Canine and feline viral dermatoses with a particular emphasis to papillomavirus infections

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Canine and feline viral dermatoses with a particular emphasis to papillomavirus infections
(Virale Dermatosen bei Hunden und Katzen unter spezieller Berücksichtigung der Papillomavirus-
Infektionen)

HABILITATIONSSCHRIFT

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Chapter 1

General introduction
Aims and scope of the thesis
Skin lesions are a predominant feature of many viral diseases in humans and large domestic mammals [1, 2]. In comparison, viral dermatoses are rarely described in dogs and cats [3]. However, they may be underdiagnosed due to the difficulty in detecting viruses. The development and increased availability of diagnostic techniques such as electron microscopy, immunohistochemistry, viral amplification (PCR) is making detection more routine [4]. To complement advances in diagnosis, several effective antiviral agents for treating at least some viral dermatoses have been developed in the last few years [1]. Consequently, making the correct diagnosis may actually have important consequences for therapy as well as for prognosis.

**Definition and classification:**

Viruses form a diverse group of non-cellular infectious agents that share a distinct composition and a unique mode of replication. These agents lack much of the enzymatic machinery necessary for their multiplication. They are consequently obligate intracellular parasites that multiply inside cells and use the synthetic apparatus of the host cell to produce their own components [1].

The animal viruses are divided in several families according to their shape, structure of virion and the type of nucleic acid within it [5-7]. In fact, in the virion, the genome of the virus consists of only one type of nucleic acid (DNA or RNA). The viral particle (virion) is made of the nucleic acid surrounded by a protective protein core called the capsid. Some viruses do additionally possess an envelope which play a major in the infection of host cells [5-7].

**Viral Replication and host response:**

Viruses make use of the host-cell machinery to synthesize and assemble viral particles (replication) because they do not possess the required enzymes. Viruses encode for structural (capsid) proteins and non structural proteins that usually regulate the viral replication. Some
of these non-structural proteins (transforming proteins) are responsible of the oncogenic potential of tumour viruses, like high-risk papillomavirus [8, 9]. Some viruses, like papillomaviruses depend on epithelial cell differentiation for completion of their replication [10]. After an initial inoculation in the basal layer of the epithelium, non-structural (early) proteins are expressed in the suprabasal layers and in the stratum spinosum. Capsid (late) proteins are subsequently produced in the stratum spinosum and the viral particles assembled and released in the upper stratum granulosum and stratum corneum, respectively [10].

Epithelial host-cells are infected by papillomaviruses through inoculation but for most other epithelium-infecting viruses, the replication cycle begins with an attachment phase called adsorption [1, 11]. This attachment requires specific interaction between host-cell receptors and virus. Cells lacking virus-specific receptors are not susceptible to infection. Following adsorption and penetration, viral envelopes and capsids are destroyed (uncoating) and viral genome can therefore instruct the host-cell machinery to produce its own proteins. Viral particles of most viruses infecting epithelial cells (herpes, papillomavirus, poxvirus) are produced inside the cells and released after cell death or cytolysis [1].

Each virus has its own site of replication: Herpesviruses and papillomaviruses replicate in the nuclei whereas poxviruses multiply in the cytoplasm. Viral replication usually causes gross cytopathic changes and host-cells sometimes die. These cytopathic effects may be pathognomonic of one specific viral infection (viral inclusions and pseudo-inclusions, syncytium formation (herpesvirus, retrovirus, paramyxovirus), modified keratinisation process (papillomaviruses).

Some viruses, like papillomaviruses or distemper virus, can however, at least in some instances, replicate without causing irreversible damage to the host keratinocytes (true commensality, chronic infections)[12-15].

Another form of non cytocydal infection is the latency (herpesviruses, papillomaviruses). In this instance, very few or no virion are produced in the infected cells but reactivation of the infection can occur at any moment.

Last but not least, some viruses (papillomaviruses, retroviruses) are able to induce host-cell immortalization and neoplastic transformation. Most of the time, transformed host-cells loose their ability to sustain productive infection [8, 9].
**Viral infection and skin lesions:**

Virus-induced skin lesions are usually the direct consequence of the virus replication. Examples of these direct effects are wart formations associated with papillomaviruses infections, pock formation in poxviruses infections or vesiculation associated with herpesvirus infections. Some skin lesions are however due to the host response or to the interaction of replication and host response. Erythema multiforme, for example, are exanthematous skin lesions associated with herpesvirus infection in humans and cats and may be, with parvovirus infections in dogs [16-18]. This reaction is considered to be due to the destruction of infected keratinocytes by cytotoxic T-cells. Finally, viruses may also modify skin biology and cause indirect changes, like in the so-called “hard pad disease” [15, 19-21].

**Diagnosis of viral infections:**

Several approaches are now available to diagnose viral infection: virus isolation and culture, microscopy, serology or detection of viral antigens or nucleic acids. As these techniques demonstrate increasing sensitivity, results should always be interpreted in the context of the clinical and histological setting. In fact, one must always keep in mind that virus infection can be fortuitous and unrelated to the disease. Cultivation of the virus and/or direct identification (pathognomonic cytopathic effects) from the clinical material represent the “gold standard” for viral diagnosis because they establish at the same time that the virus is present and actively replicates in the lesional sample. These techniques are however limited by the low sensitivity and the difficulty to cultivate some viruses like papillomaviruses. The recent development of techniques that allow the amplification and multiplication of viral nucleic acids (PCR) has dramatically increased the sensitivity of virus detection. The main pitfall of PCR is however its great sensitivity itself, as false-positive assays may result from the amplification of minute amount of nucleic acid of viruses unrelated to the disease. Electron microscopy and immunohistological identification of viral antigens in lesional samples are also available. As they detect productive infections, these techniques may be regarded as more specific. They are however less sensitive than PCR techniques.
Serologic studies to detect antiviral antibodies are important for epidemiological studies, to determine the prevalence of one specific virus in a population, and to detect individuals that have been previously affected by the condition or in contact with the virus.

**Aims and scope of the thesis:**

As mentioned above, viral dermatoses are rarely reported in domestic carnivores [3, 22]. The aim of this thesis was to report some unusual aspects of viral infections of the skin of domestic carnivores and to show that at least some of these viral cutaneous infections remained under-diagnosed. Furthermore we aimed to broaden our knowledge of the genetic diversity of carnivores papillomaviruses and to demonstrate that these latter viruses may contribute to the development of skin cancer in these species.

We have first shown that canine parvovirus 2 is able to induce, in some instances, clinical and histological changes that mimic human erythema multiforme (EM) [17]. Similar changes have already been described in dogs but were, most of time, attributed to drug reactions [23]. On the contrary, true EM is virtually always associated in Man with herpesvirus infections and almost never with drug reaction [16]. Feline EM has also been shown to be due to herpesvirus infections [24]. The disclosure of canine EM associated with virus infection should encourage veterinary dermatologists to look for virus antigens or nucleic acids in skin samples of dogs affected by this condition.

We have described and studied two cases of FeLV-induced skin conditions [25]. We have first shown that FeLV, like other retroviruses, is able to induce syncytium formation in the skin of infected cats. Similar cases have already been described but with a very different clinical phenotype [26]. More interestingly, FeLV antigens and nucleic acids were uncovered in cutaneous lymphoma samples in a serologically negative cat. These findings suggest that focal skin FeLV replication may occur in some instances.

Dogs treated with cyclosporine A sometimes develop lichenoid plaques and warts of unknown origin. We have evaluated such lesions in nine affected dogs and demonstrated that the majority of these plaques are not papillomavirus-induced. Some however harbour papillomavirus DNA and antigens and are probably due to the reactivation of a latent PV-infection of the skin [27].

Anecdotal reports have suggested that PV could play a role in the development of skin squamous cell carcinomas in dogs and cats [28-33]. We have consequently tried to amplify
PV DNA from skin samples of canine and feline squamous cell carcinomas and demonstrated that nucleic acids of these viruses are present in a significant amount of such samples [34, 35]. Furthermore, amplified PV sequences revealed that these samples are infected by PV of great genetic diversity. These findings suggest that PV could play an active role in the development of such cancers in dogs and cats and that domestic carnivore, like humans, may be infected by numerous different PVs. A last study carried out on a subset of feline squamous cell carcinomas (Bowenoid in situ carcinomas: BISC) in situ has shown that BISC are often infected by PVs and that these viruses actively replicate in such lesion [36]. These results further suggest an active role of these viruses in the development of such lesions.

32. Teifke, J.P., C.V. Lohr, and H. Shirasawa, Detection of canine oral papillomavirus-DNA in canine oral squamous cell carcinomas and p53 overexpressing skin


Chapter 2

Virale Dermatosen bei Hunden und Katzen

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Virale Dermatosen bei Hund und Katze

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Zusammenfassung:

Key words: Virus – dermatosis – dog – cat

Summary:
Viral dermatoses are considered rare in domestic animals but are probably often underdiagnosed. The purpose of this review is to present the clinical and histological features of viral dermatoses in domestic animals. It will focus on conditions directly or indirectly caused by viruses of the genera Papillomavirus, Ortho- and Para-Poxvirus, Herpesvirus, Retrovirus, Lentivirus, Morbillivirus and Parvovirus. New techniques such as nucleic acid amplification enable the detection of minute amounts of viral DNA and RNA. Diagnoses have consequently been facilitated. However, one must keep in mind that detection of viral nucleic acid in skin lesions does not prove that the virus is the cause of the disease. It is mandatory to distinguish diseases that are directly caused by viruses and conditions that are sometimes associated with viruses. The latter will only be mentioned when they are frequently reported in the literature. Some dermatoses are caused indirectly by viruses that modify the proliferation properties or the antigenicity of skin cells. These modifications may be associated with cancerization or immunologic reactions such as erythema multiforme.

Einleitung


In der folgenden Übersichtsarbeit liegt das Schwer gewicht auf der klinischen Präsentation, der Histologie und den Behandlungsmöglichkeiten der häufigsten nachgewiesenen viralen Dermatosen bei Hunden und Katzen.

Papillomaviren

Ätiologie und Pathogenese

Papillomaviren (PV) gehören zu den DNA-Viren und zeigen einen ausgeprägten Plattenepithel-Tropismus (52). Das Virus ist...
normalerweise speziesspezifisch, ansteckend und wird oft durch Mikroläsionen übertragen (74). Bei Hunden wird die Mehrheit der Infektionen durch das kanine orale PV (COPV) verursacht. Allerdings ließen sich auch andere Stämme aus kaninen Hautläsionen isolieren (60, 103). Bei Katzen ist diese Krankheit selten und wird durch feline oder bovine Papillomaviren hervorgerufen (85, 98). Bisher konnte erst bei einem kaninen (COPV) und einem felenen (FpPV-1) Papillomavirus das Genom aufgeschlüsselt werden (99, 102).

Die Replikation des Virus läuft parallel zur Differenzierung des Plattenepithels ab und kann in eine „Early Phase“ (E) und eine „Late Phase“ (L) unterteilt werden. Während der E-Phase im Stratum spinosum und der suprabasalen Schicht werden die viralen Proteine synthetisiert. Im Stratum granulosum findet während der L-Phase die Synthese der Kapsidproteine statt. Erst im oberen Bereich des Stratum granulosum wird das Kapsid zusammengebaut. Die Freisetzung des Virions erfolgt gleichzeitig mit der Desquamation (52).

Papillomaviren induzieren auch die Synthese der so genannten transformierenden Proteine E6 und E7, die für die proliferativen und karzinogenen Eigenschaften des Virus verantwortlich sind (45).

Eine Infektion durch ein unproduktives Papillomavirus ist ebenfalls möglich und wird mit der Entwicklung eines felenen Sarkoids oder eines Fibropapilloms assoziiert (85). Es wird angenommen, dass es sich hierbei um das Pendant des equinen Sarkoids handelt. In der Mehrzahl der Fälle gelang es, in solchen Läsionen PV-DNA nachzuweisen, wobei die Blast-Analyse der L1-Sequenz starke Homologien mit einem bovinen PV aufwies (42, 85, 105).

**Klinik**

**Hund**

Es existieren verschiedene klinische Präsentationen, die unterschiedlichen Viren zugeschrieben werden, deren komplettes Genom jedoch noch nicht entschlüsselt ist. Dennoch ist es möglich, dass jede einzelne klinische Präsentation (z. B. orale Infektion oder invertiertes Papillom) mit einem spezifischen PV assoziiert ist (9, 30).


**Papillomatosen des adulten Hundes:** Bei diesen Läsionen kommt es zu keiner spontanen Rückbildung. Hunde jeden Alters können betroffen sein. Die Veränderungen treten oftmals an muskuläten Übergängen, vor allem in der Maulhöhle (hier am häufigsten und gravierendsten), dem Gesicht und zwischen den Zehen auf (Abb. 2). Es handelt sich um multiple typische Warzen, die mit einer dicken Keratinschicht bedeckt sein können (Abb. 3). In vielen Fällen sind die Läsionen pigmentiert, manchmal stehen sie dicht nebeneinander stehend und können bis in den Pharynx reichen (117), wo sie zu Schluckstörungen führen. Die Ätiologie ist noch ungeklärt, doch werden vielfach Immundefizienzen als Ursache angenommen (7, 63, 73, 75, 100, 117).
**Invertierte Papillome:** Diese ungewöhnlichen Hautveränderungen treten vor allem am Abdomen und am Kinn junger adulter Hunde auf (Abb. 4). Eine Streuung über den gesamten Körper ist ebenfalls möglich. Die aufgewölbten Veränderungen weisen einen Durchmesser von 1–2 cm auf und besitzen im Zentrum eine Pore (9). Das ursächliche PV scheint sich vom COPV-1 zu unterscheiden (9).

