation. Compared to conventional PCR the  $\Delta\Delta Cq$  method has the  
42 advantage to establish a possible role of the virus in the outcome of infection.

43

## 44 Introduction

45 Detection of Feline papillomaviruses (FcaPVs) from feline cutaneous  
46 lesions has been reported worldwide<sup>1,2</sup> and these viruses are considered liable  
47 of causing several feline skin conditions.<sup>1-5</sup> These include a wide spectrum of  
48 proliferative skin lesions ranging from non-neoplastic viral plaques (VP) to pre-  
49 neoplastic Bowenoid *in situ* carcinoma (BISC), and invasive squamous cell  
50 carcinoma (SSC).<sup>6</sup> A progression from non neoplastic viral induced lesions to  
51 overt neoplasia has also been documented to occur.<sup>7</sup> Four FcaPV types  
52 (FcaPV-1 to FcaPV-4) have been so far described in cats, having different  
53 anatomical distribution and presentation.<sup>1,8-11</sup> The most frequent FcaPV  
54 (FcaPV-2, belonging to *Dyothetapapillomavirus* genus) was isolated from a  
55 cutaneous pigmented plaque,<sup>12</sup> and later related to cutaneous SCC.<sup>13-15</sup>  
56 FcaPV-2 seems to play a role in a high proportion of nasal planum squamous  
57 cell carcinomas and, animals with tumours associated to papillomaviral  
58 aetiology with p16 upregulation, show increased survival compared with those  
59 attributable to UV radiation.<sup>16</sup> The relevance of FcaPV-2 in the pathogenesis of  
60 both premalignant and malignant lesions has been recently investigated by  
61 concurrently assessing number of DNA viral copies and viral gene expression.<sup>5-</sup>  
62 <sup>7</sup> However, the effective role of FcaPV-2 in the development of malignant  
63 lesions is still a debated question, especially in view of recent results indicating

64 a high FcaPV-2 prevalence in healthy domestic cats.<sup>7,17</sup> The role of other feline  
65 PV types, namely FcaPV-1, 3, and 4, remains obscure. FcaPV-1, belongs to the  
66 *Lambdapapillomavirus* genus and has been rescued from a hyperkeratotic  
67 cutaneous lesion in a Persian cat by Tachezy et al.<sup>9</sup> In the same year another  
68 FcaPV was also identified in a shorthair domestic cat with papillomatosis.<sup>11</sup>  
69 More recently the virus was detected in multiple small sessile raised lesions on  
70 the ventral surface of the tongue in two 13-year-old domestic cats.<sup>18</sup> FcaPV-3 is  
71 a *Taupapillomavirus*, isolated for the first time from a cutaneous in situ  
72 carcinoma.<sup>3</sup> Recently FcaPV-3 has been detected from a BISC with a novel  
73 histological feature and a benign clinical behaviour.<sup>19</sup> The same  
74 histopathological changes were observed in a feline basal cell carcinoma, from  
75 which DNA sequences of a novel PV closely related to FcaPV-3 were detected  
76 by the authors.<sup>4</sup> FcaPV-4 belongs to genus *Taupapillomavirus*, and has been  
77 detected in oral cavity following a severe gengivitis.<sup>10</sup> Pathogenicity of FcaPV-1  
78 and FcaPV-4 is still unclear and these viruses are rarely detected in oral  
79 inflammatory as well as neoplastic lesions. Therefore, an active role of FcaPV-1  
80 and FcaPV-4 in carcinogenesis is still debated.

81 Based on literature, it emerges that most cases of FcaPVs infection have been  
82 documented in domestic felids from New Zealand, North and South America  
83 and, to a lesser extent from Europe. This might just reflect the active research

84 done in these countries without any epidemiological meaning. In Europe PVs  
85 infections have been reported in Switzerland in nine cases,<sup>12,20</sup> in one case in  
86 Germany<sup>21</sup> and in one case in Italy.<sup>22</sup> Here we assess by a combination of  
87 histology, immunohistochemistry and q-PCR the concurrent presence of  
88 FcaPV-1, 2, 3 and 4 viral DNA and p16 immunostaining in 29 feline lesions  
89 collected in Italy including non neoplastic and preneoplastic, viral and non viral  
90 induced skin lesions namely: VP, BISC, invasive SSC and actinic keratosis  
91 (AK). Two methods were applied to analyse the viral load data: absolute  
92 quantification (AQ) and relative quantification (RQ). The AQ was applied to  
93 determine the viral copy number per  $\mu\text{g}$  of DNA; however, by this method wide  
94 range of values can be obtained without a cut-off value that would allow to  
95 discriminate FcaPVs presence as innocent bystander or as responsible of viral  
96 lesion. To address this problem we approached the RQ by  $\Delta\Delta\text{Cq}$  calculation.<sup>23</sup>  
97 This method measures viral genome presence by relating the obtained values  
98 of each of the 4 FcaPVs in each lesion classified as VP or BISC to those of  
99 SCC and AK.

## 100 **Materials and methods**

### 101 *Biopsies*

102 Feline skin biopsies have been identified from dermatopathological databases

103 and 29 cases with a diagnosis of VP, BISC, invasive SCC and AK were  
104 selected. The retrieved formalin fixed cases were routinely processed for  
105 histopathology and 4 µm thick paraffin embedded tissues sections were stained  
106 with haematoxylin and eosin (HE). Before inclusion in the study, the selected  
107 samples were subjected to critical re-evaluation of diagnosis by defining clear  
108 distinctive criteria, such as carcinomatous vs non carcinomatous, in situ vs  
109 invasive (if carcinomatous), and viral vs actinic. The established criteria were  
110 defined as described in the scientific literature and are summarized in table  
111 1.<sup>14,20,24</sup> Data from signalment and lesion distribution, when available, were  
112 included.

### 113 *P16 immunohistochemistry*

114 Cases were evaluated for the detection of p16 by immunohistochemistry  
115 employing an ABC system (Vector Laboratories, Burlingame, USA). Antigen  
116 unmasking was carried out at 120°C for 3 min in a pressure cooker with a TRIS-  
117 EDTA buffer (1,2 g/L Tris, 0,36 g/L EDTA, pH 9.0). Sections were pre-treated  
118 for 10 min with 1% H<sub>2</sub>O<sub>2</sub> in 0.1 M phosphate buffered saline (PBS), pH 7.4 to  
119 quench endogenous peroxidase activity, and blocked for 30 min at room  
120 temperature (rt) in PBS with a 2% normal horse serum (PK-7200, Vector  
121 Laboratories, Burlingame, USA) and 0.05% TritonX-100. Sections were then  
122 incubated overnight at 4°C with a 1:100 diluted monoclonal primary antibody

123 p16-INK4a (mouse monoclonal IgG, BiorByt, San Francisco, USA). After  
124 washing in PBS, an incubation with a secondary universal biotinylated anti-  
125 mouse/rabbit antibody (PK-7200, Vector Laboratories, Burlingame, USA) was  
126 performed for 1h at rt. Staining was visualized with a diaminobenzidine (SK-  
127 4105, Vector Laboratories, Burlingame, USA) solution under a light microscope  
128 (Eclipse 80i, Nikon Tokyo, Japan).

