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Investigation of multiple *Felis catus* papillomavirus types (-1/-2/-3/-4/-5/-6) DNAs in feline oral squamous cell carcinoma: a multicentric study

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ABSTRACT. Recent evidence suggests a possible association of *Felis catus* papillomavirus type 2 (FcaPV-2) DNA with feline oral squamous cell carcinoma (FOSCC). In this study, type-specific PCR targeting two genes (L1/E6 or E1/E6) of FcaPV-1/-2/-3/-4/-5/-6 was performed to detect viral DNA in a large amount of FOSCC samples collected in Italy and Austria. FcaPV-1/-2/-3/-4/-5 were detected in 7/113 (6.2%), 7/93 (7.5%), 6/113 (5.3%), 1/113 (0.9%) and 2/113 (1.8%) specimens, respectively, with different prevalences in Italian *vs.* Austrian samples, whilst FcaPV-6 went undetected. Our results confirms that FcaPV-2 is the most prevalent in FOSCC, followed by FcaPV-1/-3 and suggest that FcaPVs have variable circulation rates in European countries.

KEYWORDS: cat, *Felis catus* papillomavirus, oral squamous cell carcinoma, PCR

Papillomaviruses (PV) are oncogenic viruses that infect cutaneous and mucosal epithelia in human and animal species, causing development of proliferative lesions ranging from warts to squamous cell carcinoma (SCC) [17].

In domestic cat (*Felis catus*), six FcaPV types (-1/-2/-3/-4/-5/-6) have been characterized, so far [17]. FcaPV-2 is recognized as the causative agent of cutaneous SCC and its precursors, since it is widely detectable and transcriptionally active in tumor samples and displays transforming properties *in vitro* through the molecular activities of its E6/E7 oncogenes [2, 4, 26]. Alongside FcaPV-2, FcaPV-1/-3/-4/-5/-6 are rarely associated with skin lesions, however their role in cancer development is not fully understood [8, 10, 14, 23, 27, 30].

In humans, 20–25% of oral SCCs are causally associated with infection by Human PV-16 (HPV-16), whilst in cats the possible association between PV infection and oral cancer is still debated [17, 24]. Indeed, most of the studies, based on consensus PCR on formalin-fixed paraffin-embedded (FFPE) tissues, yielded scarce to null detection of viral sequences in feline oral SCC (FOSCC) [18, 21, 22]. However, our recent work pointed out that the use of type-specific primers amplifying short sequences increased PCR performance, leading to detection of FcaPV-2 in 10/32 (31%) FOSCC samples in Italy [6]. Moreover, studies conducted with similar methods in Japan found FcaPV-2 in 12/28 (42.9%) tumors, strengthening the possibility of a biologically significant association [29].

The aim of this work was to assess, by using the appropriate PCR strategy, the presence of FcaPV-1/-2/-3/-4/-5/-6 in a large cohort of FOSCC samples collected in two different countries (Italy and Austria), in order to obtain a reliable estimation of their possible association with these tumors.

To this purpose, a total of 113 FFPE-FOSCC samples (T1–T113) were collected: samples T1–T32 (32/113) were retrieved from the archives of the "Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri"", Rome, Italy (years 2013–2020). Of these, 20/32 (T1–T19, T32) belonged to the cohort of samples analyzed for the presence of FcaPV-2 in our previous work and 9/20 had tested positive (T2–T7, T10–T11, T32) [6]; samples T33–T113 (81/113) were retrieved from the archive of the Research Group Oncology (RGO) or the Institute of Pathology of the University of Veterinary Medicine, Wien, Austria (years 2001–2019). All the samples were from household cats; the ages of 100 affected cats were known, ranging from 5 to 19 years

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(mean 13.27). Sections of 5/10 μm thickness were cut with separate microtome blades and stored in sterile tubes for subsequent molecular analysis. Additionally, 3 FOSCC cell lines (SCCF1 and FcaPV-2 positive SCCF2 and SCCF3), cultured as indicated in literature, were included in the study [5, 12, 13, 25].