**Multiple pigmentierte papillomatöse Plaques** (Abb. 5): Sie sind selten, kommen jedoch bei einigen Rassen (z. B. Mops) häufig vor und wurden mit immunsuppressiven Therapien in Zusammenhang gebracht (60, 72, 96, 111). In manchen Fällen ähneln diese Plaques klinisch und histopathologisch der humanen Epidermodysplasia verruciformis (72, 111). Andere dagegen weisen einzigartige histologische Strukturen mit eosinophilen zytoplasmatischen Granula auf (60). Bei nicht immunsuppressivierten Hunden konnte eine spontane Abheilung beobachtet werden. Bei manchen Hunden führte das Absetzen der immunsuppressiven Therapie zur kompletten Abheilung, wiederum bei anderen kam es zu einer malignen Transformation (54, 72, 96, 111).

**Plattenepithelkarzinom** (Abb. 6): In der Veterinärmedizin finden sich nur wenige Berichte über eine maligne Transformation von COPV-induzierten Veränderungen in Plattenepithelkarzinome (101, 104, 112). Bei einem Hund wird über ein multizentrisches Karzinom in situ (Bowen’s Disease) berichtet (35). Eine virale Ätiologie ist zwar anzunehmen, wurde bis dato aber nicht bestätigt.

**Katze**

Bei den meisten Katzen äußert sich diese Krankheit in Form von schuppigen, pigmentierten Plaques (Abb. 7) (12, 20, 62, 98). Perserkatzen und immunsuppressivierte Tiere sind prädisponiert. Bis heute wurde erst ein einziges katzenspezifisches PV entdeckt und sein Genom komplett entschlüsselt (FdPV-1) (102).

Regelmäßig wird über Katzen mit multizentrischen Karzinomen in situ berichtet (Abb. 8) (2, 67). In einer diesbezüglichen Arbeit wurde PV bei bis zu 40% der untersuchten Proben nachgewiesen (59). Solche Veränderungen werden auch mit *Demodex*-Infektionen in Zusammenhang gebracht (38).

Die letzte Form der felinen PV-Hautinfektionen ist das Fibropapillom oder feline Sarkoid (42, 85, 105). Diese Läsionen im Gesicht oder an den Extremitäten bestehen aus festen Knoten in der Dermis, wahrscheinlich aufgrund einer nichtproduktiven PV-Infektion.

Diagnosestellung


Epidermale Hypermelanose, unregelmäßige Akanthose und Hypergranulose mit verklumpten Keratozytengranula werden normalerweise bei Vorliegen von caninen multiplet pigmentierten papillomatösen Plaques gesehen (72, 96, 111). In einigen Fällen konnten virale Einschlusskörperchen nachgewiesen werden (60, 96). Bei Katzen liegen ähnliche histopathologische Veränderungen vor, wobei virale Einschlusskörperchen häufiger beobachtet werden (12, 20, 62, 98).

Histologische Untersuchungen von felinen Sarkoiden ergaben eine pseudokarzinomatöse Akanthose und dicht gepackte mesenchymale Zellen, die Kollagenbündel umgaben (85, 105).

Therapie


Aktuelle Fortschritte in der Prävention und der Immuntherapie der humanen Papillomatose, speziell beim Papillomavirusinduzierten zervikalen Karzinom der Frau, könnten als Basis für immuntherapeutische Behandlungen von PV-Infektionen dienen (61).

Bis dato wurde nicht über eine erfolgreiche Behandlung kaniner pigmentierter Plaques berichtet, doch kann eine spontane Regression auftreten. Zusätzlich empfiehlt es sich, die Ursache der
Immunsuppression bei nicht prädisponierten Rassen zu identifizieren, da es bei deren Therapie zur Abheilung der Hautläsionen kommen kann (96). Die übrigen Formen kaniner und feliner Papillomaviren-Infektionen sollten chirurgisch behandelt werden.

**Pockenviren**

**Ätiologie**


Über eine *Parapoxviren*-Infektion (Orf, Ecthyma contagiosum) beim Hund findet sich nur ein einziger Fallbericht (116).

**Pathogenität der Kuhpocken-Infektion**


**Klinik der Pockenviren-Infektion**

**Hund**


**Kuhpocken-Infektion bei Katzen**


**Diagnose von Pockenviren-Infektionen**

Herpesviren-Infektionen

Ätiologie


Canine Herpesvirus-Infektion auf Haut und Schleimhaut


Aujeszky'sche Krankheit/Pseudotollwut


Feline Herpesviren-Infektionen


Felines Erythema multiforme bedingt durch Herpesviren


Retroviren-Infektionen

Ätiologie

Diese häufigen Erkrankungen bei Katzen werden vor allem durch zwei Viren verursacht: das feline Leukämievirus (FeLV, Genus Gammaretrovirus) und das feline Immundefizienzvirus (FIV, Genus Lentivirus). FIV induziert eine Immundefizienz. Die meisten der FIV-assoziierten Hautveränderungen sind jedoch nicht spezifisch, weshalb diese Krankheit im vorliegenden Artikel nur kurz erwähnt wird. Im Gegensatz dazu wurde in der veterinärmedizinischen Literatur häufig über FeLV-assoziierte Dermatosen und Hauttumoren berichtet. Die meisten der folgenden Aussagen beziehen sich auf das FeLV-Virus.

Infektion


Klinik (FeLV)

Über FeLV-induziertes Lymphom, Leukämie und Knochenmarksuppression liegen ausführliche Berichte vor (16). Im vorliegenden Artikel werden diese Erkrankungen nicht näher besprochen.

Retroviren-assoziierte Hautveränderungen bei Katzen

Hautveränderungen assoziiert mit hoher Rate an FeLV/FIV-positiven serologischen Befunden


Hautveränderungen assoziiert mit Immundefizienz

FIV-positive Katzen leiden häufig unter verschiedenen Infektionen wie zum Beispiel Abszesse, Pyodermien, Dermatomyositen, Kryptokokkose und Demodikose (86).

Diagnose retroviraler Infektionen


Prävention und Therapie einer FeLV-Infektion

Zur Prävention einer FeLV-Infektion wurden verschiedene wirksame Impfstoffe entwickelt. Bei allen handelt es sich um Tot- oder Vektorvakzinen (basiert auf genetisch entwickeltem Protein gp70).

Zur Behandlung von FeLV-induzierten Lymphomen und FeLV-induzierter Knochenmarksuppression existieren verschiedene Arbeiten, auf die im vorliegenden Artikel nicht weiter eingegangen wird.

Staupe und “Hard Pad Disease”

Ätiologie und Pathogenese

Staupe wird durch ein RNA-Virus der Familie Paramyxoviridae verursacht (70). Der breite Einsatz von wirksamen Impfprogrammen hat zu einer drastischen Reduktion des Auftretens dieser
Krankheit geführt. Nach wie vor ist es jedoch wichtig, auch die kutanen Manifestationen dieser Infektion zu erkennen.


Klinik


Diagnose

Ohne passende Anamnese kann die „Hard Pad Disease“ als Autoimmunkrankheit (Pemphigus, Lupus), Malnutrition (zinkresponsive Dermatitis), kongenitale Erkrankung (familiäre idiopathische nasodigitale Hyperkeratose), Papillomatose oder hepatokutanes Syndrom fehldiagnostiziert werden. Histopathologisch erkennt man eine deutliche ortho- oder parakeratotische Hyperkeratose des Pfortenballens und des Planum nasale mit azidophilen zytoplasmatischen und nur selten intranukleären Einschlüssen von variabler Größe und Form (Lentz Bodies) in den Keratinozyten auf folliculärer Ebene. Teilweise können auch synzytiale Riesenzenlen beobachtet werden (119). Da virale Einschlüsse nicht immer vorhanden sind, werden auch andere Techniken wie RT-PCR zur Diagnosestellung herangezogen (26, 57).

Staupeimpfungen


Parvovirose

Bei einer zweimonatigen Dogge wurde im Zusammenhang mit Parvovirose vom Auftreten eines Erythema multiforme (EM) be-

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Chapter 3

Parvovirus Infection of Keratinocytes as a Cause of Canine Erythema Multiforme

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BRIEF COMMUNICATIONS AND CASE REPORTS

Parvovirus Infection of Keratinocytes as a Cause of Canine Erythema Multiforme

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Abstract. Erythema multiforme major was diagnosed in a dog with necrotizing parvoviral enteritis. Skin lesions consisted of ulceration of the footpads, pressure points, mouth, and vaginal mucosa; vesicles in the oral cavity; and erythematous patches on the abdomen and perivulvar skin. Microscopic examination of mucosal and haired skin specimens revealed lymphocyte-associated keratinocyte apoptosis at various levels of the epidermis. Basophilic cytoplasmic inclusions were seen in basal and suprabasal keratinocytes. Immunohistochemical staining, performed with canine parvovirus-2-specific monoclonal antibodies, confirmed the parvovirus nature of the inclusions in the nucleus and cytoplasm of oral and skin epithelial cells. This is the first case of canine erythema multiforme reported to be caused by a viral infection of keratinocytes. This case study indicates that the search for epitheliotropic viruses should be attempted in cases of erythema multiforme in which a drug cause cannot be identified.

Key words: Canine parvovirus-2 (CPV-2); dog; immunology; infection; skin; virus.

In humans, the classification of erythema multiforme (EM) variants recently has been revised with an emphasis on clinical manifestations. The relevance of this modified clinical nosology has been supported by subsequent epidemiologic and pathologic case studies. Dermatoses described clinically as EM minor and major most commonly seem to be caused by viral infections leading to lymphocyte-mediated keratinocyte apoptosis. Human EM generally is caused by herpes simplex virus, but it also can be triggered by other infectious agents such as parvovirus B19.

In 1998, the consensus clinical classification used for human EM was adapted to the canine species. That case study established that, in contrast to previous reports, canine cases of EM (minor or major) rarely were associated with previous drug exposure. In non-drug-related cases, offending causes could not be determined but a viral etiology was considered plausible. The purpose of the present paper is to describe a canine case of EM major in which parvovirus infection of epidermal and mucosal keratinocytes led to lymphocyte-associated apoptosis and clinical signs of EM major.

A 2-month-old female Great Dane puppy was presented, 3 days after adoption, with acute-onset diarrhea, vomiting, dehydration, and skin lesions. Because parvovirus enteritis had been diagnosed recently at the facility of the dog’s breeder, parvovirus was suspected as the cause of diarrhea. However, 6 days before the initial presentation, the dog had received a tetravalent vaccine (distemper, parvovirus, parainfluenza, and hepatitis). Dermatologic examination revealed well-demarcated ulceration of the footpads (Fig. 1) and pressure points, as well as mouth and vaginal mucosae. Vesicles were seen in the oral cavity. Erythematous patches were present on the abdomen and chin. In spite of fluid therapy and intravenous cephalixin and metoclopramide, the dog died 2 days after presentation.

A necropsy was performed and necrotic lesions were seen throughout all intestinal sections. Histopathologic analysis of small intestine specimens consisted of severe segmental necrotizing enteritis suggestive of an acute infection due to canine parvovirus-2 (CPV-2). Viral inclusions were not identified in the intestinal specimens, presumably because of the severe necrosis of the digestive epithelium. Skin biopsy

Fig. 1 Skin, footpad; dog. A sharp-edged ulcer is present.
specimens were obtained from lesional skin and oral mu-
cosa. Focal mononuclear interface gingivitis was identified
in biopsy samples collected from the gum. Additionally, con-
fluent basal keratinocyte vacuolation progressing to vesicu-
lation with epithelial ulceration and neutrophil accumulation
was observed. Prominent lymphocyte exocytosis was present
in preblistered mucosal epithelium. Keratinocyte apoptosis,
often in close contact with lymphocytes (e.g., satellitosis),
was observed at all levels of the epithelium. In some spec-
imens, basophilic inclusions were observed in the cytoplasm
of basal and suprabasal keratinocytes. Examination of haired
skin specimens revealed varying degrees of the same path-
ologic process. The epidermis exhibited focal hyperplasia,
crusting, and erosion. Lymphocyte exocytosis and keratino-
cyte apoptosis with satellitosis were restricted to sites of epidermal hyperplasia (Figs. 2, 3). Numerous basophilic cyto-
plasmic inclusions were seen in the lower third of the hy-
perplastic epidermis (Figs. 2, 3).
To verify the viral origin of cytoplasmic inclusions, a
three-step avidin–biotin–peroxidase immunohistochemical
technique was performed as previously described.9 Immuno-
staining of paraffin-embedded sections was done with two
monoclonal antibodies specific for CPV-2 (CPV2c2A and
CPV103B10A, 1:2,000 dilution, Mérial, Lyon, France). Ex-
amination of negative controls, consisting of sections im-
umnostained with irrelevant monoclonal antibodies, was un-
remarkable. In mucosal specimens, multiple intracellular
parvovirus inclusions were seen throughout the epithelium.

Fig. 2 Haired skin; dog. Clusters of lymphocytes (white arrowheads) are located in the immediate vicinity of apoptotic
eratinocytes (black arrows). Viral inclusions are visible in an intracellular vacuole (black arrowhead). H.E. Scale bar =
18 \mu m.

Fig. 3 Haired skin; dog. A lymphocyte (white arrowhead) is situated near an apoptotic keratinocyte (black arrow) that
contained viral inclusions (black arrowheads). H.E. Scale bar = 4 \mu m.