#### 129 *Sampling material for q-PCR*

130 Three 10 µm thick sections from the selected samples were cut with a  
131 microtome, place onto slides and left unfixed. These slides were observed  
132 under an optic microscope. Tissue that was not relevant for the study was  
133 scraped off and the remaining tissue directly collected in a DNase free 1,5 ml  
134 tube. To prevent carryover of contaminating DNA the microtome overlay was  
135 covered with a new piece of adhesive tape and a new blade was used for each  
136 sample.

#### 137 *DNA extraction*

138 DNA extraction was performed using DNeasy Blood and Tissue kit (Qiagen,  
139 Hilden, Germany) following manufacturer's instruction and applying a  
140 preliminary removal of paraffin by extraction with xylene. DNAs were eluted in  
141 30 µl, and each sample concentration was quantified using the Qubit

142 spectrophotometer (ThermoFisher Scientific, Waltham, USA), DNA samples  
143 were stored at -20°C until analysis.

#### 144 *q-PCR*

145 The extracted DNAs were amplified using four specific set of primers amplifying  
146 a portion of the four types of FcaPVs L1 gene so far identified in the feline  
147 species (Table 2). To normalize the amount of DNA used for each sample so to  
148 achieve a correct quantification of viral copy number, a q-PCR under the same  
149 condition as for FcaPV but with a specific set of primers was run in parallel for  
150 the reference gene albumin (ALB).<sup>25</sup> The number of copy of the target viral  
151 gene, measured as Cq value, generates a  $\Delta Cq$  value when compared to the  
152 corresponding Cq value of the reference gene. Moreover, a  $\Delta\Delta Cq$  value was  
153 calculated comparing the  $\Delta Cq$  value of the sample of interest to the mean  $\Delta Cq$   
154 value obtained from the SCC and AK group, considered as the negative control  
155 group. The  $\Delta\Delta Cq$  value represents a normalized measure of DNA viral quantity  
156 and was calculated using REST software.<sup>26</sup> In this work, a RQ using the  $2^{-\Delta\Delta Cq}$   
157 method<sup>23</sup> was adapted to estimate in each sample the fold change of FcaPV-1,  
158 2, 3 and 4 target viral gene copies relative to the albumin reference gene.  
159 Melting curve analysis was performed in conjunction with each four specific  
160 FcaPV amplification protocol to determine if non specific products were

161 amplified during reaction. The specificity of the melting curve was compared to  
162 melting curves values obtained from a plasmid (pFcaPV) containing amplicon of  
163 the four FcaPVs spanning the real-time products. The plasmid was generated  
164 by inserting the four FcaPVs gene segments into pMA-T vector using GeneArt  
165 technology (Thermo Fisher Scientific, Waltham, USA). Serial dilutions of  
166 pFcaPV plasmid ranging from  $10^6$  to  $10^2$  copies/5 $\mu$ l were used to calculate the  
167 efficiency of q-PCR for each FcaPV set of primers and compared to the  
168 efficiency calculated on five points of a 2-fold serial dilution of a quantified DNA  
169 template for ALB gene. The efficiency of each q-PCR assay was similar and  
170 ranging from 91% to 98% (average 94,6%). The lesions identified by  
171 histological classification as SCC-AK were presumed to serve as the negative  
172 control group and each sample belonging to this group was also individually  
173 tested by  $\Delta\Delta Cq$  analysis for each FcaPV type. Samples that had eventually  
174 scored a positive  $\Delta\Delta Cq$  value were excluded. In addition, the viral copy  
175 number/ $\mu$ l of input DNA for each FcaPV type in each sample tested was  
176 calculated (AQ). The assays were performed in Rotorgene thermocycler  
177 (Corbett Research, Sydney, Australia) using SSCO SYBR Green master mix  
178 (Biorad, Hercules, USA) and 5  $\mu$ l of extracted DNA. All samples were tested in  
179 duplicate and the results were calculated using the mean Cq values. Samples  
180 positive for only one replica were considered as negative. All samples with a

181 positive  $\Delta\Delta Cq$  values were submitted to sequence analysis (BMR genomics,  
182 Padova, Italy).

### 183 **Results**

184 Histologically all VPs were recognized for areas of focal epidermal  
185 dysplasia with koilocytes and keratinocytes with cytoplasm enlarged by blue-  
186 greyish material. Regarding BISC diagnosis, the presence of koilocytes or  
187 koilocyte-like cells pointed to the viral origin in 17/20 cases while in the  
188 remaining 3 cases diagnosis was formulated by other morphological details  
189 listed in Table 1. SCC and AK showed the morphological alterations listed in  
190 Table 1. Among the 20 BISCs cases 5 VPs were also detected in the  
191 adjacent skin by histology and in other 5, sites of penetration of keratinocytes  
192 through the basement membrane into the dermis were observed indicative of  
193 progression toward SCC.

194 Clinical reports for each of the 29 cases fulfilling the established  
195 histological criteria were recorded. Breed, age, gender and anatomical  
196 distribution are summarised in Table 3. Twenty-five subjects were shorthair  
197 domestic cats, two persian and two main coons. Mean age at presentation  
198 varied between 6 and 20 years (median 11,6), 14 out of 29 were female and  
199 15 were male. A total of 4 VPs, 20 BISCs, 4 SCCs and 1 AK were detected.

200 Lesions were observed in densely haired skin regions in 9 cats (dorsum,  
201 flank, and shoulder), in areas non-exposed to sunlight in 1 cat (groin) and in  
202 hypotrichotic and solar exposed skin in 18 cats (ears, nose, eyelids, temporal  
203 region). All cases of SCC were observed in sun-exposed areas. Clinically  
204 VPs were few millimeters wide, alone or grouped, slightly raised, pigmented  
205 and non-pruritic lesions; BISCs were clinically larger than VPs, usually more  
206 than 1 cm of diameter, multifocally coalescing, raised and often verrucous,  
207 crusted and hyperpigmented. In SCC, erosive and ulcerative crusted lesions  
208 were clinically detected. In the only case in which AK was diagnosed,  
209 erythema, scales and crust were seen (Figure 1a, b, c).

210 P16 was immunolocalized in all VPs epidermis and in the epidermis and  
211 follicular wall of the BISCs. No signal or only faint staining was detected in  
212 the SCC and AK lesion. Immunoreactivity was either nuclear and  
213 cytoplasmic; in VPs and BISCs the signal was strong while in the SCCs  
214 immunoreactivity was faint and therefore considered as no significant  
215 (Figure 1d).

216 By q-PCR we were able to detect the albumin reference gene in 28 out of 29  
217 DNA samples. Only one sample classified as BISC was not suitable to  
218 molecular analysis and therefore it was excluded from further analysis.

219 By AQ, the presence of FcaPVs has been detected in low copies in almost all  
220 cases (Table 4). By RQ ( $\Delta\Delta Cq$  method) a reduced number of samples was  
221 linked to viral lesions. In details, FcaPVs positive  $\Delta\Delta Cq$  values were detected  
222 in all VPs (4/4) and in 8/19 BISCs (Table 3). VPs were positive to FcaPV-2 in  
223 three samples and to FcaPV-1 in one sample. BISCs were positive to FcaPV-  
224 2 in three samples, FcaPV-4 and FcaPV-1 in one sample each. Interestingly,  
225 as never reported before, in BISCs lesion we found three cases of double  
226 infection. In two of those, FcaPV-1 and 2 were detected simultaneously and  
227 in one case FcaPV-2 was present along with FcaPV-4 (Table 3).

