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

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:	Abstract 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). 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. 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. 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.

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

Abstract

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).

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 $\Delta\Delta$Cq values, which represent a normalized measure of DNA viral quantity. Samples with a positive $\Delta\Delta$Cq values were submitted to sequence analysis.

Results: 4 VPs, 19 BISCs 4 SCCs and 1 AK were included. By $\Delta\Delta$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.

Conclusion and relevance: Using ΔΔCq 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 ΔΔCq investigation. Compared to conventional PCR the ΔΔCq method has the advantage to establish a possible role of the virus in the outcome of infection.

Introduction

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

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

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

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

Materials and methods

Biopsies

Feline skin biopsies have been identified from dermatopathological databases

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

P16 immunohistochemistry

Cases were evaluated for the detection of p16 by immunohistochemistry employing an ABC system (Vector Laboratories, Burlingame, USA). Antigen unmasking was carried out at 120°C for 3 min in a pressure cooker with a TRIS-EDTA buffer (1,2 g/L Tris, 0,36 g/L EDTA, pH 9.0). Sections were pre-treated for 10 min with 1% H_2O_2 in 0.1 M phosphate buffered saline (PBS), pH 7.4 to quench endogenous peroxidase activity, and blocked for 30 min at room temperature (rt) in PBS with a 2% normal horse serum (PK-7200, Vector Laboratories, Burlingame, USA) and 0.05% TritonX-100. Sections were then incubated overnight at 4˚C with a 1:100 diluted monoclonal primary antibody

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

Sampling material for q-PCR

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

DNA extraction

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

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

q-PCR

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

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

positive ΔΔCq values were submitted to sequence analysis (BMR genomics, Padova, Italy).

Results

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

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

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

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

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

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

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

Discussion

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

238 and one VP.[3,22] 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".[7] The hypothesis that the
247 presence of high viral loads likely represents an infectious state of PV is
248 supported by Thomson et al.[7] 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

major PV type implicated in skin preneoplastic and neoplastic lesions in cats.[7] Since the genome organization and the role of viral proteins within the replication cycle are considered similar even between different genera belonging to the *Papillomaviridae* family we assumed that a similar link is maintained for feline PVs other than FcaPV-2. Importantly we reported positive ΔΔCq values for FcaPV-1 and 4; two FcaPV types rarely reported. [9–11,18]

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

Failure to demonstrate PV DNA in every BISC have been previously explained through a carcinogenesis model in which papillomaviruses cause transformation but are only for a short period present within the lesion. [29] Surprisingly, cases in which koilocytes were detected did not show any ΔΔCq positive value; this 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 ΔΔCq analysis was necessary to associate the FcaPVs as
281 biological agent potentially causing the lesions. ΔΔCq positive values for the
282 different PV types were detected in all VPs and in 42% of BISCs.
283 Noteworthy, mean ΔΔCq 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 ΔΔCq 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.[9,10,18,30] None of the lesions was associated to
291 FcaPV-3 when adopting ΔΔCq 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

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

Conclusion

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

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LEGEND

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

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

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

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

Abstract

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

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 ΔΔCq values, which represent a normalized measure of DNA viral quantity. Samples with a positive ΔΔCq values were submitted to sequence analysis.

Results: 4 VPs, 19 BISCs 4 SCCs and 1 AK were included. By ΔΔCq analysis we found that all VPs were positive for FcaPV-1 or FcaPV-2; and 8 BISCs for

FcaPV-1, 2, 4. ~~The ΔΔCq detected 3 out of 4 types of FcaPVs. ΔΔCq detected FcaPV-1 in 1 VP and 3 BISCs, FcaPV-2 in 3 VPs and 6 BISCs, FcaPV-4 in 2 BISCs.~~ FcaPV-2 was the most prevalent among the group of VPs and BISCs.

Conclusion and relevance: Using ΔΔCq 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 ΔΔCq investigation. Compared to conventional PCR the ΔΔCq method has the advantage to ~~allow~~ establish a possible role of the virus in the outcome of infection.