Fig. 4 Haired skin, abdomen; dog. Dark-staining viral inclusions fill the cytoplasm of basal and juxtabasal keratinocytes.
Viral aggregates are occasionally present in the superficial dermis. Small inclusions are visible within keratinocyte nuclei
(black arrow). Avidin–biotin–peroxidase immunohistochemistry, aminoethylcarbazole chromogen, hematoxylin counter-
stain, CPV2c2A parvovirus-specific monoclonal antibodies. Scale bar = 11 \mu m.
In haired skin samples, parvovirus inclusions were seen most commonly coalescing in basal and juxtbasal keratinocytes of hyperplastic epidermis (Fig. 4). The smallest viral inclusions were identified in the keratinocyte’s nucleus (Fig. 4). Inclusions further aggregated and filled-up the cytoplasm of epithelial cells leading to displacement of the nucleus to the cell’s periphery and subsequent cell degeneration. Immuno-staining of digestive specimens similarly demonstrated CPV-2 particles in the epithelial crypts of the small intestine. Furthermore, viral inclusions of skin and mucosal sections were negative when immunohistochemistry was performed using monoclonal antibodies specific for either distemper virus (1:50, Merial, Lyon, France) or papillomavirus-group–specific antigens (AR087–5R, undiluted, Biogenex, San Ramon, CA).

According to the recently proposed classification of canine EM, the skin lesions exhibited by this patient fit the criteria for a clinical diagnosis of EM major (erythematous patchy lesions with ulcerations on less than 10% of the body surface and with more than one mucosa affected). 5 Our histologic and immunohistochemical investigations suggested CPV-2 infection of mucosal and epidermal keratinocytes as the primary cause of EM in this dog. Remarkably, most parvoviral inclusions were identified in the cytoplasm of keratinocytes, whereas only rare viral particles were seen in cell nuclei. However, these observations are identical to those described in glossal specimens of dogs naturally infected with CPV-2. 2 Indeed, viral replication initially occurs in the nucleus but large virion clusters appear as cytoplasmic aggregates. However, these inclusions still are surrounded by the nuclear membrane and should be referred to as pseudocytoplasmic. 3 A viral infection of keratinocytes suggests a logical pathogenesis of EM lesions in this dog. We hypothesize that an infection of stem cells and transient amplifying keratinocytes most likely occurred following hematogenic dissemination of the parvovirus. Viral peptides could be presented by I major histocompatibility complex molecules at the surface of epithelial cells. Recognition of viral antigens by T-lymphocytes, possibly sensitized by the previous parvovirus vaccination, would trigger these cytotoxic cells to induce the apoptosis of virus-infected keratinocytes.

The present case study supports the concept that a viral etiology is possible in some forms of canine EM. We propose that a search for epitheliotropic viruses (e.g., distemper, papilloma-viruses, parvoviruses, and herpesviruses) should be attempted in cases of canine EM in which a causative drug cannot be clearly established.

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References


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Chapter 4

Two cases of FeLV-associated dermatoses

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Case report

Two cases of FeLV-associated dermatoses

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Abstract Two cases of feline leukaemia virus (FeLV)-associated dermatosis are described. The first cat was affected by an ulcerative dermatitis identified as a giant-cell dermatosis. The second case was a cutaneous lymphoma. In both cases, FeLV antigens and FeLV genome were demonstrated in the affected skin immunologically and with polymerase chain reaction, respectively. The first case suggests that, like other retroviruses, at least some strains of FeLV can induce syncytium formation. As FeLV antigens and genome were demonstrated in a serologically negative cat, the second case suggests that focal skin FeLV replication may occur. FeLV-associated dermatoses are rare skin conditions that may be under-diagnosed.

INTRODUCTION

Feline leukaemia virus (FeLV), a member of the oncornavirus subfamily of retroviruses, occurs worldwide and replicates in many tissues including respiratory epithelium, salivary gland and bone marrow. It causes approximately one-third of feline lethal cancers and numerous cats die of anaemia or infectious diseases as a consequence of the immunosuppressive effects of the virus. FeLV infection has also been associated with numerous infectious dermatoses of fungal, parasitic and/or bacterial aetiology. A direct cytopathic effect of the virus in the skin, however, has been rarely demonstrated, but is associated with two different syndromes: giant-cell dermatosis and epidermal horns. Lymphoma accounts for about 90% of the haematopoietic tumours in cats and is often a consequence of FeLV infection. Cutaneous lymphomas, however, are rare and usually occur in older FeLV-negative cats.

The purpose of this article is to present two new cases of FeLV-induced dermatoses with evidence of viral antigens and proviral sequences in the skin: one of T-cell lymphoma in a serologically negative cat and one case of giant-cell dermatosis in a serologically positive cat.

MATERIAL AND METHODS

Animals Two castrated domestic indoor–outdoor male cats (the first aged 3 and the second aged 15 years), in reduced general condition and with skin plaques and ulcerations, were presented at the Clinic for Small Animal Internal Medicine, Dermatology Unit, Vetsuisse Faculty, Zürich. Both cats were given clinical and dermatological examinations.

Serological examination The presence of plasma FeLV p27 antigen as a measure for viraemia was determined using double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) as previously described. Plasma samples were tested for feline immunodeficiency virus (FIV) by ELISA, measuring antibodies against the FIV transmembrane protein.

Histological and immunohistological examination Skin samples for histopathological examination were taken (during the first examination of both cats) by biopsy from the lesions with a 6-mm skin punch, fixed in 4% neutral buffered formalin and embedded in paraffin wax. Sections were cut and either stained with haematoxylin and eosin or used for immunohistological examination.

Skin lesions were examined immunohistologically for FeLV antigens using a cocktail of mouse monoclonal antibodies against the FeLV envelope protein gp70 and the group-specific protein p27 (clones C11D82i and PF12J-10A; Custom Monoclonals, Sacramento, CA, USA). The peroxidase–antiperoxidase method was used as chromogen.
was applied as previously described. In case 2, the skin lesions were also stained for the pan-T-cell marker CD3 and the pan-B-cell marker CD45R as previously described.

**Real-time polymerase chain reaction (real-time PCR) for exogenous FeLV and feline herpesvirus-1 (FHV-1)**

Polymerase chain reaction (PCR) analysis was performed on skin with lesions after deparaffinization of two 20-µm thick sections of the same lesional tissues as mentioned previously and DNA extraction using the DNeasy tissue kit. FeLV provirus was detected by real-time PCR with primers that recognize the unique region (U3) of the long-terminal repeat (LTR) of exogenous FeLV-A,-B,-C as described previously. Examination for feline herpesvirus (FHV-1) sequences was undertaken as described elsewhere.

**RESULTS**

**Case 1**

The cat was presented with a pruritic dermatosis of 3 months’ duration and a previous history including vaccination for FeLV during the first year of life and booster injection in the second year. Physical examination revealed well-demarcated ulcerative lesions of the head, limbs and paws (Fig. 1). The cat was also depressed and febrile (39.7 °C). A staphylococcal infection was identified by cytological and bacteriological examination (*Staphylococcus intermedius*), but multiple skin scrapings were negative for parasites and fungal culture was negative. A nonregenerative normochrome normocytic anaemia (hematocrit: 18% (reference range: 33–45%) reticulocytes: 0.6%) was diagnosed. The ELISA for FeLV antigen was positive, whereas that for FIV antibodies was negative. The cat was started on cefalexin (25 mg kg⁻¹ twice daily) therapy.

Histological examination revealed an ulcerative dermatitis with folliculitis, dyskeratotic keratinocytes and syncytia formation within the epidermis and the sebaceous glands (Figs 2 and 3). The epidermis was acanthotic and hyperkeratotic. Multiple giant keratinocytes and scattered apoptotic cells were present in the superficial epidermis, sebaceous glands and hair follicles. In the dermis, a severe perifollicular to diffuse inflammatory infiltration with numerous lymphocytes, plasma cells and neutrophils was present.

Immunohistological analyses revealed numerous epithelial cells that expressed viral proteins with variable intensity (Fig. 4) in the epidermis of the skin surface, hair follicles and sebaceous glands. Lymphocytes in the dermal infiltrates were often positive as well. A 131-bp long proviral FeLV DNA was amplified from skin samples of this cat. Skin samples evaluated for FHV-1 DNA were deemed negative. A diagnosis of FeLV-induced giant-cell dermatosis was made.

Despite the treatment and a marked but temporary improvement of the skin lesions, the general condition deteriorated and the cat was euthanized. Necropsy was not permitted.

**Case 2**

The cat was presented with a dermatosis of 2 months’ duration and weight loss. It had previously been treated with megestrol acetate and prednisolone (variable doses) on the basis of a tentative diagnosis of eosinophilic plaques. The cat was depressed and febrile (39.6 °C). Physical examination of the skin revealed multiple nodules.
and ulcerated lesions that affected the face, feet and abdomen (Fig. 5). Multiple skin scrapings were negative for parasites. Additionally, the cat was anaemic (Ht. 23%) and lymphopenic (170 lymphocytes per microlitre). Serum ELISA tests for FeLV antigens and FIV antibodies were both negative. Radiographic examination of the thorax revealed a nodular opacity in the left lung. Sonographic examination of the abdomen showed the presence of a small nodule in the liver. Fine needle aspirates of pulmonary and liver nodules were, however, unremarkable.

Histological examination of the skin lesions revealed extensive superficial ulceration and focally extensive dense dermal infiltration by pleomorphic round cells, resembling lymphoblasts with round to indented nuclei containing fine chromatin and one single medium-sized nucleolus (Fig. 6). Cellular atypia such as anisocytosis and anisocaryosis were observed, multiple large nucleoli and abnormal mitoses were also seen (Fig. 7). A large proportion of neoplastic cells exhibited peripheral and/or cytoplasmic CD3 expression (Fig. 8). Based on these results a diagnosis of cutaneous non-epitheliotropic T-cell lymphoma was made. Immunohistology for FeLV antigen revealed variably intense viral protein expression by numerous neoplastic cells and weak expression by epidermal cells in all layers (Fig. 9). A 131-bp long proviral FeLV DNA fragment was amplified from skin samples of this cat.

Despite Lomustine therapy (10 mg once daily) started after histological diagnosis, the cat’s general condition deteriorated and it was euthanized. Necropsy was not permitted so the nodular liver and pulmonary lesions could not be further evaluated.

**DISCUSSION**

This report describes two FeLV-associated skin conditions in cats, presenting clinically as dermatoses with poor response to treatment: giant-cell dermatosis and cutaneous lymphoma. The presence of proviral FeLV sequences as well as FeLV antigens in the skin with...
lesions of both cats was demonstrated by PCR and immunohistological analysis, respectively.

So far, six cases of FeLV-induced giant-cell dermatosis have been described in the literature. Most presented as scaling and crusting dermatoses affecting mainly the face and the neck. Vesicular and ulcerative lesions were also reported with involvement of the footpads and mucous membranes. The clinical and histological presentation of this case was similar to those previously described, although more ulcerative and less hyperkeratotic. Mucous membranes were unremarkable. Affected cats usually decline quickly with death or euthanasia days to weeks after initial presentation.

The histological hallmark of giant-cell dermatosis is the presence of syncytial keratinocytes and dyskeratotic cells. The former have also been observed in FeLV-infected cats in association with cutaneous horns. Multinucleated keratinocytes are observed in humans in association with neoplastic (squamous cell carcinoma), infectious (alpha-herpesvirus infections) and immunologic disorders such as lupus erythematosus, Hailey–Hailey disease and psoriasis. Retroviruses including human lentiviruses such as human immunodeficiency virus and feline gammaretroviruses (e.g. FeLV), possess fusion proteins and are sometimes seen to induce syncytium formation in lymphoid tissues. However syncytial keratinocytes are mainly observed in AIDS patients that are concomitantly infected with herpesvirus or papillomavirus. Gross and coworkers suggested that syncytium formation observed in feline cases is not a consequence of a direct cytopathic effect of the virus but of carcinomatous transformation of the epidermis. HIV-induced squamous cell carcinoma, however, has rarely been observed in humans. The oncogenic potential of FeLV is much greater than that of HIV but Rohn and coworkers have also demonstrated that FeLV variants do exhibit various pathogenic and cytopathic effects, including syncytium formation in one strain. It thus appears possible that FeLV-induced giant-cell dermatosis is the result of a specific and probably rare viral variant. Confirmation of this hypothesis needs further investigation.

Lymphomas are frequent neoplasms in cats and often a consequence of FeLV infection. Cutaneous lymphomas, however, are rare and usually occur in older serologically FeLV-negative cats. Attempts to identify FeLV genomic sequences and antigens in epitheliotropic and nonepitheliotropic cutaneous
lymphomas are occasionally successful and confirm FeLV involvement in the development of at least some cutaneous lymphomas in cats. \(^{19,20}\) Tobey and co-workers suggested definitive or latent infection as they did not detect FeLV antigens in the neoplastic cells of a cutaneous lymphoma. \(^{21}\) In the case reported here, both proviral genome and antigens were demonstrated. The presence of viral antigens in neoplastic cells and keratinocytes but not in the peripheral blood suggests focal productive infection of both cell types. As the greater sensitivity of RT-PCR detects proviral DNA in the serum of cats with undetectable antigenemia, negative ELISA does not rule out generalized infection. Localized FeLV replication has been reported after experimental infection with viral antigens in the spleen, bone marrow, lymph nodes or small intestine. \(^{22}\) Restricted, localized FeLV replication was also shown in another study in naturally infected cats. The same organs were examined but no viral antigen was found. \(^{19}\) This case report supports the hypothesis that infections restricted to the skin may sometimes occur in cats and subsequently induce cutaneous lymphomas.