228 In all cases with a positive  $\Delta\Delta Cq$  value, the viral load was always greater  
229 than  $10^2$  copies / $\mu g$  DNA (FcaVP-1: min 167 – max 310 Standard Error (SE):  
230 33,0; FcaPV-2: min 100 – max 1150 SE: 163,9; FcaPV-4 min 391 – max  
231 3700 SE: 1654,5) (Table 4). Sequence analysis confirmed the FcaPV types  
232 as indicated by q-PCR  $\Delta\Delta Cq$  analysis.

### 233 **Discussion**

234 In this retrospective study, we detected the concurrent presence of different  
235 types of PVs, namely of FcaPV-1, 2 and 4 in feline VPs and BISCs by  $\Delta\Delta Cq$   
236 method using specific primers. To the best of our knowledge only the  
237 concurrent presence of FcaPV-2 and 3 have been reported from one BISC

238 and one VP.<sup>3,22</sup> However differently from previous reports, here we report the  
239 presence of FcaPV-1 and FcaPV-4, alone or in association to FcaPV-2.

240 A limitation when performing PCR-based studies on PVs infection is  
241 that the viral DNA is often detected and cannot be directly linked to an  
242 effective role of virus in pathogenesis and therefore it is not possible to fully  
243 establish whether the presence of a viral genome is uneventful (subclinical  
244 lesion). In particular, FcaPV-2 has been recently detected in a number of skin  
245 swabs from healthy cats, making difficult to discern whether PVs are causing  
246 cancer or are merely an “innocent bystander”.<sup>7</sup> The hypothesis that the  
247 presence of high viral loads likely represents an infectious state of PV is  
248 supported by Thomson et al.<sup>7</sup> who recently showed that the finding of high  
249 copy numbers of FcaPV-2 DNA within a lesion suggests that the detected  
250 virus may be responsible for it. In fact, while high viral copy numbers were  
251 associated with E6/E7 gene expression, no gene expression was detected in  
252 association of low copy numbers, an indication of an incidental finding.

253 For the above-mentioned reasons, for this study we used a q-PCR  
254 protocol applying a  $\Delta\Delta Cq$  method to investigate if and which FcaPVs have  
255 induced the lesion in cats. In this sense the obtained results are partially in  
256 line with the scientific literature which indicates so far that FcaPV-2 is the

257 major PV type implicated in skin preneoplastic and neoplastic lesions in  
258 cats.<sup>7</sup> Since the genome organization and the role of viral proteins within the  
259 replication cycle are considered similar even between different genera  
260 belonging to the *Papillomaviridae* family we assumed that a similar link is  
261 maintained for feline PVs other than FcaPV-2. Importantly we reported  
262 positive  $\Delta\Delta Cq$  values for FcaPV-1 and 4; two FcaPV types rarely reported.<sup>9–</sup>  
263 <sup>11,18</sup>

264 A comparison between our data with those obtained from other studies  
265 is difficult for two reasons: i) type of PCR primers used (specific vs  
266 consensus); ii) type of PCR used (conventional vs quantitative). A general  
267 prevalence ranging from 24 to 100% of FcaPV-2 both BISCs and VPs has  
268 been reported.<sup>7,8,27,28</sup> In our study, by using specific primers in a q-PCR  
269 analysis applying RQ method, we obtained a prevalence for FcaPV-2 in  
270 BISCs of 31,6% (6/19) and for all FcaPV types of 42,10% (8/19).

271 Failure to demonstrate PV DNA in every BISC have been previously explained  
272 through a carcinogenesis model in which papillomaviruses cause transformation  
273 but are only for a short period present within the lesion.<sup>29</sup> Surprisingly, cases in  
274 which koilocytes were detected did not show any  $\Delta\Delta Cq$  positive value; this  
275 result is likely due to: i) the detection of koilocytes in very focal lesions that

276 could have been no more present when additional serial paraffin sections were  
277 prepared for DNA extraction and PCR analysis; ii) the presence of a PCR  
278 undetectable PV variant.

279 Low viral copies for all PV types have been detected in almost all cases,  
280 however the  $\Delta\Delta Cq$  analysis was necessary to associate the FcaPVs as  
281 biological agent potentially causing the lesions.  $\Delta\Delta Cq$  positive values for the  
282 different PV types were detected in all VPs and in 42% of BISCs.  
283 Noteworthy, mean  $\Delta\Delta Cq$  of positive samples differed among PV types being  
284 62.5, 189.1 and 3083.5 for FcaPV-1, FcaPV-2 and FcaPV-4 respectively.  
285 Studies considering a larger panel of cases might be necessary to establish a  
286 further  $\Delta\Delta Cq$  cut off value.

287 Here we document the presence of FcaPV-2 with high prevalence in both  
288 VPs and BISCs while we rescued FcaPV-1 and FcaPV-4 less frequently;  
289 these last two PVs are rarely described as playing an active role in skin and  
290 oral mucosa lesions in cats.<sup>9,10,18,30</sup> None of the lesions was associated to  
291 FcaPV-3 when adopting  $\Delta\Delta Cq$  method, confirming the rare occurrence of this  
292 FcaPV type also in Italy.

293 Either cytoplasmic or nuclear p16 signals were detected in all cases where  
294 cytopathic effects were found (VP, BISC) while p16 immunolocalisation was

295 present with faint and mainly cytoplasmic signal in cases of SCCs and no p16-  
296 immunoreactivity was found in the case of AK. Despite the difficulties of  
297 interpretation when the signal was faint, these results are in line with what  
298 already documented previously. About half of the SCC studied by Thomson et  
299 al.<sup>7</sup> were negative to p16 immunostaining. A strong association between  
300 FcaPV-2 E6/E7 gene expression and p16 immunostaining have been recently  
301 found in feline SCC with 18 of 20 (90%) E6/E7-positive SCCs being also  
302 positive for p16 compared to 13 of 40 (33%) E6/E7-negative SCCs.<sup>7</sup> E6/7 gene  
303 expression investigation has not been carried out in our study but our results  
304 might reflect this correlation.

### 305 **Conclusion**

306  $\Delta\Delta Cq$  analysis has proved to be necessary to associate the FcaPVs as  
307 biological agent potentially causing the lesions. Based on this method the  
308 presence of FcaPV-2 is confirmed to be the most representative FcaPV in  
309 feline skin lesions referable to diagnosis of VP and BISC in Italy. To a lesser  
310 extent also FcaPV-1 and FcaPV-4 has been detected in our examined  
311 samples.