Introduction

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

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

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

done in these countries without any epidemiological meaning. In Europe PVs infections have been reported in Switzerland in ~~only~~ nine cases,[12,20] in one case in Germany [21] and in ~~another~~ one case in Italy.[22] Here we assess by a combination of histology, immunohistochemistry and q-PCR the concurrent presence of FcaPV-1, 2, 3 and 4 viral DNA and p16 immunostaining in 29 feline lesions collected in Italy including non neoplastic and preneoplastic, viral and non viral induced skin lesions namely: VP, BISC, invasive SSC and actinic keratosis (AK). Two methods were applied to analyse the viral load data: absolute quantification (AQ) and relative quantification (RQ). The AQ was applied to determine the viral copy number per µg of DNA; however, by this method wide range of values can be obtained without a cut-off value that would allow to discriminate FcaPVs presence as innocent bystander or as responsible of viral lesion. To address this problem we approached the RQ by ΔΔCq calculation. [23] This method measures viral genome presence by relating the obtained values of each of the 4 FcaPVs in each lesion classified as VP or BISC to those of SCC and AK.

Materials and methods

Biopsies

Feline skin biopsies have been identified from dermatopathological databases

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

P16 immunohistochemistry

Cases were evaluated for the detection of p16 by immunohistochemistry employing an ABC system (Vector Laboratories, Burlingame, USA). Antigen unmasking was carried out at 120°C for 3 min in a pressure cooker with a TRIS-EDTA buffer (1,2 g/L Tris; 0,36 g/L EDTA; pH 9.0). Sections were pre-treated for 10 min with 1% H_2O_2 in 0.1 M phosphate buffered saline (PBS), pH 7.4 ~~10 min~~ to quench endogenous peroxidase activity, and blocked for 30 min at room temperature (rt) in PBS with a 2% normal horse serum (PK-7200, Vector Laboratories, Burlingame, USA) and 0.05% TritonX-100. Sections were then incubated overnight at 4˚C with a 1:100 diluted monoclonal primary antibody

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

Sampling material for q-PCR

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

DNA extraction

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

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

q-PCR

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

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

positive ΔΔCq values were submitted to sequence analysis (BMR genomics, Padova, Italy).

Results

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

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

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

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

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

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

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

In all cases with a positive ΔΔCq value, the viral load was always greater than 10^2 copies /µg DNA (FcaVP-1: min 167 – max 310 Standard Error (SE):

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

Discussion

In this retrospective study, we detected the concurrent presence, ~~we investigated the presence~~ of different types of PVs, namely of FcaPV-1, 2, and 4 in feline VPs and BISCs by ΔΔCq method using specific primers. To the best of our knowledge only the concurrent presence of FcaPV-2 and 3 have been reported from one BISC and one VP.[3,22]

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

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

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

belonging to the *Papillomaviridae* family we assumed that a similar link is maintained for feline PVs other than FcaPV-2. Importantly we reported positive ΔΔCq values for FcaPV-1 and 4; two FcaPV types rarely reported. [9–11,18] ~~It is noteworthy the observation that also FcaPV-1 and FcaPV-4 showed positive ΔΔCq values however no data are available on these two types for comparison.~~

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

Failure to demonstrate PV DNA in every BISC have been previously explained through a carcinogenesis model in which papillomaviruses cause transformation but are only for a short period present within the lesion. [29] Surprisingly, cases in

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

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

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

316 FcaPV-3 when adopting ΔΔCq 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.[7] 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.[7] E6/7 gene
328 expression investigation has not been carried out in our study but our results
329 might reflect this correlation.