As PCR assays and immunohistology for FeLV are not routinely carried out on cutaneous lymphomas, the frequency of this association is unknown and may be underestimated. It is possible, however, that FeLV tumorigenesis of dermal T cells is rare and caused of particular FeLV variants. A previous study failed to detect FeLV nucleic acid in a portion (20%) of feline lymphoma samples and suggested that FeLV lymphomagenesis can be associated with clearance of viral nucleic acids from cancer cells. \(^{23}\) This ‘hit and run’ mechanism has already been associated with other conditions induced by retroviruses. \(^{24}\) This study was, however, carried out with a single-round PCR system \(^{23}\) that has a lower diagnostic sensitivity than the more recently developed FeLV-specific nested and TaqMan PCR systems. \(^{10,11}\) As real-time TaqMan PCR can detect fewer nucleic acid copies compared to conventional PCR we may be able to detect FeLV in a larger portion of lymphosarcoma cases.

In conclusion, this report suggests that FeLV-induced dermatoses are probably due to particular viral variants and their frequency might be underestimated.

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Chapter 5

Evaluation of papillomaviruses associated with cyclosporine-induced hyperplastic verrucous lesions in dogs

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Evaluation of papillomaviruses associated with cyclosporine-induced hyperplastic verrucous lesions in dogs

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Objective—To determine whether cyclosporine A-induced hyperplastic skin lesions of dogs were associated with papillomavirus infections.

Animals—9 dogs that were treated with cyclosporine A and developed hyperplastic skin lesions.

Procedure—History and clinical and histopathologic data were collected. Paraffin-embedded skin biopsy specimens from hyperplastic skin lesions were immunostained for common papillomavirus genus-specific structural antigens by use of a polyclonal rabbit anti-bovine papillomavirus type 1 antiserum. Sections from each tissue block underwent DNA extraction, and polymerase chain reaction (PCR) assays were performed with several sets of primers to amplify a wide range of papillomavirus DNA from humans and other animals.

Results—In 7 of 9 dogs, there were more than 10 hyperplastic skin lesions that microscopically resembled those of psoriasiform lichenoid dermatitis. In those dogs, results of testing for papillomavirus via immunohistochemical analyses and PCR assays were negative. In the other 2 dogs, there were only 1 and 3 verrucous lesions, and in those dogs, histologic evaluation revealed koilocytes and nuclear viral inclusions that were immunoreactive for papillomavirus antigens. Papillomavirus DNA was amplified from both dogs. One of the sequences was characteristic for the canine oral papillomavirus, whereas the other had similarities with the recently described canine papillomavirus 2.

Conclusions and Clinical Relevance—In dogs, hyperplastic skin lesions occasionally develop during treatment with cyclosporine A. Most of the lesions resemble those of psoriasiform lichenoid dermatitis, although papillomavirus can be detected in some instances. (Am J Vet Res 2005;66:1764–1769)

Cyclosporine A is a potent immunosuppressive agent that acts primarily by selectively inhibiting helper T lymphocytes. In humans, cyclosporine A has been used for more than a decade to prevent transplant rejection and for treatment for dermatologic conditions that include severe psoriasis and atopic dermatitis. The usefulness of cyclosporine A in treatment for atopic dermatitis in dogs has been reported, and the drug has also been approved for treatment for immune-mediated conditions such as peripheral joint disease and sebaceous adenitis.

Hyperplastic skin lesions are known adverse effects of long-term treatment with cyclosporine A in humans. Most lesions appear to be papillomavirus-induced verruca vulgaris, but malignant carcinomatous transformations are also possible. Similar lesions have also been described in dogs, but evidence for causative involvement by papillomavirus is lacking. Most lesions in dogs resemble those reported as psoriasiform lichenoid dermatitis, and skin nodules usually regress spontaneously or in response to antimicrobial treatment. Because papillomavirus has been detected in most cyclosporine A-induced hyperplastic skin lesions in humans, a similar role for papillomavirus in the development of similar lesions in dogs warrants investigation. We observed that these drug-induced nodules appear to be heterogeneous in nature. Most lesions are numerous and resemble those of psoriasiform lichenoid dermatitis, with staphylococci in the stratum corneum and absence of detectable papillomavirus DNA and antigens. In 2 dogs, however, cyclosporine A administration was associated with the eruption of few skin nodules diagnosed as viral papillomomas. The objective of this study was to determine whether cyclosporine A-induced hyperplastic skin lesions in dogs contained papillomavirus DNA and genus-specific structural antigens.

Materials and Methods

History and clinical information regarding 9 dogs that were treated with cyclosporine A and developed verrucous skin lesions were recorded retrospectively. Limited clinical and pathologic information regarding 1 of the dogs has already been published.

Histologic and immunohistochemical evaluations—Punch biopsy specimens of the skin were obtained from dermatologic lesions of the 9 dogs. Biopsy specimens were fixed in formalin, embedded in paraffin, and processed routinely for histologic assessment. Five-micrometer-thick skin sections were stained with H&E and Gram stains by use of standard methods.

For the detection of papillomavirus antigens, a 3-step immunohistochemical method was used. The primary immunoreagent was a polyclonal rabbit antiserum directed against chemically disrupted bovine papillomavirus type 1.
This reagent detects papillomavirus genus-specific common structural antigens regardless of the host species. The primary antisera was used at a 1:200 dilution, and other reagents (ie, biotinylated goat anti–canine rabbit IgG and streptavidin peroxidase) were diluted at 1:40. Diaminobenzidine was used as a chromogen. The positive control consisted of paraffin-embedded sections from a dog with canine oral papillomavirus (COPV)-induced oral papillomas, whereas the negative control antisera consisted of normal rabbit serum.

Skin sections stained with H&E, Gram stain, and immunostain for papillomavirus were coded and evaluated by an author (TO) who was unaware of the origins of the specimens. Sections stained with H&E were examined for changes typically associated with papillomavirus infection in dogs, and those changes were recorded as present or absent, including epidermal dysplasia, hypergranulosis, coalescing keratohyalin granules, koilocytes, intranuclear viral inclusions in the stratum spinosum and stratum granulosum, intrafollicular or intraepidermal pustules, and bacteria in the stratum corneum. Sections stained with Gram stain were evaluated for epidermal bacteria. Sections immunostained for papillomavirus group-specific antigens were evaluated for staining in keratinocytes of the stratum spinosum, stratum granulosum, and stratum corneum. Transmission electron microscopy was performed on the stratum corneum of 1 specimen.

Polymerase chain reaction assays—Amplification of papillomavirus DNA via polymerase chain reaction (PCR) assays was performed on formalin-fixed paraffin-embedded specimens. Thirty-micrometer-thick sections were cut from tissue blocks by use of a new disposable microtome blade for each block to avoid cross-contamination between samples. Each section was deparaffinized twice with 1.2 mL of xylene at 20°C for 10 minutes, washed with 100% ethanol, and air-dried. Desiccated samples were suspended in a lysis buffer (50mM Tris-HCl [pH, 8.5], 1mM EDTA, 2.8% sodium dodecyl sulfate, and 20 mg of proteinase K/mL) and incubated for 10 hours at 56°C on a rocking platform. After lysis, samples were transferred to a spin column and centrifuged to reduce viscosity. Viral DNA was precipitated with absolute ethanol and extracted by use of a commercially available kit.

Phylogenetic studies have revealed that COPV and feline papillomavirus are closely related and that this group of viruses is closest to some genera of human papillomavirus than to papillomaviruses in other animals, including bovine papillomavirus. Therefore, 2 sets of primers were designed. The first set of primers (PapE1-forward and PapE1-reverse) amplified DNA from COPV and feline papillomavirus, whereas the second set of primers (CP4, CP5, and PPF1) amplified genomic sequences of as many different papillomaviruses as possible, the CP4, CP5, PPF1 primer set was selected because of its ability to detect the nucleic acids of up to 64 human papillomaviruses. The PCR reactions were performed in 20-mL volumes containing 1 µL of genomic DNA, 50mM KCl, 3mM KCl2, 200µM of each dNTP, 0.3µM each of consensus sense and antisense primers, and 2.5 units of DNA polymerase. Amplification involved an initial denaturation at 95°C for 4 minutes and 30 cycles at 95°C for 1 minute, 50°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 74°C for 5 minutes. Reaction mixture with no DNA served as a negative control, and COPV-positive papilloma DNA samples and feline papilloma-positive DNA samples were used as positive controls. The PCR products were resolved via electrophoresis in 2% agarose gel stained with ethidium bromide. Amplified DNA was sequenced by use of fluorescent sequencing and fluorescent dye terminator.

Detection of papillomavirus with CP4, CP5, and PPF1 primers—To amplify the DNA of as many different papillomaviruses as possible, the CP4, CP5, PPF1 primer set was selected because of its ability to detect the nucleic acids of up to 64 human papillomaviruses. The PCR reactions were performed in 30-mL volumes containing 1 µL of genomic DNA, 50mM KCl, 3mM KCl2, 200µM of each dNTP, 0.45µM of the CP4 and CP5 primers, 0.3µM of the PPF1 primer, and 2.5 units of DNA polymerase. Amplification involved an initial denaturation at 95°C for 10 minutes and 40 cycles at 95°C.

Phylogenetic studies have revealed that COPV and feline papillomavirus are closely related and that this group of viruses is closest to some genera of human papillomavirus than to papillomaviruses in other animals, including bovine papillomavirus. Therefore, 2 sets of primers were designed. The first set of primers (PapE1-forward and PapE1-reverse) amplified DNA from COPV and feline papillomavirus, whereas the second set of primers (CP4, CP5, and PPF1) amplified human papillomaviruses, including the oncogenic strains. To amplify genomic sequences of canine, feline, or closely related papillomaviruses in clinical samples, nucleotide sequences conserved among known canine and feline papillomaviruses were reviewed and sequences encoding the E1 early gene were found to be the most highly conserved. Therefore, E1 sequences of feline (LOCUS AF480454) and canine (LOCUS NC001619) papillomaviruses were aligned with the aim of designing consensus primer pairs able to amplify an approximate 341-bp fragment of both genomes. The forward primer was 5’-ATGCCGGMTARAAAAGGTA-3’ and the reverse primer was 5’-AACAGCTGYTTTTTARCYTTTTP-3’. Internal control was made by use of the same forward primer with the reverse primer PapE1 5’-ACAGTTGCAAGGAAGGTC-3’ to amplify an internal 184-bp fragment. The PCR reactions were performed in 30-mL volumes containing 1 µL of genomic DNA, 50mM KCl, 3mM KCl2, 200µM of each dNTP, 0.3µM each of consensus sense and antisense primers, and 2.5 units of DNA polymerase. Amplification involved an initial denaturation at 95°C for 4 minutes and 30 cycles at 95°C for 1 minute, 50°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 74°C for 5 minutes. Reaction mixture with no DNA served as a negative control, and COPV-positive papilloma DNA samples and feline papilloma-positive DNA samples were used as positive controls. The PCR products were resolved via electrophoresis in 2% agarose gel stained with ethidium bromide. Amplified DNA was sequenced by use of fluorescent sequencing and fluorescent dye terminator.

Figure 1—Photomicrographs of a section of an exophytic papilloma from a dog. Notice hypergranulosis, koilocytes, and intranuclear viral inclusions (arrowheads) in keratinocytes of the stratum spinosum, stratum granulosum, and lower stratum corneum. H&E stain; bar = 1 mm (A) and 25 µm (B).

Figure 2—Photomicrographs of a section of a papilloma in a dog. Notice hypergranulosis, koilocytosis, and intranuclear viral inclusions (arrowheads) predominantly in the stratum spinosum. H&E stain; bar = 0.1 mm (A) and 25 µm (B).
for 1 minute, 47°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 75°C for 5 minutes. Reaction mixture with no DNA served as a negative control, and COPV-positive papilloma DNA samples and feline papilloma-positive DNA samples were used as positive controls. The PCR products were resolved via electrophoresis in 2% agarose gel stained with ethidium bromide. Amplified DNA was sequenced on an automated sequencer with fluorescent dye terminator, and sequences were compared with those included in the GenBank database by use of alignment software.

Samples were considered to have positive results for detection of papillomavirus DNA if they had a band of the expected size after gel electrophoresis and if amplified DNA was sequenced and the protein encoded by the sequence had homology with the E1 protein of a previously established papillomavirus sequence. Comparisons were made with alignment software.

Results
Clinical information—In 8 of the 9 dogs, administration of cyclosporine A at the median dosage of 5 mg/kg every 24 hours was associated with the eruption of multiple hyperplastic and verrucous skin lesions. A single lesion developed in the other dog. The duration of treatment with cyclosporine A before development of lesions varied from 1 to 24 months (median, 4 months). Eight breeds were represented, and there were 2 West Highland White Terriers; 8 dogs were male, and 1 dog was female. Age at the time hyperplastic skin lesions developed ranged from 6 months to 9 years (median, 3 years). Two dogs (dogs 1 and 2) had 1 to 3 lesions with the typical appearance of a papilloma. The other dogs (dogs 3 to 9) had numerous variably pigmented, slightly raised verrucous papules on the trunk and limbs. In dog 1, the skin nodules were removed surgically. In 5 dogs, the lesions regressed after the dose of cyclosporine A was reduced and administration of antimicrobials was instituted. In the remaining 3 dogs, lesions regressed spontaneously after cyclosporine A administration was discontinued or the dose was tapered.

Histopathology—In dogs 1 and 2, examination of H&E-stained sections of biopsy specimens revealed severe focal epidermal hyperplasia and dysplasia, koilocytosis, and intranuclear viral inclusions with margination of chromatin (Figures 1 and 2). Focal hypergranulosis and coalescing keratohyalin granules also were observed in 1 of those dogs. Such changes were absent in sections from the other dog, suggesting that the differing cytopathic effects seen in the 2 dogs resulted from infection with different viruses.