312

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413 carcinomas is associated with an increased survival time and the  
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415

416

#### 417 LEGEND

418 Figure 1. Multiple symmetrical raised, hyperpigmented and crusted plaques  
419 on the face of a cat with Bowenoid in situ carcinoma (a). Histopathology from  
420 a viral plaque in a cat. Focal epidermal hyperplasia, absence of follicular wall  
421 involvement, evident viral cytopathic effects (koilocytes) (bar=50µm) (b);

422 Histopathology from a Bowenoid in situ carcinoma in a cat. Epidermal and  
423 follicular dysplasia with upward in situ keratinocyte proliferation and a few  
424 koilocytes (bar=50 $\mu$ m) (c); p16 immunohistochemistry, groups of  
425 keratinocytes show nuclear and cytoplasmic positivity (ABC system, bar=100  
426  $\mu$ m) (d).

427

For Peer Review

1 **Papillomavirus induced skin lesions in cats from Italy.**

2 **A study of multiple *Felis catus* Papillomavirus types (FcaPV-1, 2, 3, 4) in**  
3 **cat skin lesion in Italy by q-PCR.**

4

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18

19 **Key words:** feline papillomavirus, skin, viral plaque, *in situ* Bowenoid  
20 carcinoma, squamous cell carcinoma, actinic keratosis, q-PCR

## 21 **Abstract**

22 **Objective:** Aim of the study is to investigate by q-PCR the presence of PV  
23 Papillomavirus in feline viral plaques (VP), Bowenoid *in situ* carcinoma (BISC),  
24 squamous cell carcinoma (SCC), and actinic keratosis (AK).

25 **Methods:** Twenty-nine cases with previously established diagnosis of feline VP,  
26 BISC, invasive SCC, and AK were selected from a dermatopathological  
27 database. A critical re-evaluation of diagnosis was performed by defining clear  
28 criteria toward carcinomatous vs non carcinomatous, *in situ* vs invasive (if  
29 carcinomatous) and viral vs actinic. Cases were evaluated for p16  
30 immunolocalisation. The presence of the target viral genes for FcaPV-1, 2, 3  
31 and 4 was determined by q-PCR. Data generated  $\Delta\Delta Cq$  values, which  
32 represent a normalized measure of DNA viral quantity. Samples with a positive  
33  $\Delta\Delta Cq$  values were submitted to sequence analysis.

34 **Results:** 4 VPs, 19 BISCs 4 SCCs and 1 AK were included. By  $\Delta\Delta Cq$  analysis  
35 we found that all VPs were positive for FcaPV-1 or FcaPV-2; and 8 BISCs for

36 FcaPV-1, 2, 4. The  $\Delta\Delta Cq$  detected 3 out of 4 types of FcaPVs.  $\Delta\Delta Cq$  detected  
37 FcaPV-1 in 1 VP and 3 BISCs, FcaPV-2 in 3 VPs and 6 BISCs, FcaPV-4 in 2  
38 BISCs. FcaPV-2 was the most prevalent among the group of VPs and BISCs.

39 **Conclusion and relevance:** Using  $\Delta\Delta Cq$  method we report the first evidence of  
40 FcaPV-1, 2 and 4 in Italy, FcaPV-2 was the most frequently detected. To a  
41 lesser extent also FcaPV-1 and FcaPV-4 has been detected in our examined  
42 samples while FcaPV-3 was never associated to viral induced lesions by  $\Delta\Delta Cq$   
43 investigation. Compared to conventional PCR the  $\Delta\Delta Cq$  method has the  
44 advantage to allow establish a possible role of the virus in the outcome of  
45 infection.

46

## 47 Introduction

48 Detection of Feline papillomaviruses (FcaPVs) from feline cutaneous  
49 lesions has been reported worldwide<sup>1,2</sup> and these viruses are considered liable  
50 of causing several feline skin conditions.<sup>1-5</sup> These include a wide spectrum of  
51 proliferative skin lesions ranging from non-neoplastic viral plaques (VP) to pre-  
52 neoplastic Bowenoid *in situ* carcinoma (BISC), and ~~to sarcoid and~~ invasive  
53 squamous cell carcinoma (SSC).<sup>6</sup> A progression from non neoplastic viral  
54 induced lesions to overt neoplasia has also been documented to occur.<sup>7</sup> Four  
55 FcaPV types (FcaPV-1 to FcaPV-4) have been so far described in cats, having  
56 different anatomical distribution and presentation.<sup>1,8-11</sup> The most frequent  
57 FcaPV (FcaPV-2, belonging to *Dyothetapapillomavirus* genus) was isolated  
58 from a cutaneous pigmented plaque,<sup>12</sup> and later related to cutaneous SCC.<sup>13-15</sup>  
59 FcaPV-2 seems to play a role in a high proportion of nasal planum squamous  
60 cell carcinomas and, animals with tumours associated to papillomaviral  
61 aetiology with p16 upregulation, show increased survival compared with those  
62 attributable to UV radiation.<sup>16</sup> The relevance of FcaPV-2 in the pathogenesis of  
63 both premalignant and malignant lesions has been recently investigated by  
64 concurrently assessing number of DNA viral copies and viral gene expression.<sup>5-</sup>  
65 <sup>7</sup> However, the effective role of FcaPV-2 in the development of malignant  
66 lesions is still a debated question, especially in view of recent results indicating

67 a high FcaPV-2 prevalence in healthy domestic cats.<sup>7,17</sup> The role of other feline  
68 PV types, namely FcaPV-1, 3, and 4, remains obscure. FcaPV-1, belongs to the  
69 *Lambdapapillomavirus* genus and has been rescued from a hyperkeratotic  
70 cutaneous lesion in a Persian cat by Tachezy et al.<sup>9</sup> In the same year another  
71 FcaPV was also identified in a shorthair domestic cat with papillomatosis.<sup>11</sup>  
72 More recently the virus was detected in multiple small sessile raised lesions on  
73 the ventral surface of the tongue in two 13-year-old domestic cats.<sup>18</sup> FcaPV-3 is  
74 a *Taupapillomavirus*, isolated for the first time from a cutaneous in situ  
75 carcinoma.<sup>3</sup> Recently FcaPV-3 has been detected from a BISC with a novel  
76 histological feature and a benign clinical behaviour.<sup>19</sup> The same  
77 histopathological **cell** changes were observed in a feline basal cell carcinoma,  
78 from which DNA sequences of a novel PV closely related to FcaPV-3 were  
79 detected by the authors.<sup>4</sup> FcaPV-4 belongs to genus *Taupapillomavirus*, and  
80 has been detected in oral cavity following a severe gingivitis.<sup>10</sup> Pathogenicity of  
81 FcaPV-1 and FcaPV-4 is still unclear and these viruses are rarely detected in  
82 oral inflammatory as well as neoplastic lesions. Therefore, an active role of  
83 FcaPV-1 and FcaPV-4 in carcinogenesis is still debated.