330 **Conclusion**

331 ΔΔCq 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

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

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LEGEND

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

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

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-greish 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 downword keratinocyte proliferation, keratonocyte invasion to the dermis through the basal lamina, mitosis;
AK	Epidermal dysplasia and atipia 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	CCCAGGAACCTCTGTTCATT	3855	

Table 3.
Signalment, lesion distribution, p16 immunolocalisation and FcaPV type assessed by ΔΔCq 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	+-	-	

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

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

Table 4. Absolute viral load quantification expressed as viral copy number/μg of input DNA

Cases	Type of lesion	FcaPV-1	FcaPV-2	FcaPV-3	FcaPV-4
1	VP	10	**153**	10	20
2	VP		**100**		6
3	VP	**167**	33	23	28
4	VP		**1130**		
5	BISC	30	48		
6	BISC	18	55	26	**3700**
7	BISC	13	2		4
8	BISC	**201**			
9	BISC	42	12		
10	BISC		15		
11	BISC	87	**1150**	59	**391**
12	BISC		10		
13	BISC	33	55	28	120
14	BISC				
15	BISC	14	**436**		
16	BISC	**176**	**210**	13	
17	BISC		18		
18	BISC		**1120**	79	45
19	BISC	3			
20	BISC	33	10		
21	BISC	6	**193**		
22	BISC				
23	BISC	**310**	**1140**	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		
Mean viral load		*63*	*247.7*	*2.4*	*366.2*
Mean viral load of ΔΔCq positive samples		*213.5*	*625.8*		*2045.5*
Standard error of ΔΔCq positive samples		*33*	*163.9*		*1654.5*

Bold and underlined numbers indicate ΔΔCq positive samples.

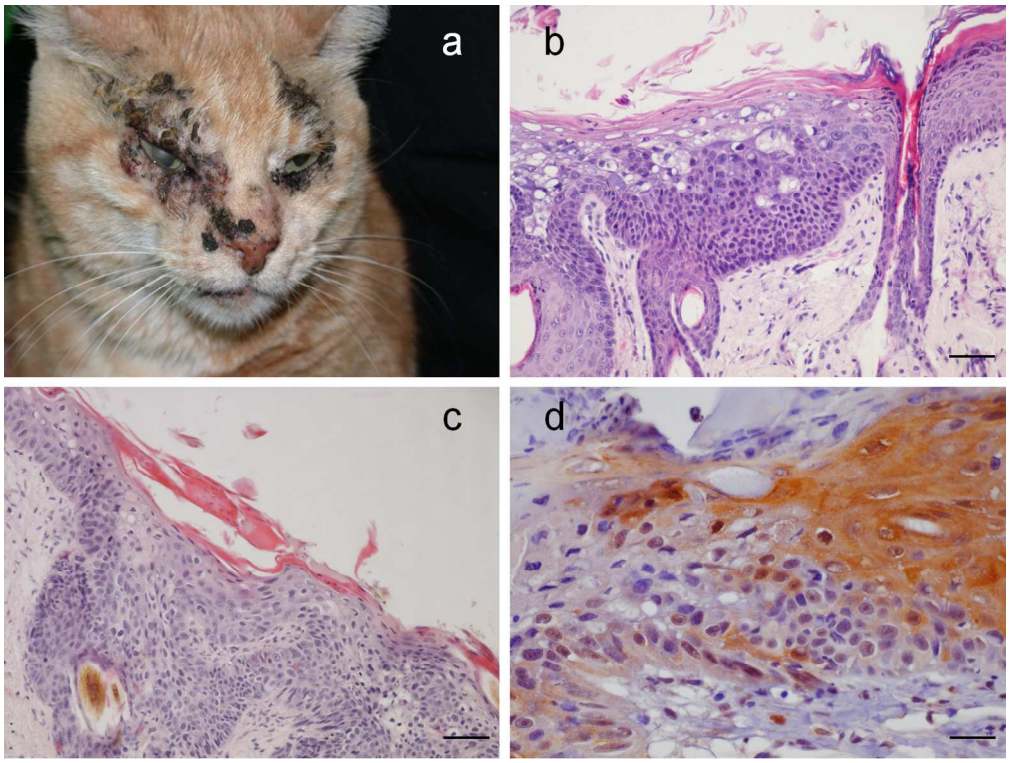

Figure 1

136x103mm (300 x 300 DPI)