Among the remaining 7 dogs, microscopic findings in H&E-stained sections were similar. Findings included epidermal acanthosis without dysplasia, hypergranulosis, variable lymphocyte exocytosis, intraepidermal or intrafollicular pustules, and bacteria in the stratum corneum (Figure 3). The upper portion of the dermis contained bands of lymphocytes and plasma cells, although a true interface dermatitis was not detected. These features were considered similar to changes referred to as psoriasiform lichenoid dermatitis. Examination of stained sections revealed gram-negative rods in epidermal crypts in 1 dog and clusters of gram-positive cocci in the stratum corneum of hair follicle infundibula in dogs 3 to 9. Transmission electron microscopy revealed bacteria with features similar to those of staphylococci.

Figure 3—Photomicrographs of a section of a hyperplastic verrucous skin lesion in a dog. Notice irregular epidermal hyperplasia with luminal (open arrowheads) and mural (solid arrowhead) folliculitis and a band of lymphocytes and plasma cells in the superficial portion of the dermis; lymphocytes are also in the lower epidermal layers. H&E stain; bar = 0.1 mm (A) and 25 µm (B).

Figure 4—Photomicrograph of a portion of an exophytic papilloma from a dog. Notice that intranuclear inclusions in keratinocytes in the stratum granulosum are immunohistochemically stained for papillomavirus group-specific antigens. Diaminobenzidine chromogen with hematoxylin counterstain; bar = 10 µm.
Immunohistochemical analyses—In dogs 1 and 2, the results of immunohistochemical staining confirmed the intranuclear keratinocyte viral inclusions to be derived from papillomavirus. In dog 1, papillomavirus inclusions were in nuclei in the stratum granulosum and lower stratum corneum, whereas in dog 2, the inclusions were in cells from the stratum spinosum to the lower stratum corneum (Figure 4). In dogs 3 to 9, intrakeratinocyte staining with the papillomavirus-specific antiserum was not detected. However, there was bacterial uptake of stain in the stratum corneum (Figure 5).

PCR assay—The PCR assay detected papillomavirus DNA from sections of the 3 positive controls with both sets of primers, and the sequence of amplicons was 99% homologous with that of the canine COPV E1 gene. In dog 1, papillomavirus DNA was amplified with the CP4, CP5, PPF1, and PapE1 primers (Figure 6). The amplified sequence was 98% homologous with that of COPV. In dog 2, papillomavirus DNA was amplified with the CP4, CP5, and PPF1 primers. The amplified sequence was 97% homologous with that of a recently described canine papillomavirus (GenBank No. AY725239). Moreover, this sequence was homologous at the predicted amino-acid level with E1 protein of human papillomavirus 63 (76% homology), bovine papillomavirus 3 (74% homology), and feline papillomavirus (72% homology). Papillomavirus DNA was not amplified from any other specimens from dogs 3 to 9.

Discussion

In humans, administration of cyclosporine A is often associated with numerous cutaneous adverse effects.9,17,18 Most of those changes are associated with the development of viral, bacterial, or fungal infections. Papillomaviruses are the most frequently reported virus detected in the associated infections, but herpes simplex and molluscum contagiosum virus infections also have been recorded.9,17,19 Noninfectious changes such as hyperpigmentation, skin tags, lichen simplex, acne, cysts, and sebaceous hyperplasia are reported less frequently.17,18,20 Follicular dystrophy, increased hair growth (hypertrichosis),21,22 and gingival hyperplasia are frequently recorded.23 Furthermore, compared with the general population, the incidence of squamous cell carcinoma is higher in human patients treated with cyclosporine A for longer than 2 years.24

In dogs, lesions resembling psoriasiform lichenoid dermatitis have been reported in association with administration of cyclosporine A.7,10,25 Gingival hyperplasia and hypertrichosis have been reported to be rare adverse drug events.7,10,25 Results of our study indicated that psoriasiform lichenoid dermatitis was the most common diagnosis for hyperplastic verrucous skin lesions in 9 dogs treated with cyclosporine A. It has
been hypothesized\(^1\) that this reaction is induced by staphylococcal infection, a premise that was supported by the observation of cocci in 6 of 9 specimens in our study. However, in 3 of 8 dogs, all lesions regressed without the administration of antimicrobials after discontinuation or decreasing the administration of cyclosporine A. Immunohistochemical staining for papillomavirus with the polyclonal antiserum stained bacteria in the stratum corneum. In 1 dog with such bacteria, transmission electron microscopy revealed bacteria with features consistent with staphylococci in the stratum corneum but no papillomavirus. Thus, care must be taken in the interpretation of immunostaining for papillomavirus with this reagent.

Several pharmacologic properties of cyclosporine A can explain the development of hyperplastic lesions in the skin of dogs and humans. Cyclosporine A modifies cytokine secretions in several cell types.\(^2\) In humans, development of gingival hyperplasia is caused by increased production of extracellular matrix in association with secretion of transforming growth factor-\(\beta\).\(^3\) Moreover, inhibition by cyclosporine A of the calcineurin-nuclear factor of activated T-cell 1 pathway in follicular keratinocytes stimulates hair growth and induces hypertrichosis.\(^4\)

Results of our study also suggested that cyclosporine A-induced verrucous skin lesions can sometimes be associated with infections with various papillomaviruses, for which emergence might be promoted by suppression of cell-mediated immunity. However, it is not known whether treatment with cyclosporine A favors recurrence of a latent infection or promotes de novo infection. In dog 1, clinical findings, histologic findings, and results of immunohistochemical and PCR testing were consistent with those typically associated with infection with COPV\(^5\) (LOCUS NC001619). In dog 2, results of histologic and immunohistochemical staining were different from those in dog 1, mainly with regard to koilocytes in the stratum spinosum and the absence of hypergranulosis or coalescing keratohyalin granules.\(^6\) These differences may have indicated infection with a virus other than COPV. Moreover, DNA from papillomavirus was only amplified by use of the CP4, CP5, PPF1 set of primers; the subsequent amplification indicated infection with a recently described papillomavirus (GenBank No. AY725239). In humans, the use of degenerated primers has broadened the spectrum of capability of PCR assays and enabled detection of unknown papillomaviruses.\(^7\) It is possible that the epidermis of affected dogs may be infected by papillomaviruses that are difficult to detect by use of traditional techniques that amplify COPV DNA. Likewise, it is possible that the cyclosporine A-induced hyperplastic skin lesions in dogs 3 to 9 might have been caused by papillomaviruses that were undetectable with available techniques. In humans, DNA of papillomavirus is often amplified from lesions of psoriasis, a dermatologic condition with similarities to canine psoriasiform lichenoid dermatosis. It has been hypothesized\(^8\) that papillomavirus induces autoimmune reactions in the epidermis and could play a role in the development of such lesions. Finally, it must be kept in mind that cyclosporine A-induced skin lesions develop most frequently in humans and dogs treated with high doses, for extended periods of time, or with a combination of immunosuppressive drugs.\(^9,10\) Most of those changes regress after treatment is discontinued.

Our results suggested that hyperplastic and verrucous skin lesions observed in dogs after cyclosporine A administration may have multiple causes. In most dogs, the lesions are typical of those characterized as lichenoid psoriasiform dermatosis, but infection by papillomaviruses may develop in some dogs. In all instances, decreasing or discontinuing administration of cyclosporine A, with or without concurrent administration of antimicrobials, resulted in regression of the lesions.

References


Chapter 6

Detection of Novel Papillomaviruses in Canine Mucosal, Cutaneous and in situ Squamous Cell Carcinomas

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Detection of novel papillomaviruses in canine mucosal, cutaneous and in situ squamous cell carcinomas

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Abstract Papillomavirus (PV) DNA is frequently uncovered in samples of human skin squamous cell carcinomas (SCC). However, the role of these viruses in the development of such cancers in canine species remains controversial. While approximately 100 human PVs are known, only one single canine oral PV (COPV) has been identified and studied extensively. Therefore, we applied a narrow-range polymerase chain reaction (PCR) suitable for the detection of classical canine and feline PVs, as well as a broad-range PCR, which has been used for the detection of various novel PVs in humans, in order to analyse 42 paraffin-embedded samples, representing three different forms of canine SCCs. Ten samples of skin tissues with various non-neoplastic conditions served as controls. While none of the negative controls reacted positively, PV DNA was discovered in 21% of the tested SCC samples. Interestingly, the classical COPV was amplified from only one sample, while the other positive cases were associated with a variety of thus far unknown PVs. This study suggests that a fraction of canine SCC is infected with PVs and that a genetic variety of canine PVs exists. Therefore, these results will facilitate the future study of the role of PVs in the development of canine skin cancers.

INTRODUCTION

Squamous cell carcinomas (SCC) are malignant tumours that arise from the squamous epithelium of the skin and the mucous membranes. Squamous cell carcinomas account for up to 5% of the skin tumours in dogs and are the most frequent malignant canine tumours of the digits, tongue and gingiva. Cutaneous SCCs may be either exophytic or ulcerative, whereas mucous membrane SCCs are usually exophytic. Aside from these two classical forms of invasive SCC, a case of multifocal in situ SCC arising from pigmented papules and plaques has been described in a dog. In this case, tumoral cells were confined to the epidermis, while the basal membrane remained unaffected. Furthermore, cases of canine multiple pigmented plaques that evolved into SCC have been reported.

In humans, solar keratosis, Bowen’s disease and Bowenoid papulosis are regarded as forms of in situ SCC. Some of them subsequently develop into invasive SCCs. Mucosal forms of SCCs also affect the cervical, anal and oral mucous membranes. Furthermore, *Epidermodysplasia verruciformis* is a rare genetic predisposition to develop viral warts with high risk of carcinomatous transformation.

The aetiology of canine skin and mucous membrane SCC remains largely unknown, although environmental factors such as sunlight exposure or burns have been implicated. The role of papillomaviruses (PV) also remains unclear and very few reports have confirmed the association between PV and SCC in dogs. Additionally, the role of these viruses in the development of cancer has not been established and the genomes of potentially causative viruses have not yet been cloned and analysed.

Similarly, the role of human PVs (HPV) in the development of human skin SCC remains controversial. HPV DNA is, however, frequently uncovered in at least three types of human skin SCCs: *Epidermodysplasia verruciformis*, Bowen’s disease and Bowenoid papulosis. Additionally, links between HPVs and cervical and anal human SCCs have been well demonstrated and the causality established.

Papillomaviruses are host-specific epitheliotropic DNA viruses that infect skin and mucous membranes. The complete genome and the biological properties of only one canine papillomavirus are well known. However, the existence of up to six different types has been suggested.

In contrast, classification of HPVs has recently been reviewed, and nearly 100 HPV types have been described based on isolation and sequencing of complete genomes. de Villiers has additionally proposed criteria to define genera, species, types, subtypes and variants within the *papillomaviridae* family. It has also recently been shown that phylogenetic classification based on the L1 gene of PVs correlates, at least partially, with the biological and pathological properties.
Papillomavirus infection may either be subclinical, or induce microlesions or benign neoplasias. Additionally, a subset of HPVs and animal PVs is clearly implicated in the development of cancer in humans and animals. In humans, these PVs cause mucosal carcinomas and are referred to as high-risk PVs.

The nature and the biological properties of canine PVs, as well as the aetiology of canine SCC, remain largely unknown. As HPVs that induce benign warts in humans are usually different from those that induce cancer, it can be speculated that the well known canine oral PV (COPV) does not usually induce SCC in dogs and that some other unknown canine PVs, the canine counterparts of the high-risk HPVs, may be responsible or contribute to such development.

The purpose of our study was therefore to detect a broad spectrum of PV DNAs in samples from three forms of canine SCC (cutaneous invasive, cutaneous in situ and mucosal invasive) and to sequence the amplified DNA.

**MATERIALS AND METHODS**

*Materials*

Fifty-seven samples of paraffin-embedded skin were included in the study:

- seventeen samples of canine invasive cutaneous SCC;
- twenty-three samples of canine mucosal SCC;
- two samples of canine in situ SCC;
- ten samples of canine skin with various nontumoral conditions;
- three samples of virus-induced canine wart;
- one sample of virus-induced canine in situ SCC; and
- one sample of virus-induced bovine fibropapilloma.

Except for one case (#2 provided by Dr T. L. Gross), all samples were selected by one board-certified pathologist (BH) at the Institute of Veterinary Pathology of the University of Zürich, Switzerland.

*Methods*

The study was carried out using a PCR technique on formalin-fixed, paraffin-embedded samples. Thirty-µm-thick sections of each sample were cut from each tissue block, using a new disposable microtome blade for thick sections of each sample were cut from each tissue block. Rigorous precautions were taken in order to avoid cross-contamination between samples.

**DNA extraction.** Each section was deparaffinized twice with 1.2 mL xylene at room temperature for 10 min, washed with ethanol 100% and then dried. The desiccated samples were suspended in an ATL lysis buffer (50 mM Tris-HCl, pH 8.5, 1 mM ethylenediaminetetraacetic acid, 2.8% sodium dodecyl sulphate and 20 µg/mL Proteinase K) and incubated at 56°C on the rocking platform overnight. After lysis, samples were transferred to a QIA shredder™ column (Quiagen, Basel, Switzerland) and centrifuged in order to reduce viscosity. Desoxyribonucleic acid was precipitated with absolute ethanol and extracted with the QIAamp® DNA Mini Kit (Quiagen).