84 Based on literature, it emerges that most cases of FcaPVs infection have been  
85 documented in domestic felids from New Zealand, North and South America  
86 and, to a lesser extent from Europe. This might just reflect the active research

87 done in these countries without any epidemiological meaning. In Europe PVs  
88 infections have been reported in Switzerland in **only** nine cases,<sup>12,20</sup> in one case  
89 in Germany <sup>21</sup> and in **another one** case in Italy.<sup>22</sup> **Here we assess by a**  
90 **combination of histology, immunohistochemistry and q-PCR the concurrent**  
91 **presence of FcaPV-1, 2, 3 and 4 viral DNA and p16 immunostaining in 29 feline**  
92 **lesions collected in Italy including non neoplastic and preneoplastic, viral and**  
93 **non viral induced skin lesions namely: VP, BISC, invasive SSC and actinic**  
94 **keratosis (AK). Two methods were applied to analyse the viral load data:**  
95 **absolute quantification (AQ) and relative quantification (RQ). The AQ was**  
96 **applied to determine the viral copy number per  $\mu\text{g}$  of DNA; however, by this**  
97 **method wide range of values can be obtained without a cut-off value that would**  
98 **allow to discriminate FcaPVs presence as innocent bystander or as responsible**  
99 **of viral lesion. To address this problem we approached the RQ by  $\Delta\Delta\text{Cq}$**   
100 **calculation.** <sup>23</sup> **This method measures viral genome presence by relating the**  
101 **obtained values of each of the 4 FcaPVs in each lesion classified as VP or**  
102 **BISC to those of SCC and AK.**

## 103 **Materials and methods**

### 104 *Biopsies*

105 Feline skin biopsies have been identified from dermatopathological databases

106 and 29 cases with a diagnosis of VP, BISC, invasive SCC and AK were  
107 selected. The retrieved formalin fixed cases were routinely processed for  
108 histopathology and 4 µm thick paraffin embedded tissues sections were stained  
109 with haematoxylin and eosin (HE). Before inclusion in the study, the selected  
110 samples were subjected to critical re-evaluation of diagnosis by defining clear  
111 distinctive criteria, such as carcinomatous vs non carcinomatous, in situ vs  
112 invasive (if carcinomatous), and viral vs actinic. The established criteria were  
113 defined as described in the scientific literature and are summarized in table  
114 1.<sup>14,20,24</sup> Data from signalment and lesion distribution, when available, were  
115 included.

#### 116 *P16 immunohistochemistry*

117 Cases were evaluated for the detection of p16 by immunohistochemistry  
118 employing an ABC system (Vector Laboratories, Burlingame, USA). Antigen  
119 unmasking was carried out at 120°C for 3 min in a pressure cooker with a TRIS-  
120 EDTA buffer (1,2 g/L Tris; 0,36 g/L EDTA; pH 9.0). Sections were pre-treated  
121 for 10 min with 1% H<sub>2</sub>O<sub>2</sub> in 0.1 M phosphate buffered saline (PBS), pH 7.4 10  
122 min to quench endogenous peroxidase activity, and blocked for 30 min at room  
123 temperature (rt) in PBS with a 2% normal horse serum (PK-7200, Vector  
124 Laboratories, Burlingame, USA) and 0.05% TritonX-100. Sections were then  
125 incubated overnight at 4°C with a 1:100 diluted monoclonal primary antibody

126 p16-INK4a (mouse monoclonal IgG, BiorByt, San Francisco, USA). After  
127 washing in PBS, an incubation with a secondary universal biotinylated anti-  
128 mouse/rabbit antibody (PK-7200, Vector Laboratories, Burlingame, USA) was  
129 performed for 1h at rt. Staining was visualized with a diaminobenzidine (SK-  
130 4105, Vector Laboratories, Burlingame, USA) solution under a light microscope  
131 (Eclipse 80i, Nikon Tokyo, Japan).

#### 132 *Sampling material for q-PCR*

133 Three 10 µm thick sections from the selected samples were cut with a  
134 microtome, place onto slides and left unfixed. These slides were observed  
135 under an optic microscope. Tissue that was not relevant for the study was  
136 scraped off and the remaining tissue directly collected in a DNase free 1,5 ml  
137 tube. To prevent carryover of contaminating DNA the microtome overlay was  
138 covered with a new piece of adhesive tape and a new blade was used for each  
139 sample. and the microtome overlay covered with a new piece of adhesive tape.

#### 140 *DNA extraction*

141 DNA extraction was performed using DNeasy Blood and Tissue kit (Qiagen,  
142 Hilden, Germany) following manufacturer's instruction and applying a  
143 preliminary removal of paraffin by extraction with xylene. DNAs were eluted in  
144 30 µl, and each sample concentration was quantified using the Qubit Nanodrop

145 spectrophotometer (ThermoFisher Scientific, Waltham, USA), DNA samples  
146 were stored at -20°C until analysis.

#### 147 *q-PCR*

148 The extracted DNAs were amplified using four specific set of primers amplifying  
149 a portion of the four types of FcaPVs L1 gene so far identified in the feline  
150 species (Table 2). To normalize the amount of DNA used for each sample so to  
151 achieve a correct quantification of viral copy number, a q-PCR under the same  
152 condition as for FcaPV but with a specific set of primers was run in parallel for  
153 the reference gene albumin (ALB).<sup>25</sup> The number of copy of the target viral  
154 gene, measured as Cq value, generates a  $\Delta Cq$  value when compared to the  
155 corresponding Cq value of the reference gene. Moreover, a  $\Delta\Delta Cq$  value was  
156 calculated comparing the  $\Delta Cq$  value of the sample of interest to the mean  $\Delta Cq$   
157 value obtained from the SCC and AK group, considered as the negative control  
158 group. The  $\Delta\Delta Cq$  value represents a normalized measure of DNA viral quantity  
159 and was calculated using REST software.<sup>26</sup> In this work, a RQ using the  $2^{-\Delta\Delta Cq}$   
160 method<sup>23</sup> was adapted to estimate in each sample the fold change of FcaPV-1,  
161 2, 3 and 4 target viral gene copies relative to the albumin reference gene.  
162 Melting curve analysis was performed in conjunction with each four specific  
163 FcaPV amplification protocol to determine if non specific products were

164 amplified during reaction. The specificity of the melting curve was compared to  
165 melting curves values obtained from a plasmid (pFcaPV) containing amplicon of  
166 the four FcaPVs spanning the real-time products. The plasmid was generated  
167 by inserting the four FcaPVs gene segments into pMA-T vector using GeneArt  
168 technology (Thermo Fisher Scientific, Waltham, USA). Serial dilutions of  
169 pFcaPV plasmid ranging from  $10^6$  to  $10^2$  copies/5 $\mu$ l were used to calculate the  
170 efficiency of q-PCR for each FcaPV set of primers and compared to the  
171 efficiency calculated on five points of a 2-fold serial dilution of a quantified DNA  
172 template for ALB gene. The efficiency of each q-PCR assay was similar and  
173 ranging from 91% to 98% (average 94,6%). The lesions identified by  
174 histological classification as SCC-AK were presumed to serve as the negative  
175 control group and each sample belonging to this group was also individually  
176 tested by  $\Delta\Delta Cq$  analysis for each FcaPV type. Samples that had eventually  
177 scored a positive  $\Delta\Delta Cq$  value were excluded. In addition, the viral copy  
178 number/ $\mu$ l of input DNA for each FcaPV type in each sample tested was  
179 calculated (AQ). The assays were performed in Rotorgene thermocycler  
180 (Corbett Research, Sydney, Australia) using SSCO sybr green master mix  
181 (Biorad, Hercules, USA) and 5  $\mu$ l of extracted DNA. All samples were tested in  
182 duplicate and the results were calculated using the mean Cq values. Samples  
183 positive for only one replica were considered as negative. All samples with a

184 positive  $\Delta\Delta Cq$  values were submitted to sequence analysis (BMR genomics,  
185 Padova, Italy).