DNA extraction and PCR reactions were performed as previously described [6]. The quality of DNA samples was ensured by PCR for feline β-globin or β-actin as reported elsewhere [3, 7]. Amplification of FcaPV-1 E1 and FcaPV-2/-3/-4 L1 was carried out by employing the validated type-specific primer pairs FPV1F/R, JMPF/R, JMY2F/R, JMY3F/R, respectively, whilst new primers amplifying FcaPV-5/-6 L1 were designed for this study [16, 19, 20]. PCR for FcaPV-2 E6 was performed as previously described, additional primer pairs were synthesized to specifically amplify E6 from FcaPV-1/-3/-4/-5/-6 [4]. Primers details including names, sequences, annealing temperatures and intended product sizes are summarized in Supplementary Table 1. For each PCR reaction, samples with no DNA template were included as negative control. Cloned FcaPV-1 and FcaPV-2 genomes, DNA from an FcaPV-3 positive SCC, gBlocks Gene Fragments (Integrated DNA Technologies, Coralville, IA, USA) mimicking the FcaPV-4/-5/-6 L1 and E6 intended amplicons were employed as positive controls. PCR products were visualized by agarose gel electrophoresis and UV light as previously described [6]. Randomly chosen amplicons from E6 PCR were purified by using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and submitted to sequence analysis (BMR-Genomics, Padova, Italy). The obtained sequences were aligned to viral genes using the Basic Local Alignment Search Tool (NCBI/BLAST). FOSCC samples and cell lines were tested for the presence of viral DNAs by amplifying two genes of each viral type, such as E1/E6 for FcaPV-1 and L1/E6 for FcaPV-2/-3/-4/-5/-6; samples T103-T113 could be analyzed only for E6, T32 only for E1/L1. For a comprehensive view of PCR results, see Table 1.

FcaPV-1 was detected in 7/113 (6.2%) FOSCC samples, of these 1/113 (0.9%) was positive only for E1 (T39), 6/113 (5.3%) only for E6 (T34, T48, T61, T64, T74, T103). In the case of FcaPV-2, 20/113 samples (T1-T19, T32), accounting for most of Italian cohort (20/32), were neither analyzed nor included in the prevalence rates, given their belonging to data already published in our previous study [6]. Among the rest of the samples, 7/93 (7.5%) tested positive for FcaPV-2 DNA, of which 3/93 (3.2%) for L1 (T37, T39, T42), 5/93 (5.4%) for E6 (T39, T85, T93, T102, T112), 1/93 (1.3%) for both genes. FcaPV-3 was detected in 6/113 (5.3%) FOSCC samples, of these 5/113 (4.4%) were positive for L1 (T4, T32, T38, T52, T63), 4/113 (3.5%) for E6 (T3, T4, T38, T63), 3/113 (2.6%) were double-positive. FcaPV-4 and FcaPV-5 DNAs were amplified in 1/113 (0.9%; T66) and 2/113 (1.8%; T22, T28) samples, respectively, solely by L1 PCR. FcaPV-6 was not found in any of the analyzed specimens. Four co-infections could be evidenced: T39 was positive for FcaPV-1/-2, three FcaPV-3 positive FOSCCs (T3, T4, T32) were known to harbor also FcaPV-2 from Altamura *et al.*, 2020 [6]. Sequencing of randomly chosen E6 PCR products confirmed the identity of the amplicons. Representative PCR gels are shown in Supplementary Figs. 1 and 2. There was no difference in the age between PV-positive and negative cases.

When splitting results according to geographical area (Table 2), it came out that in Austrian samples FcaPV-1 and -2 shared the highest prevalence (7/81, 8.6% for both), followed by FcaPV-3 (3/81, 3.7%) and -4 (1/81, 1.2%), whilst FcaPV-5 went undetected. In samples from Italy, FcaPV-1/-4 were not found, whilst FcaPV-3 and -5 were revealed with a prevalence of 9.3% (3/32) and 6.2% (2/32), respectively. Negative FcaPV-2 PCR results (0/12) and thus null prevalence in Italian samples analyzed *ex novo* in this study (12/32) were not deemed to be representative due to the small sample size, therefore they were not further considered throughout the manuscript.

No viral DNA was amplifiable in SCCF1, and no additional viral types were detected in FcaPV-2 positive SCCF2/3 cell lines.

This is the first study specifically testing the presence of each FcaPV type in such a numerous sample of FOSCC and cell lines. A possible limitation may be DNA fragmentation occurring in FFPE samples, which might have influenced PCR results; however,

Table 1. Detection of *Felis catus* papillomavirus (FcaPV) type -1/-2/-3/-4/-5 in feline oral squamous cell carcinoma by PCR

	FcaPV-1			FcaPV-2			FcaPV-3			FcaPV-4			FcaPV-5			FcaPV-6		
	E1	E6	E1/E6	L1	E6	L1/E6	L1	E6	L1/E6	L1	E6	L1/E6	L1	E6	L1/E6	L1	E6	L1/E6
Per gene	1/113 (0.9%)	6/113 (5.3%)	0/113 (0%)	3/93 (3.2%)	5/93 (5.4%)	1/93 (1.3%)	5/113 (4.4%)	4/113 (3.5%)	3/113 (2.6%)	1/113 (0.9%)	0/113 (0%)	0/113 (0%)	2/113 (1.8%)	0/113 (0%)	0/113 (0%)	0/113 (0%)	0/113 (0%)	0/113 (0%)
Total	7/113 (6.2%)			7/93 (7.5%)			6/113 (5.3%)			1/113 (0.9%)			2/113 (1.8%)			0/113 (0%)		

For each viral type, single-positive (E1 or L1 and E6), double-positive (E1/E6 and L1/E6) and total PCR positive ratios (%) are indicated.