**Papillomavirus detection and sequencing**

Phylogenetic studies have shown that COPV and feline PV are closely related and that this group is closer to some human PVs, including oncogenic PVs, than to PV in other animals, such as bovine PV. The investigators consequently selected two sets of primers: the first one (PapE1-Forward, PapE1-Reverse) is designed to amplify specifically COPV and FdPV DNA and the second one (CP4, CP5, PPF1) is designed to amplify various HPV DNA, especially the oncogenic ones.

**Narrow-range PCR with PapE1 primers**

All samples were coded before being assayed. The sequences encoding E1 are most highly conserved amongst canine, feline or closely related PVs. Therefore, the E1 sequences of feline [LOCUS AF480454] and canine [LOCUS NC001619] papillomaviruses were aligned with the aim of designing degenerated consensus primer pairs able to amplify an estimated 341-bp fragment from both phylogenetically related genomes. The forward primer used was 5'-ATGGCGGMMTARAAAGGTAT'-3' and the reverse primer used was 5'-AACAGCTGYTTTTTARCYTTTTT'-3'. To amplify an internal 184-bp fragment using the same primer forward, a second reverse primer 5'-GAAACA- GTTGCAGGGAAAGTC'-3' was designed.

The PCR reactions were performed in 30-µL volumes, containing 1 µL of genomic DNA, 50 mM KCl, 3 mM KCl2, 200 µM of each dNTP, 0.3 µM each of consensus sense and antisense primers and 2.5 U of PfuTurboDNA polymerase (Stratagene, CA, USA). Polymerase chain reaction amplification involved an initial denaturation step at 95°C for 4 min, followed by 30 cycles at 95°C for 1 min, 50°C for 1 min and 74°C for 1 min, with a final elongation step at 74°C for 5 min. Reaction mixture with no DNA served as negative control, and COPV-positive papilloma DNA samples and feline papilloma positive DNA samples were used as positive controls. The PCR products were resolved by electrophoresis in 2% agarose gel stained with ethidium bromide. Amplified DNA was sequenced using AB-3100-based fluorescent sequencing and BigDye™ terminator chemistry.

**Broad-range PCR with CP4, CP5 & PPF1 primers**

In order to amplify as many different PVs as possible, the CP4, CP5, PPF1 was selected, because of its ability to uncover up to 64 different HPVs.

The PCR reactions were performed in 30-µL volumes, containing 1 µL of genomic DNA, 50 mM KCl, 3 mM KCl2, 200 µM of each dNTP, 0.45 µM of the CP4 and CP5 primers and 0.3 µM of the PPF1 primer, and 2.5 U of PfuTurboDNA polymerase (Stratagene). Polymerase chain reaction amplification involved an
initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 1 min, 47 °C for 1 min and 74 °C for 1 min, with a final elongation step at 75 °C for 5 min. Reaction mixture with no DNA served as negative control, and COPV-positive papilloma DNA samples and feline papilloma positive DNA samples were used as positive controls. The PCR products were resolved by electrophoresis in 1% agarose gels stained with ethidium bromide. Amplified DNA was sequenced using AB-3100-based fluorescent sequencing and BigDye™ terminator chemistry, and obtained sequences were compared with entries in the GenBank database.

Interpretation of the results and sequence analyses
Samples were deemed positive if the two following criteria were fulfilled:

- samples exhibited a band of the expected size after gel electrophoresis;
- the amplified DNA exhibited a significant homology with DNA coding for the E1 protein of a previously established PV. Comparisons were made with the BLAST software (GenBank – National Center of Biotechnology Information: NCBI).

Sequences were subsequently compared to each other on the amino acid sequence level in order to establish their homology on the protein level.

RESULTS

Clinical and histological criteria
Available SCC cases were assigned to clinical and histological groups according to the following criteria. Two dogs (cases 1 and 2) exhibited numerous hyperpigmented, scaly maculae, plaques and nodules. One of the plaques of case 1 ulcerated and was subsequently biopsied (Fig. 1). Case 2 exhibited several ulcerated nodules that were biopsied. Histopathological examination of the two cases revealed marked acanthosis, orthokeratotic hyperkeratosis and hypergranulosis (Fig. 3) with keratohyalin granule clumping (Fig. 3). The epidermis was disorganized, with numerous atypical cells and premature keratinization. Proliferation of basaloid cells was observed in some areas but most of the atypical cells were of the squamous type (Fig. 3). Atypia consisted of macrokaryosis, anisokaryosis (Fig. 3), hyperchromasia, prominent nucleoli, multinucleated cells and abnormal mitoses (Fig. 4). However, the basement membrane was intact and the dermis was not affected (Figs 2, 3 and 4). Therefore, these dogs...
Detection of novel papillomaviruses were considered to represent cases of *in situ* SCC (Table 1).

Seventeen additional cases represented the group of skin-derived invasive SCC. Some cases exhibited a proliferative, exophytic and invasive pattern (Fig. 5), whereas others were also invasive but more ulcerative (Table 1). Histologically, they all consisted of cords or islands of atypical cells that invaded the dermis (Fig. 6). Large nuclei with prominent nucleoli were present in all cases, as well as numerous mitoses (Fig. 7). Premature keratinization and intercellular bridges were also present in most of the samples. Finally, 23 cases of mucous membrane-derived invasive SCC were available, which histologically all exhibited similar changes as those described above for skin-derived SCCs. Seventeen of the latter lesions arose from the gingiva, four from the tongue, one from the nasal mucosa and one from the lips.

Three canine papillomas, one feline squamous cell carcinoma *in situ* and one bovine fibropapilloma were included as positive controls. The bovine fibropapilloma and the canine papillomas exhibited changes typical of papillomavirus infections, such as koilocytosis, clumping of the keratohyalin granules and viral inclusion bodies (data not shown). The feline *in situ* SCC (data not shown) had previously been shown by immunohistochemistry to react positively with papillomavirus-specific antibodies.

**PCR studies**
Papillomavirus DNA was detected in the positive control tissues but not in the negative control tissues. The narrow-range PCR, optimized to detect known feline and canine papillomaviruses, reacted positively with extracts from the canine papillomas as well as with the immunohistologically positive case of feline *in situ* SCC. In contrast, this set of primers was unable to discover bovine papillomavirus in extracts from the bovine fibropapilloma tissue. However, the broad-range PCR detected papillomavirus DNA in both carnivorous and bovine positive control tissues.
The results of the test samples included in this study are presented in Table 2. Interestingly, the narrow-range PCR detected only one case of SCC associated with a papillomavirus infection, namely a mucous membrane-derived SCC (case 20). However, this case and eight additional cases were detected with the broad-range technique, including 2/2 samples from in situ SCC, 2/17 invasive skin SCCs, and 5/23 cases of mucous membrane-derived invasive SCC.

**Sequencing studies**
The above PCR results strongly suggested that thus far unknown papillomaviruses had been detected with the broad-range PCR. To address this issue, the nucleotide sequence of the papillomavirus-positive cases was compared with those available in GenBank.

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**Table 1. Identification and properties of clinical SCC cases**

<table>
<thead>
<tr>
<th>Case #</th>
<th>Group</th>
<th>Breed</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>In situ</em></td>
<td>Rhodesian ridgeback</td>
<td>M</td>
<td>4</td>
<td>Diffuse</td>
</tr>
<tr>
<td>2</td>
<td><em>In situ</em></td>
<td>Retriever mix</td>
<td>F</td>
<td>5</td>
<td>Abdomen, limbs</td>
</tr>
<tr>
<td>3</td>
<td>Skin</td>
<td>German shepherd dog</td>
<td>F</td>
<td>13</td>
<td>Limb</td>
</tr>
<tr>
<td>4</td>
<td>Skin</td>
<td>Flatcoat retriever</td>
<td>F</td>
<td>12</td>
<td>Clawbed</td>
</tr>
<tr>
<td>5</td>
<td>Skin</td>
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<td>M</td>
<td>13</td>
<td>Nasal planum</td>
</tr>
<tr>
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<td>F</td>
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<tr>
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<tr>
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<td>Clawbed</td>
</tr>
<tr>
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<td>12</td>
<td>Limb</td>
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<tr>
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<td>Flatcoat retriever</td>
<td>M</td>
<td>8</td>
<td>Limb</td>
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<td>Skin</td>
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<td>10</td>
<td>Limb</td>
</tr>
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**Table 2. Papillomavirus-positive cases**

<table>
<thead>
<tr>
<th>Case #</th>
<th>Group*</th>
<th>Breed</th>
<th>Sex</th>
<th>Age</th>
<th>Localization</th>
<th>Related to†</th>
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<td>1</td>
<td><em>In situ</em></td>
<td>Rhodesian ridgeback</td>
<td>M</td>
<td>4</td>
<td>Diffuse</td>
<td>HPV65‡</td>
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<tr>
<td>2</td>
<td><em>In situ</em></td>
<td>Retriever mix</td>
<td>F</td>
<td>5</td>
<td>Abdomen, limbs</td>
<td>BPV1</td>
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<tr>
<td>11</td>
<td>Invasive skin</td>
<td>Golden retriever</td>
<td>M</td>
<td>12</td>
<td>Limb</td>
<td>HPV85</td>
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<tr>
<td>18</td>
<td>Invasive skin</td>
<td>Weimaraner</td>
<td>M</td>
<td>8</td>
<td>Face</td>
<td>HPV9</td>
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<tr>
<td>20</td>
<td>Mucous membrane</td>
<td>Cocker</td>
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<td>7</td>
<td>Nose</td>
<td>COPV§</td>
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<tr>
<td>33</td>
<td>Mucous membrane</td>
<td>West Highland white terrier</td>
<td>F</td>
<td>12</td>
<td>Gingiva</td>
<td>HPV65</td>
</tr>
<tr>
<td>36</td>
<td>Mucous membrane</td>
<td>Mongrel</td>
<td>M</td>
<td>13</td>
<td>Gingiva</td>
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</tr>
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<td>40</td>
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<td>M</td>
<td>6</td>
<td>Gingiva</td>
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<tr>
<td>42</td>
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<td>Golden retriever</td>
<td>M</td>
<td>4</td>
<td>Gingiva</td>
<td>HPV4</td>
</tr>
</tbody>
</table>

*Clinical SCC type; †closest relative detected by NCBI-BLAST analysis; ‡unless otherwise stated, detected exclusively by broad-range PCR; §detected by both narrow-range and broad-range PCR.

The results of the test samples included in this study are presented in Table 2. Interestingly, the narrow-range PCR detected only one case of SCC associated with a papillomavirus infection, namely a mucous membrane-derived SCC (case 20). However, this case and eight additional cases were detected with the broad-range technique, including 2/2 samples from *in situ* SCC, 2/17 invasive skin SCCs, and 5/23 cases of mucous membrane-derived invasive SCC.
sequences of the amplification products of the individual PCRs were determined and compared by BLAST analysis to known papillomavirus sequences. If available (7542 dogs), several independent samples from each dog were used for PCR and sequencing, and sequencing results were identical for different locations of the lesions from each individual dog. However, the sequences differed largely between different dogs. As expected, the sequence obtained from case 20 turned out to be closely related to the published sequence of canine oral papillomavirus. In contrast, the remaining eight positive cases were more closely related to other papillomaviruses, such as human and bovine papillomaviruses (Table 2). Although the classification of papillomavirus is based on the L1 sequences, these results strongly support that novel canine papillomaviruses were detected in canine SCCs throughout this study. However, 78% of the tested samples still remained negative, which indicated that either a large proportion of PVs of great genetic diversity.

Breed, sex and age distribution
These data are included in Tables 1 and 2. Although schnauzers appeared to be over-represented in the total sample, retrievers (flatcoated and golden) (19% of the sample but 33% of papillomavirus-positive individuals) emerged as a possible breed group with a tendency to SCC caused by papillomavirus. The total sex distribution resulted in 60% male and 40% female dogs. However, 70% of the papillomavirus-positive dogs were male. The average age of dogs with SCC was 9.25 years, with in situ SCC, 4.5 years, with invasive skin SCC, 9.38 years, and of dogs with mucous SCC, 9.57 years. The average age of papillomavirus-positive dogs with SCC was 7.89 years.

DISCUSSION
In this study, it was possible to uncover PV DNA in 9/42 samples of canine SCC (21.4%) with a broad-range PCR-assay and in 1/42 (2.3%) with a narrow-range PCR-assay designed to amplify DNA from COPV, FdPV and closely related PVs. Interestingly, the last figure is in line with that of the only other study which fulfilled by the canine in situ SCCs. The clinical relevance of the presence of PVs in invasive mucosal and skin SCC lesions is another important question. Assuming that PVs play a role in the development of canine SCC, the absence of PV DNA in numerous canine SCC can be explained by the ‘hit and run’ model, which postulates an initial transformation of the infected cell and a subsequent loss of PV-DNA. Interestingly, in a previous study in canines, a PV antigen-negative SCC arose from a PV antigen-positive Epidermodysplasia verruciformis-like lesion. This model, however, implies a deletion of viral genes during integration of the viral DNA in the host chromosomes. Such a deletion has only been demonstrated in humans with the E2 gene. As human high-risk papillomaviruses are genetically different from low-risk HPVs one can postulate that canine high-risk PV (provided they do exist) should be genetically different from COPV. As we have only used sets of primers that were designed to uncover COPV DNA and high-risk HPV DNA, we cannot rule out that the DNA of some unknown canine PV remained undetectable.