## 186 **Results**

187 Histologically all VPs were recognized for areas of focal epidermal  
188 dysplasia with koilocytes and keratinocytes with cytoplasm enlarged by blue-  
189 greyish material. Regarding BISC diagnosis, the presence of koilocytes or  
190 koilocyte-like cells pointed to the viral origin in 17/20 cases while in the  
191 remaining 3 cases **diagnosis was formulated by** other morphological details  
192 listed in Table 1. **were used to formulate the diagnosis.** SCC and AK showed  
193 the morphological alterations listed in Table 1. Among the 20 BISCs cases 5  
194 VPs were also detected in the adjacent skin by histology and in other 5, sites  
195 of penetration of keratinocytes through the basement membrane into the  
196 dermis were observed indicative of progression toward SCC.

197 Clinical reports for each of the 29 cases fulfilling the established  
198 histological criteria were recorded. Breed, age, gender and anatomical  
199 distribution are summarised in Table 3. Twenty-five subjects were shorthair  
200 domestic cats, two persian and two main coons. Mean age at presentation  
201 varied between 6 and 20 years (median 11,6), 14 out of 29 were female and  
202 15 were male. A total of 4 VPs, 20 BISCs, 4 SCCs and 1 AK were detected.

203 Lesions were observed in densely haired skin regions in 9 cats (dorsum,  
204 flank, and shoulder), in areas non-exposed to sunlight in 1 cat (groin) and in  
205 hypotrichotic and solar exposed skin in 18 cats (ears, nose, eyelids, temporal  
206 region). All cases of SCC were observed in sun-exposed areas. Clinically  
207 VPs were few millimeters wide, **unique alone** or grouped, slightly raised,  
208 pigmented and non-pruritic lesions; BISCs were clinically larger than VPs,  
209 usually more than 1 cm of diameter, multifocally coalescing, raised and often  
210 verrucous, crusted and hyperpigmented. In SCC, erosive and ulcerative  
211 crusted lesions were clinically detected. In the only case in which AK was  
212 diagnosed, erythema, scales and crust were seen (Figure 1a, b, c).

213 P16 was immunolocalized in all VPs epidermis and in the epidermis and  
214 follicular wall of the BISCs. No signal or only faint staining was detected in  
215 the SCC and AK lesion. Immunoreactivity was either nuclear and  
216 cytoplasmic; in VPs and BISCs the signal was strong while in the SCCs  
217 immunoreactivity was faint and therefore considered as no significant  
218 (Figure 1d).

219 By q-PCR we were able to detect the albumin reference gene in 28 out of 29  
220 DNA samples. Only one sample classified as BISC was not suitable to  
221 molecular analysis and therefore it was excluded from further analysis.

222 By AQ, the presence of FcaPVs has been detected in low copies in almost all  
223 cases (Table 4). By RQ ( $\Delta\Delta Cq$  method) a reduced number of samples was  
224 linked to viral lesions. In details, FcaPVs positive  $\Delta\Delta Cq$  values were detected  
225 in all VPs (4/4) and in 8/19 BISCs (Table 3). VPs were positive to FcaPV-2 in  
226 three samples and to FcaPV-1 in one sample. BISCs were positive to FcaPV-  
227 2 in three samples, FcaPV-4 and FcaPV-1 in one sample each. Interestingly,  
228 as never reported before, in BISCs lesion we found three cases of double  
229 infection. In two of those, FcaPV-1 and 2 were detected simultaneously and  
230 in one case FcaPV-2 was present along with FcaPV-4 (table 3).

231 ~~The presence of FcaPVs has been detected in low copies in almost all cases~~  
232 ~~(Table 4 ). By  $\Delta\Delta Cq$  analysis a reduced number of cases was identified:~~  
233 ~~FcaPVs positive  $\Delta\Delta Cq$  value were detected in all VPs (4/4) and in 8/19~~  
234 ~~BISCs (table 3). The PV type most frequently detected was FcaPV-2 that~~  
235 ~~was the unique PV in 3 out of 4 VPs and in 3 BISCs; in 2 BISCs FcaPV-2~~  
236 ~~was associated to FcaPV-1, and in 1 case FcaPV-2 was associated to~~  
237 ~~FcaPV-4. FcaPV-1 was the other detected unique PV type in 1 VP and 1~~  
238 ~~BISC, while FcaPV-4 was the unique PV type in only 1 BISC (Table 3).~~

239 In all cases with a positive  $\Delta\Delta Cq$  value, the viral load was always greater  
240 than  $10^2$  copies / $\mu\text{g}$  DNA (FcaVP-1: min 167 – max 310 Standard Error (SE):

241 33,0; FcaPV-2: min 100 – max 1150 SE: 163,9; FcaPV-4 min 391 – max  
242 3700 SE: 1654,5) (Table 4). Sequence analysis confirmed the FcaPV types  
243 as indicated by q-PCR  $\Delta\Delta Cq$  analysis. ~~Number of  $\Delta\Delta Cq$  FcaPV1-4 positive~~  
244 ~~samples for each type of lesion are summarized and showed in~~  
245 ~~supplementary material.~~

## 246 Discussion

247 In this retrospective study, we detected the concurrent presence, we  
248 ~~investigated the presence of~~ different types of PVs, namely of FcaPV-1, 2,  
249 and 4 in feline VPs and BISCs by  $\Delta\Delta Cq$  method using specific primers. To  
250 the best of our knowledge only the concurrent presence of FcaPV-2 and 3  
251 have been reported from one BISC and one VP.<sup>3,22</sup>

252 . The data obtained on concurrent presence of more than one PV type  
253 is not unexpected since multiple PVs infection has been previously detected  
254 in single skin samples from cats; FcaPV-2 and FcaPV-3 have been  
255 concurrently detected in a feline BISC by Munday et al and from a viral  
256 plaque by Alberti et al. However differently from previous reports, here we  
257 report the presence of FcaPV-1 and FcaPV-4, alone or in association to  
258 FcaPV-2.

259 A limitation when performing PCR-based studies on PVs infection is  
260 that the viral DNA is often detected and cannot be directly linked to an  
261 effective role of virus in pathogenesis and therefore it is not possible to fully  
262 establish whether the presence of a viral genome is uneventful (subclinical  
263 lesion). In particular, FcaPV-2 has been recently detected in a number of skin  
264 swabs from healthy cats, making difficult to discern whether PVs are causing  
265 cancer or are merely an “innocent bystander”.<sup>7</sup> The hypothesis that the  
266 presence of high viral loads likely represents an infectious state of PV is  
267 supported by Thomson et al.<sup>7</sup> who recently showed that the finding of high  
268 copy numbers of FcaPV-2 DNA within a lesion suggests that the detected  
269 virus may be responsible for it. In fact, while high viral copy numbers were  
270 associated with E6/E7 gene expression, no gene expression was detected in  
271 association of low copy numbers, an indication of an incidental finding.