Table 2. PCR positive ratios (%) for each *Felis catus* papillomavirus (FcaPV) type splitted according to geographical area of feline oral squamous cell carcinoma (FOSCC) sampling (Italy *vs.* Austria)

	FcaPV-1	FcaPV-2	FcaPV-3	FcaPV-4	FcaPV-5	FcaPV-6
FOSCC Italy	0/32 (0%)	0/12 (0%)	3/32 (9.3%)	0/32 (0%)	2/32 (6.2%)	0/32 (0%)
FOSCC Austria	7/81 (8.6%)	7/81 (8.6%)	3/81 (3.7%)	1/81 (1.2%)	0/81 (0%)	0/81 (0%)

in agreement with previous studies, our data confirm that the use of type-specific primers amplifying short viral sequences allows detection of FcaPVs in FFPE-FOSCCs, likely overcoming this issue and ensuring a better yield with respect to consensus oligos amplifying longer sequences [6, 18, 21, 22, 29]. FcaPV-2 was found to be the most prevalent and confirmed as the unique viral type associated with SCCF2/3 cell lines [5]. These data are in line with the growing evidence supporting a role for FcaPV-2 in development of a subset of FOSCC, such as detection of viral DNA and transcriptional activity in tumor samples and its biological similarities with mucosal HPV-16 in experimental models [4, 5, 26, 29]; nevertheless, FcaPV-2-associated FOSCC has been proposed as a spontaneous animal model of human counterpart [1].

Here we also report, to a lesser extent, detection of FcaPV-1 (6.2%) and FcaPV-3 (5.3%). Our results seem to corroborate some previous studies that reported the presence of FcaPV-1 in 1/36 FOSCC via PCR and FcaPV-3 in 1/20 samples via virome sequencing approach [9, 16]. Additional epidemiological and experimental studies are warranted to eventually confirm whether these two FcaPV types may be a possible rare "cause" of FOSCC. In contrast, prevalence data on FcaPV-4/-5 (0.9%/1.8%) revealed sporadic detection of unlikely biological meaning in FOSCC.

In co-infections, FcaPV-1 or FcaPV-3 were always co-detected along with FcaPV-2, therefore it is likely that this latter plays a major role; however, recent studies suggest that the "causative" agent in skin lesions associated with multiple FcaPVs is identifiable by a higher viral load [14, 27]. Future studies would clarify whether this scenario is conceivable in FOSCC as well.

Interestingly, E6 PCR greatly increased the positive ratios with respect to E1 for FcaPV-1. Studies in human and animal species suggest that this may be due to PV integration into host genome, leading to disruption of E1 gene and thus resulting in loss of some PCR positivity [11, 15]; similarly, the occurrence of E6 single-positive samples for other viral types might be related to integration events which interrupts L genes [28]; however, viral kinetics of FcaPVs are currently unknown, therefore further investigations are needed to clarify this point.

A recent report from Asian countries suggests that the rate of detection of FcaPV-2 in FOSCC may vary according to its general prevalence in a given geographical area; indeed, authors found it in 11/19 tumors from Taiwan *vs.* 1/9 samples from Japan, where in fact FcaPV-3 and -4 are more prevalent [29, 30]. Consistently, when pooling data on FcaPV-1/-3/-4/-5 obtained in the current study (0/32, 3/32, 0/32, 2/32, respectively) with those on FcaPV-2 from our recent work (10/32), this latter appeared as the most frequently detectable in FOSCC from Italy, in agreement with previous studies reporting FcaPV-2 as highly prevalent over all viral types in this country [6, 14, 27]. Therefore, it is likely that the different detection rates of each FcaPV in Austrian samples compared with Italian ones might be representative of diverse general prevalences. However, the possibility that the different sizes of samples groups (81 *vs.* 32) may have influenced some results should be taken into account. Finally, this work hints that FcaPV-6, originally isolated in Australia, would be neither circulating in Austria nor in Italy and/or would not exert mucosal tropism [8].

In conclusion, this study confirms that type-specific PCR allows detection of FcaPV-2 and, to a lesser extent, of FcaPV-1/-3 in a subset of FOSCC, thus encouraging further work aimed at unveiling their possible role in oral carcinogenesis and reveals that differences in the prevalence of each viral type may exist among European countries.

CONFLICT OF INTEREST. The authors declare that they have no conflict of interest.

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