The amplified DNA sequences suggest the presence of several different PVs in the canine SCCs. Furthermore, eight out of nine positive samples were infected by these unknown PVs. Moreover, our findings confirm that dogs, as well as humans, can be infected with PVs of great genetic diversity.
The detection of PV DNA in SCC tissues and not in normal skin or skin affected by other conditions might result from the increased replication of latent virus in response to tumoral cell cytokine secretions.\(^1\)

Mitsuishi \textit{et al.} have uncovered HPV DNA in 74 and 67\% of the samples of human actinic keratosis and Bowen’s diseases, respectively.\(^9\) In the same study, no differences in p53, p21, Ki 67 and PCNA were found between HPV-positive and HPV-negative samples. This set of data suggests that HPVs probably play a role in the pathogenesis of both conditions but that the viruses alone are not able to induce cancer transformation.

Human SCCs also occur many years after the initial infection, which usually has a benign course, even with oncogenic HPV types.\(^4\) Malignant transformation thus implies the presence of continuing infection and prolonged E6/E7 oncogene expression.\(^33\) Such chronic infections occur in human anal or cervical PV infections and \textit{Epidermodysplastra verruciformis} but have not been demonstrated with canine PV infections.\(^20\)

Although the present results cannot be regarded as sufficient proof for the carcinogenic potential of canine PVs, the detection of novel members of this large family of viruses is important for further research on this issue.

Importantly, the novel papillomaviruses were uncovered in the two cases of SCC \textit{in situ}. These two dogs were younger than the average age of the available dogs with SCC, which also favours a viral aetiology for this type of lesions. Indeed, the presence of PVs in such lesions has been established previously.\(^4\) Additionally, one of the lesions described by this author subsequently developed into invasive SCC and the similarities between these cases and the human \textit{Epidermodysplastra verruciformis} has already been emphasized.\(^4\) The presence of specific oncogenic PVs in a significant number of such canine lesions consequently warrants further investigation.

The study has also demonstrated the great diversity of the canine PVs. Cloning these new viruses will allow phylogenetic comparison with human commensal and high-risk PVs. These comparisons can eventually provide clues for the interpretation of past and future studies.

ACKNOWLEDGEMENTS

The authors would like to acknowledge Dr Thelma Lee Gross who has provided one of the samples (case #2).

REFERENCES

Detection of novel papillomaviruses


Résumé L’ADN de papillomavirus (PV) est fréquemment retrouvé dans des achantillons de carcinome épidermoïde (SCC) chez l’homme. Cependant le rôle de ces virus dans le développement de ces cancers est controversé. Alors qu’environ une centaine de PV sont recensés chez l’homme, un seul PV canin oral (COPV) a été identifié et étudié. Nous avons utilisé une technique de PCR spécifique des PV canin et félín, ainsi qu’une technique de PCR utilisée pour la détection des nouveaux PV humains, afin d’analyser 42 biopsies parafiânées, représentant trois formes différentes de SCC canins. 10 échantillons de tissus affectés par des maladies non néoplasiques ont servi de contrôle. Aucun des témoins négatifs n’a réagi positivement, et de l’ADN de PV a été retrouvé caj 21% des prélèvements testés de SCC. Le COPV classique n’a été amplifié qu’une seule fois, alors que les autres cas positifs étaient associés à la présence d’une variété inconnue de PV humain. Cette étude suggère qu’une fraction des SCC canins est infectée par le PV, et qu’il existe une variété génétique des PV canins. Ces résultats vont faciliter les études futures qui s’intéresseront au rôle des PV dans le développement des cancers cutanés du chien.

Resumen El ADN del virus papiloma (PV) se descubre de forma frecuente en muestas de carcinoma de células escamosas (SCC) de la piel humana. Sin embargo, el papel de estos virus en el desarrollo de estas neoplasias en el perro es aún controvertido. Mientras que se conocen aproximadamente 100 virus papiloma en humanos, tan sólo un virus papiloma canino oral (COPV) ha sido identificado y estudiado de forma exhaustiva. Por ello, para analizar 42 muestras incluidas en parafina que representaban tres formas diferentes de carcinomas de células escamosas en perros, aplicamos una reacción de polimerasa en cadena (PCR) de estrecho rango, válida para detectar virus papiloma clásicos caninos y félinos; y también una PCR de amplio rango, que ha sido utilizada para la detección de nuevos virus papiloma en humanos. Diez muestras de piel con lesiones no neoplásicas se utilizaron como controles. Mientras que ninguno de los controles negativos dio resultado positivo, ADN de virus papiloma se encontró en un 21% de las muestras de carcinoma de células escamosas. Curiosamente, el clásico virus papiloma oral canino solo se amplificó de una muestra, mientras que los otros casos positivos se asociaron con variedades hasta ahora desconocidas de virus papiloma. Este estudio sugiere que una fracción de carcinomas de células escamosas caninos está infectada con el virus papiloma, y que existe una diversidad genética de virus papiloma caninos. Por lo tanto, estos resultados facilitarán futuros estudios sobre el papel del virus papiloma en el desarrollo de cáncer de piel en perros.

Zusammenfassung Papillomavirus (PV) DNA wird häufig in Hautproben von Plattenepithelkarzinomen des Menschen gefunden. Beim Hund bleibt die Rolle dieser Viren bei der Entstehung derartiger Tumoren allerdings
Chapter 7

Detection of novel papillomavirus-like DNA sequences in paraffine-embedded samples of feline invasive and in situ squamous cell carcinomas

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Detection of novel papillomaviruslike sequences in paraffin-embedded specimens of invasive and in situ squamous cell carcinomas from cats

Gilles Nespeca, Med Vet; Paula Grest, DVM; Wayne S. Rosenkrantz, DVM; Mathias Ackermann, DVM, PhD; Claude Favrot, DrVet, MS

**Objective**—To detect and partially characterize papillomavirus (PV) DNA in squamous cell carcinoma (SCC) tumor specimens from cats.

**Sample Population**—54 formalin-fixed paraffin-embedded skin biopsy specimens were examined. Specimens originated from Bowenoid in situ SCC (BISC; n = 21), invasive SCC (22), and skin affected by miscellaneous nonneoplastic conditions (11).

**Procedures**—Samples from each tissue block underwent DNA extraction after deparaffinization, and PCR assays were performed. Two sets of primers derived from PV E7 were used. The first set of primers was designed for the narrow-range PCR assay and was able to generate amplification products of feline PV (FePV), canine oral PV, or closely related PVs. The second set of primers was selected for the broad-range PCR assay because of its ability to amplify DNA from 64 human PVS. Sequence analysis of each amplified DNA was performed.

**Results**—1 of the 21 specimens of BISC was positive for PV DNA on the basis of narrow-range PCR assay results, whereas all the other specimens (BISC, invasive SCC, and controls) had negative results for PV DNA. In contrast, 5 of 21 BISC specimens and 4 of 22 invasive SCC specimens were positive for PV DNA on the basis of broad-range PCR assay results. Sequence analysis revealed that only 1 specimen was infected by a virus closely related to classic FePV. In the 8 other specimens positive for PV DNA, DNA of unknown PVS was uncovered.

**Conclusions and Clinical Relevance**—Bowenoid in situ SCC and invasive SCC of cats may be associated with PVs of genetic diversity. (Am J Vet Res 2006;67:2036–2041)

Squamous cell carcinoma is, after basal cell carcinoma, the second most common cancer of the skin in humans,²² Squamous cell carcinoma involves cancerous changes to the cells of the middle portion of the epidermal skin layer. This cancer may begin in normal skin; in skin at the site of a burn, injury, or scar; or at a site of chronic inflammation.¹ Most often, it originates from AK, a precancerous skin growth associated with sun exposure.¹ However, AK is often regarded as a form of SCC, which is confined to the epidermis; thus, AK is also referred to as in situ SCC.²² A second form of in situ SCC, precancerous dermatitis (termed Bowen's disease), presents as 1 or more flat red scaly patches up to several centimeters wide, often found in large numbers.³⁻⁶ In situ SCC can persist as such; regress; or develop into a third, even more malignant form, invasive SCC. Similar skin cancers are also observed in veterinary medicine, specifically in cats.⁷ Squamous cell carcinoma has been linked to a variety of causative associations, which include exposure to UV or ionizing radiation; arsenic ingestion; toxic exposure to tars and oils; immunosuppression from drugs such as corticosteroids, azathioprine, and cyclosporine; and last but not least, to PV infection.⁸⁻¹⁰

Papillomaviruses are host-specific epitheliotropic DNA viruses that infect skin and mucous membranes. In general, PV infections are benign, result in a latent infection, or induce microlesions or benign neoplasias.¹¹⁻¹₄ However, a subset of HuPVs and other animal PVS is clearly implicated in the development of cancer.¹₀,¹₄⁻¹₇ Human PVSs that cause mucosal and skin carcinomas in humans are referred to as high-risk PVS or epidermodysplasia verruciformis–associated HuPV types, respectively.¹⁴

Close to 100 HuPV types have been described on the basis of isolation of complete genomes.¹₃ Knowledge on the combination of biological properties and sequence similarities led to the definition of new criteria to define genera, species, types, subtypes, and variants within the Papillomaviridae family.¹₃

In contrast to the numerous HuPVs, only a single FePV has been identified.¹⁹ However, the existence of a few other FePVs has been suggested on the basis of findings from several clinical and immunohistochemical studies.¹⁹⁻²²

Until now, little has been known about the presence of PVs in SCCs of cats. Investigators in 1 study²³

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**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Term</th>
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<tr>
<td>AK</td>
<td>Actinic keratosis</td>
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<td>SCC</td>
<td>Squamous cell carcinoma</td>
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<td>PV</td>
<td>Papillomavirus</td>
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<td>Human PV</td>
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<td>FePV</td>
<td>Feline PV</td>
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<td>BISC</td>
<td>Bowenoid in situ SCC</td>
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<td>CaPV</td>
<td>Canine PV</td>
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<td>BoPV</td>
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failed to uncover PV antigen in SCCs of cats, whereas findings in another study revealed the presence of PV antigens in 44% of tumor specimens of BISC from cats. Furthermore, PV DNA has been uncovered in tumor specimens from fibropapillomas, another type of cutaneous proliferative disease, of cats.25

Similar to the human disease types, 3 varieties of SCC have been described for cats, which are AK, BISC, and invasive SCC.25 Actinic keratosis usually occurs as a solitary lesion on sun-exposed, lightly haired areas, such as ear tips, external nares, or eyelids. White cats are predisposed for the development of such lesions. On the other hand, BISC is characterized usually by multiple well-circumscribed, hyperpigmented lesions that occur frequently on the face, neck, and limbs.17,28 To our knowledge, comparative studies of these 2 early forms of cancer have not been performed in cats.

The purpose of the study reported here was to detect PV DNA in specimens representing the various types of SCC in cats and in specimens from feline skin with various nontumor conditions. We wanted to test whether tumor specimens from cats with SCC were more often infected by PVs than nontumor skin specimens. Two types of PCR assays, narrow and broad range, were applied to extend the range of targeted PVs as far as possible.

Materials and Methods

Tissue specimens—Tissues were obtained from the collections of the Prairie Diagnostics Services, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada; the Institut de Pathologie et de génétique, Looeveral, Belgium; Rest associates, London; and, the Pathology Institute, Vetsuisse Faculty, University of Berne, Berne, Switzerland. Fifty-four formalin-fixed paraffin-embedded skin biopsy specimens were examined. Specimens originated from BISC (n = 21), invasive SCC (22), and miscellaneous skin conditions other than skin cancers (eg, allergic dermatitis; 11) that were used as negative controls. Specimens from white cats or from locations typical for AK such as ear tips and eyelids were excluded from this study. Thirty-micrometer-thick sections were cut from each tissue block, with a new disposable microtome blade for each block, before DNA extraction. Two canine warts, which had histopathologic characteristics of typical PV-induced inclusion bodies, and 1 bovine fibropapilloma served as positive controls.

DNA extraction—The protocol of Albini et al was used for DNA extraction. Briefly, each section was deparaffinized twice with 1.2 mL of xylene at room temperature (approx 20°C) for 10 minutes, washed with 100% ethanol, and then dried at 37°C for 30 minutes. Desiccated samples were suspended in a tissue lysis buffer (50mM Tris-HCl [pH 8.5], 1mM ethilenediaminetetraacetic acid, and 2.8% sodium dodecylsulfate) and proteinase K (20 mg/mL) and incubated at 56°C on a rocking platform overnight. After lysis, samples were transferred to a column and centrifuged to reduce viscosity. The DNA was precipitated with absolute ethanol and extracted with a commercial DNA kit.8

Primers—Two sets of primers were used for the PCR assay. Because the sequences encoding E1 were highly conserved, the E1 regions of FePV (GenBank accession No. AF480434) and CaPV (GenBank accession No. NC001619) were aligned to design a set of consensus primers (ie, PapF, 5′-ATGGCCGGGTMARAAAGGTA-3′ and PapR, 5′-AACAGCTGYTTTTARCYTTTTT-3′) for narrow-range PCR assay, which is able to generate amplification products of approximately 341 bp of FePV, CaPV, or closely related PVs. The second set of primers (ie, CP4, CP5, and PPF1 primers), also derived from E1, was selected for broad-range PCR assay with the objective of amplifying as many PVs as possible. With this set of primers, up to 64 HuPVs are identifiable.30 The expected size of the PCR product was approximately 450 bp.

PCR assay and agarose gel electrophoresis—Polymerase chain reaction conditions for PapF and PapR were performed. Volumes of 30 mL were used. Each reaction contained 1 µL of genomic DNA, 200 µM of each deoxynucleoside triphosphate, 0.3µM of each of the sense and antisense primers, and 2.5 units of a DNA polymerase. After an initial denaturation step at 95°C for 4 minutes, PCR assay was performed for 30 cycles at 95°C for 1 minute, 50°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 74°C for 5 minutes. Deoxyribonucleic acid extracted from canine warts served as positive control, whereas DNA- and RNA-free water was used as negative control.