272 For the above-mentioned reasons, for this study we used a q-PCR  
273 protocol applying a  $\Delta\Delta Cq$  method to investigate if and which FcaPVs have  
274 induced the lesion in cats. In this sense the obtained results are partially in  
275 line with the scientific literature which indicates so far that FcaPV-2 is the  
276 major PV type implicated in skin preneoplastic and neoplastic lesions in  
277 cats.<sup>7</sup> Since the genome organization and the role of viral proteins within the  
278 replication cycle are considered similar even between different genera

279 belonging to the *Papillomaviridae* family we assumed that a similar link is  
280 maintained for feline PVs other than FcaPV-2. Importantly we reported  
281 positive  $\Delta\Delta Cq$  values for FcaPV-1 and 4; two FcaPV types rarely reported.<sup>9–</sup>  
282 <sup>11,18</sup> It is noteworthy the observation that also FcaPV-1 and FcaPV-4 showed  
283 positive  $\Delta\Delta Cq$  values however no data are available on these two types for  
284 comparison.

285 A comparison between our data with those obtained from other studies  
286 is difficult for two reasons: i) type of PCR primers used (specific vs  
287 consensus); ii) type of PCR used (conventional vs quantitative). A general  
288 prevalence ranging from 24 to 100% of FcaPV-2 both BISCs and VPs has  
289 been reported.<sup>7,8,27,28</sup> The rate of PV detection by  $\Delta\Delta Cq$  analysis was in  
290 agreement with previous studies which reported a prevalence ranging from  
291 24 to 61% . Moreover, using  $\Delta\Delta Cq$  might have lowered the prevalence. In  
292 our study, by using specific primers in a q-PCR analysis applying RQ  
293 method, we obtained a prevalence for FcaPV-2 in BISCs of 31,6% (6/19) and  
294 for all FcaPV types of 42,10% (8/19).

295 Failure to demonstrate PV DNA in every BISC have been previously explained  
296 through a carcinogenesis model in which papillomaviruses cause transformation  
297 but are only for a short period present within the lesion.<sup>29</sup> Surprisingly, cases in

298 which koilocytes were detected did not show any  $\Delta\Delta Cq$  positive value; this  
299 result is likely due to: i) the detection of koilocytes in very focal lesions that  
300 could have been no more present when additional serial paraffin sections were  
301 prepared for DNA extraction and PCR analysis; ii) the presence of a PCR  
302 undetectable PV variant.

303 Low viral copies for all PV types have been detected in almost all cases,  
304 however the  $\Delta\Delta Cq$  analysis was necessary to associate the FcaPVs as  
305 biological agent potentially causing the lesions.  $\Delta\Delta Cq$  positive values for the  
306 different PV types were detected in all VPs and in 42% of BISCs. Low viral  
307 copies number was also detected from the SCC-AK group resulting in a not  
308 detectable  $\Delta\Delta Cq$ . Noteworthy, mean  $\Delta\Delta Cq$  of positive samples differed  
309 among PV types being 62.5, 189.1 and 3083.5 for FcaPV-1, FcaPV-2 and  
310 FcaPV-4 respectively. Studies considering a larger panel of cases might be  
311 necessary to establish a further  $\Delta\Delta Cq$  cut off value.

312 Here we document the presence of FcaPV-2 with high prevalence in both  
313 VPs and BISCs while we rescued FcaPV-1 and FcaPV-4 less frequently;  
314 these last two PVs are rarely described as playing an active role in skin and  
315 oral mucosa lesions in cats.<sup>9,10,18,30</sup> None of the lesions was associated to

316 FcaPV-3 when adopting  $\Delta\Delta\text{Cq}$  method, confirming the rare occurrence of this  
317 FcaPV type also in Italy.

318 Either cytoplasmic or nuclear p16 signals were detected in all cases where  
319 cytopathic effects were found (VP, BISC) while p16 immunolocalisation was  
320 present with faint and mainly cytoplasmic signal in cases of SCCs and no p16-  
321 immunoreactivity was found in the case of AK. Despite the difficulties of  
322 interpretation when the signal was faint, these results are in line with what  
323 already documented previously. About half of the SCC studied by Thomson et  
324 al.<sup>7</sup> were negative to p16 immunostaining. A strong association between  
325 FcaPV-2 E6/E7 gene expression and p16 immunostaining have been recently  
326 found in feline SCC with 18 of 20 (90%) E6/E7-positive SCCs being also  
327 positive for p16 compared to 13 of 40 (33%) E6/E7-negative SCCs.<sup>7</sup> E6/E7 gene  
328 expression investigation has not been carried out in our study but our results  
329 might reflect this correlation.

### 330 **Conclusion**

331  $\Delta\Delta\text{Cq}$  analysis has proved to be necessary to associate the FcaPVs as  
332 biological agent potentially causing the lesions. Based on this method the  
333 presence of FcaPV-2 is confirmed to be the most representative FcaPV in  
334 feline skin lesions referable to diagnosis of VP and BISC in Italy. To a lesser

335 extent also FcaPV-1 and FcaPV-4 has been detected in our examined  
336 samples. while FcaPV3 was never detected.

337

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440

441

## 442 LEGEND

443 Figure 1. Multiple symmetrical raised, hyperpigmented and crusted plaques  
444 on the face of a cat with Bowenoid in situ carcinoma (a). Histopathology from

445 a viral plaque in a cat. Focal epidermal hyperplasia, absence of follicular wall  
446 involvement, evident viral cytopathic effects (koilocytes) (bar=50 $\mu$ m) (b);  
447 Histopathology from a Bowenoid in situ carcinoma in a cat. Epidermal and  
448 follicular dysplasia with upward in situ keratinocyte proliferation and a few  
449 koilocytes (bar=50 $\mu$ m) (c); p16 immunohistochemistry, groups of  
450 keratinocytes show nuclear and cytoplasmic positivity (ABC system, bar=100  
451  $\mu$ m) (d).

452

Table 1. Histological criteria for the diagnosis of VP VP, BISC, SCC and AK.

Type of lesion	Histopathological criteria
VP	Focal epidermal hyperplasia, koilocytes, clear cells, giant keratoyalin granules, cytoplasm enlarged by blue-greyish fibrillar material, hyperpigmentation;
BISC	Epidermal and follicular dysplasia with upward in situ keratinocyte proliferation, loss of nuclear polarity, windblown nuclei, koilocytes, mitosis;
SCC	Epidermal dysplasia and atypia with downward keratinocyte proliferation, keratinocyte invasion to the dermis through the basal lamina, mitosis;
AK	Epidermal dysplasia and atypia with upward in situ keratinocyte proliferation, squamotisation, apoptosis

Legend: VP viral plaque; BISC Bowenoid *in situ* carcinoma; SCC squamous cell carcinoma; AK actinic keratosis

Table 2. Primers nucleotide sequences

Oligonucleotide name	Nucleotide sequence (5'-3')	5' Nucleotide position	Reference sequence accession number
FcaPV1-F	AGGATGGTGACATGGTGGAT	7143	AF480454
FcaPV1-R	TTTGCACTGTGTGTCTGCAA	7246	
FcaPV2-F	TACACGCGGTACCAATTTCA	7191	EU796884
FcaPV2-R	AGAGTGACCACGCACACTTG	7331	
FcaPV3-F	AAGATTGGTATGGCGTTTGC	5960	JX972168
FcaPV3-R	TTTGCCTTTCATCTGCTGTG	6105	
FcaPV4-F	ATGCAAATGGCCAGACTTTC	6356	KF147892
FcaPV4-R	AAAAATGGCGGCAGTACAAC	6453	
Fel Alb F	GATGGCTGATTGCTGTGAGA	3706	NC_018726.2 GPC_000001738
Fel Alb-R	CCCAGGAACCTCTGTTTCATT	3855	

1

Table 3.

Signalment, lesion distribution, p16 immunolocalisation and FcaPV type assessed by  $\Delta\Delta Cq$  analysis in cats with of VP, BISC, SCC and AK diagnosis

Case N°	Diagnosis	Breed	Years	Gender	Lesion distribution	p16	FcaPV type	$2^{-\Delta\Delta Cq}$
1	VP	DSH	10	M	Flank	+	2	119
2	VP	persian	15	F	Groin	+	2	53
3	VP	DSH	9	M	Nose	+	1	72
4	VP	main coon	10	F	Nose	+	2	271
5	BISC	DSH	10	F	Flank	+	-	
6	BISC	main coon	6	M	Shoulder	+	4	5836
7	BISC	DSH	13	M	Nose	+	-	
8	BISC	DSH	13	F	Nose	+	1	49
9	BISC	DSH	9	F	Temporal	+	-	
10	BISC	DSH	20	F	Dorsal	+	ND	
11	BISC	DSH	15	M	Temporal	+	2-4	224-331
12	BISC	DSH	15	M	Nose	+	-	
13	BISC	DSH	16	F	Shoulder	+	-	
14	BISC	DSH	12	M	Ear	+	-	
15	BISC	DSH	12	M	Dorsum	+	2	172
16	BISC	DSH	8	F	Temporal	+	1-2	58-167
17	BISC	DSH	10	F	Temporal	+	-	
18	BISC	persian	15	M	Eyelid	+	2	211
19	BISC	DSH	10	F	Ear	+	-	
20	BISC	DSH	7	F	Flank	+	-	
21	BISC	DSH	10	M	Nose	+	2	159
22	BISC	DSH	15	M	Shoulder	+	-	
23	BISC	DSH	12	M	Nose	+	1-2	71-326
24	BISC	DSH	10	M	Flank	+	-	
25	SCC	DSH	9	F	Ear	+-	-	
26	SCC	DSH	8	M	Nose	+-	-	
27	SCC	DSH	15	F	Ear	+-	-	
28	SCC	DSH	10	M	Eyelid	+-	-	
29	AK	DSH	11	F	Ear	+-	-	

2

3 Legend: VP viral plaque, BISC Bowenoid *in situ* carcinoma, SCC squamous cell carcinoma, AK actinic  
4 keratosis, DSH domestic shorthair, ND non determined.

5  $2^{-\Delta\Delta Cq}$  indicates the fold expression change of FcaPV normalised to Albumin.

6

Table 4. Absolute viral load quantification expressed as viral copy number/ $\mu\text{g}$  of input DNA

Cases	Type of lesion	FcaPV-1	FcaPV-2	FcaPV-3	FcaPV-4
1	VP	10	<b><u>153</u></b>	10	20
2	VP		<b><u>100</u></b>		6
3	VP	<b><u>167</u></b>	33	23	28
4	VP		<b><u>1130</u></b>		
5	BISC	30	48		
6	BISC	18	55	26	<b><u>3700</u></b>
7	BISC	13	2		4
8	BISC	<b><u>201</u></b>			
9	BISC	42	12		
10	BISC		15		
11	BISC	87	<b><u>1150</u></b>	59	<b><u>391</u></b>
12	BISC		10		
13	BISC	33	55	28	120
14	BISC				
15	BISC	14	<b><u>436</u></b>		
16	BISC	<b><u>176</u></b>	<b><u>210</u></b>	13	
17	BISC		18		
18	BISC		<b><u>1120</u></b>	79	45
19	BISC	3			
20	BISC	33	10		
21	BISC	6	<b><u>193</u></b>		
22	BISC				
23	BISC	<b><u>310</u></b>	<b><u>1140</u></b>	18	26
24	BISC				
25	SSC		6	2	
26	SSC	12	10		17
27	SSC	20	15	7	23
28	SSC	10	5	14	14
29	AK	12	18		
<i>Mean viral load</i>		63	247.7	2.4	366.2
<i>Mean viral load of <math>\Delta\Delta\text{Cq}</math> positive samples</i>		213.5	625.8		2045.5
<i>Standard error of <math>\Delta\Delta\text{Cq}</math> positive samples</i>		33	163.9		1654.5

Bold and underlined numbers indicate  $\Delta\Delta\text{Cq}$  positive samples.

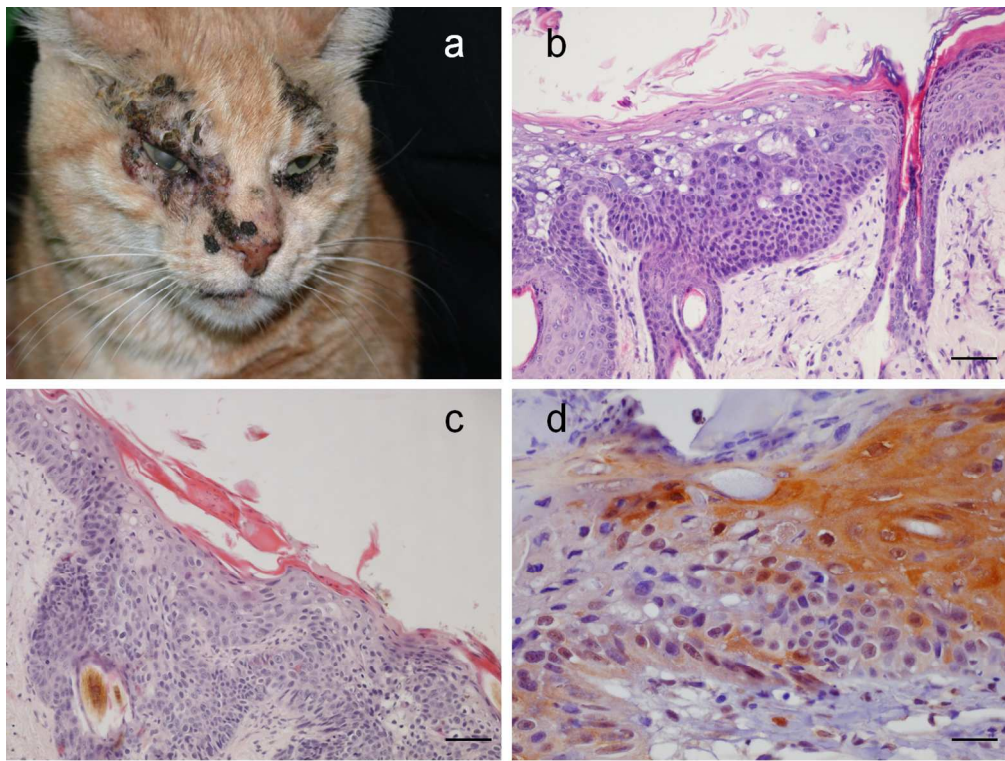


Figure 1

136x103mm (300 x 300 DPI)

review