The PCR mix with CP4, CP5, and PPF1 primers was identical to the mix for narrow-range PCR assay, except that 0.45µM of the CP4 and CP5 primers and 0.3µM of the PPF1 primer were used. The PCR assay consisted of a denaturation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 1 minute, 47°C for 1 minute, and 74°C for 1 minute, with a final elongation step at 74°C for 5 minutes. An extract from bovine fibropapilloma served as an additional positive control.

Polymerase chain reaction products were segregated by agarose gel electrophoresis, and bands were viewed under UV light after ethidium bromide staining. Bands on the gel were excised, and DNA was extracted with a gel extraction kit.4 Amplified DNA was sequenced by use of fluorescent sequencing and terminator chemistry.1

Sequence analysis—Samples were considered positive for PV DNA if they met the following requirements: they had a band of the expected size after gel electrophoresis and the sequenced DNA had homology with E1 of previously sequenced PVs. Homologous DNA sequences were searched for by use of the National Center for Biotechnology Information GenBank via a BLAST search.3 Sequence alignments and phylogenetic trees were made from the clustal algorithm obtained by use of a software program.4

Results

Macro- and microscopic analysis—A careful macroscopic selection and microscopic confirmation of affected specimens was a major prerequisite prior to the virologic analysis. Specimens representing invasive SCC had been resected from sun-exposed or white areas of 21 domestic shorthaired cats and 1 Persian cat (12 males and 10 females). Twenty-two specimens from ear tips (n = 11), nose (3), eyelids (3), digits (3), and lips (2) met criteria required for invasive SCC. These criteria included the macroscopic presence of scaly-to-crusty and erosive-to-plaque-like or ulcerative lesions (Figure 1). The growth process was always endophytic. Histologically, cords or islets of infiltrative cells were detected in all specimens. Furthermore, anisocytosis; anisokaryosis; large, hyperchromatic nuclei; prominent nucleoli; increased mitotic index; and abnormal mitoses were encountered in all specimens with variable intensity and in variable proportion. In addition, keratin pearls
and intercellular bridges were present in 16 and 12 specimens, respectively.

A second group of 21 tumor specimens met the criteria for BISC. These specimens were obtained from the face (n = 16), neck (12), and limbs (3) or were scattered (2). Thirteen domestic shorthair cats, 3 domestic longhair cats, 2 Siamese, 1 Persian, 1 Himalayan, and 1 Cornish Rex were affected. Macroscopically, the lesions were squamous crustosus and grossly circular. Two lesions had a single center, but 19 were multicentric (Figure 1). Microscopically, the following criteria were met for BISC: moderate-to-severe parakeratotic hyperkeratosis, acanthosis with papillomatous hyperplasia (n = 1) or irregular hyperplasias (2), loss of polarity, and scattered dyskeratotic keratinocytes atypia in all layers of the epidermis and usually also in the infundibulum and reaching the isthmus. Furthermore, the following types of atypia were recorded: enlarged nuclei, anisokaryosis, monster cells (bizarre multinucleated giant cells), and abnormal mitotic figures. Hyperpigmentation was found in all but 3 specimens. Fifteen of the 21 specimens had clumped keratohyalin granules, which were considered as suggestive for PV infection. However, other signs such as koilocytosis and nuclear inclusion bodies were not detected.

With the exception of the ulcerated lesions, the dermis of all samples was considered normal and not heavily inflamed (Figure 1). Thus, a total of 22 samples representing invasive SCC and 21 samples representing BISC were available for virologic analysis by PCR assay and sequencing.

PCR assays—The narrow-range PCR assay amplified PV DNA extracted from canine warts but not DNA extracted from bovine fibropapilloma (Figure 2). In contrast, the broad-range PCR assay amplified PV DNA from canine warts and bovine fibropapilloma. It was concluded that both PCR assays were able to specifically amplify selected PV DNAs.

The narrow-range PCR assay was applied to samples from invasive SCC and BISC specimens; 1 BISC sample (BISC sample No. 15; Appendix; Figure 3) had positive results for PV DNA, whereas the others had negative results. Next, the broad-range PCR assay was applied to the same samples. Interestingly, 5 of 21 BISC samples (BISC sample Nos. 2, 5, 6, 10, and 15) as well as 4 of 22 SCC samples (SCC sample Nos. 15, 24, 28, and 29) had positive results for PV DNA (Figure 2). One of the samples that had positive results for PV DNA on the broad-range PCR assay (BISC sample No. 15) also had positive results for PV DNA on the narrow-range PCR assay. These results suggested that the broad-range PCR assay was indeed able to uncover PVs that were different from the known FePV and CaPVs.

Figure 1—Macroscopic and microscopic lesions of SCCs in cats. A—Photograph of invasive SCC in a cat with ulceration of the eyelid. B—Photomicrograph of a section of the invasive SCC from panel A at low magnification. Notice invasive proliferation of atypical keratinocytes with pearl formation (white arrow) and the cornified layer (red arrow). The basal membrane is not discernible. The dermis (long arrow) is invaded by cords of atypical keratinocytes (short black arrow, pointing towards such an invasive site). H&E stain; bar = 200 µm. C—Photomicrograph of a section of the invasive SCC from panel A at high magnification. Notice islets of keratinocytes with features of malignancy, such as anisokaryosis, anisocytosis, multinucleated cells (short arrow), and abnormal mitosis (long arrow). H&E stain; bar = 50 µm. D—Photograph of BISC in a cat with circular, crusted, erosive, and hyperpigmented plaques (arrow). E—Photomicrograph of a section of the BISC from panel D at low magnification. The basal membrane (white arrow) is intact, and the dermis is not invaded. Irregular acanthosis (long black arrow) is obvious. Notice that hair follicles are affected (short arrow). H&E stain; bar = 200 µm. F—Photomicrograph of a section of the BISC from panel D at high magnification. Notice acanthosis, hyperpigmentation (brown cells), clumped keratohyalin granules (white arrow), loss of polarity, and anisokaryosis (branched arrow). H&E stain; bar = 50 µm.

Figure 2—Establishment of broad-range and narrow-range PCR assays for detection of carnivore PVs. Polymerase chain reaction products were loaded on agarose gels and stained with ethidium bromide. A—Amplification of cloned DNA by either broad-range (450 bp) or narrow-range PCR assay (341 bp). Lane 1 = Water in place of DNA added to the reaction. Lane 2 = DNA from a commercially available phagemid. Lane 3 = DNA from a tumor specimen of a cat with SCC (GenBank accession No. DQ085784). Lane 4 = DNA from FePV cloned into the phagemid. M1 = Molecular weight marker (100-bp ladder). B—The DNA was extracted from tissues before being amplified by either the narrow range or the broad-range PCR assays. M2 = 1-kilobase ladder. M1 = 100-bp ladder. Lane 1 = Negative control with no DNA added to the reaction. Lane 2 = Extract from canine wart tissue, which had typical PV-induced inclusion bodies on histologic examination. Lane 3 = Extract from a tumor specimen of a cat with SCC (GenBank accession No. DQ088784). Lane 4 = Extract from SSC sample No. 39.
like sequences had been detected. Indeed, use of the sequences were compared to assess whether novel PV-like species could be identified. A phylogenetic tree drawn from the aligned sequences divided the new sequences into 4 clusters (Figure 3). In among the HuPVs, types 4, 55, 63, 65, 71, and 74 were closely related to the newly detected viral sequences, with a relative amino acid identity of 56% to 71%. Among the BoPVs, type 5 was closest relationship to the new sequences with 58% to 61% amino acid identity. Among the BoPVs, type 5 was closest to the newly detected viral sequences, with a relative amino acid identity of 56% to 71%. Among the HuPVs, types 4, 55, 63, 65, 71, and 74 were aligned most frequently but type 71 most often had the closest relationship to the new sequences with 58% to 61% amino acid identity. Among the BoPVs, type 5 was the closest relative, having 55% to 62% amino acid identity. A phylogenetic tree drawn from the aligned sequences divided the new sequences into 4 clusters (Figure 3). In cluster 1, BISC sample No. 15 was situated most closely with CaPV and FePV. Cluster 2 was occupied by BISC sample No. 10 and was between FePV and HuPV type 71. Cluster 3 was represented by BISC sample No. 2 and found close to HuPV type 71. The remaining sequences (SCC sample Nos. 15, 24, 28, and 29 and BISC sample Nos. 5 and 6) represented a fourth cluster, which was clearly distinct from BoPV type 5 on the most distant side and HuPV type 71. FePV and CaPV on the less distant side. These results suggested the presence of thus far unidentified PVs in tissues representing invasive SCC and BISC. Interestingly, sequences obtained from specimens of 3 cats with invasive SCC (SCC sample Nos. 15, 24, and 28) had identical sequences. Furthermore, it was observed that not a single sample from invasive SCC specimens had been associated with the more classic FePV and CaPV. However, because the classification of PVs is based on the sequence of L1, the exact taxonomic position of these novel PV-like sequences could not be assigned.

Sequence analysis—The nucleotide sequence of the amplified DNA was determined and the resulting sequences were compared to assess whether novel PV-like sequences had been detected. Indeed, use of the basic local alignment search tool revealed relatedness to PV E1 sequences for all 9 samples that were positive for PV DNA. Relation to HuPV, FePV, CaPV, rat PV, and BoPV was evident. Clustal alignments revealed the various degrees of relationship of the newly determined sequences among each other as well as in comparison with known PVs. Overall, CaPVs and FePVs were most closely related to the newly detected viral sequences, with a relative amino acid identity of 56% to 71%. Among the HuPVs, types 4, 55, 63, 65, 71, and 74 were aligned most frequently but type 71 most often had the closest relationship to the new sequences with 58% to 61% amino acid identity. Among the BoPVs, type 5 was the closest relative, having 55% to 62% amino acid identity. A phylogenetic tree drawn from the aligned sequences divided the new sequences into 4 clusters (Figure 3). In cluster 1, BISC sample No. 15 was situated most closely with CaPV and FePV. Cluster 2 was occupied by BISC sample No. 10 and was between FePV and HuPV type 71. Cluster 3 was represented by BISC sample No. 2 and found close to HuPV type 71. The remaining sequences (SCC sample Nos. 15, 24, 28, and 29 and BISC sample Nos. 5 and 6) represented a fourth cluster, which was clearly distinct from BoPV type 5 on the most distant side and HuPV type 71. FePV and CaPV on the less distant side. These results suggested the presence of thus far unidentified PVs in tissues representing invasive SCC and BISC. Interestingly, sequences obtained from specimens of 3 cats with invasive SCC (SCC sample Nos. 15, 24, and 28) had identical sequences. Furthermore, it was observed that not a single sample from invasive SCC specimens had been associated with the more classic FePV and CaPV. However, because the classification of PVs is based on the sequence of L1, the exact taxonomic position of these novel PV-like sequences could not be assigned.

Discussion

The purpose of our study was to detect and partially characterize PV DNA in samples representing in situ and invasive types of SCC in cats to learn more about PV variants in cats and about possible associations of these viruses with individual forms of SCC in cats. Two types of PCR-assays, a narrow range and a broad-range PCR, were applied to extend the range of targeted PVs as far as possible.

Careful macroscopic selection and microscopic confirmation resulted in the identification of 22 samples representing invasive SCC and 21 samples representing BISC, which were available for virologic analysis by PCR assay. Papillomavirus DNA was detected in 4 of 22 samples representing invasive SCC and in 5 of 21 samples representing BISC, whereas all nontumor control samples had negative results for PV DNA. Only 1 (BISC sample No. 15) of the 9 viral DNAs had been revealed by the narrow-range PCR assay. However, the same narrow-range PCR assay amplified PV DNA extracted from canine warts, which was expected because the primers had been chosen for their homology with conserved sequences within E1 of FePV and CaPV. Yet, the restricted range of these primers was confirmed, as they proved unable to amplify DNA from the more distantly related bovine fibropapilloma virus. These results indicate that the remaining samples with positive results for PV DNA did not harbor conventional FePV or CaPV.

Eight samples, which had negative results for PV DNA on narrow-range PCR assay, had positive results for PV DNA on broad-range PCR assay. The broad-range PCR assay made use of a second set of primers that were also derived from E1 but known to uncover a large variety of HuPVs. In our study, this second set of primers also amplified DNA from bovine fibropapilloma virus as well as viral DNA from canine warts. Sequencing of the amplification products obtained from the 8 samples revealed novel PV-related DNAs, although relations to HuPV, FePV, CaPV, rat PV, and BoPV were evident. A phylogenetic tree drawn from the aligned sequences divided the new sequences into 4 clusters. Three of those clusters had close relationship to CaPV, FePV, and HuPV. Interestingly, the fourth cluster, represented by 6 amplification products, was clearly distinct from BoPV type 5 and from HuPV type 71, FePV, and CaPV. Judging from the limited sequence information available, it appeared as if this fourth cluster represented a novel group of FePVs that had not been detected previously and that may be associated with SCC in cats. Notably, all PVs detected in association with invasive SCC were found to belong to this novel cluster.

This represents, to our knowledge, the first evidence of thus far unknown PV-like sequences associated with SCC in cats. Interestingly, some of the novel sequences were found in association with invasive SCC. Notably, previous attempts to detect conventional PV antigens in such lesions had failed, which led to the hypothesis that invasive carcinomas of cats are probably not virally induced, whereas instances of Bowen’s disease in cats are probably PV-induced. Our findings clearly challenge the former opinion, although...
a causative correlation between the disease and the novel PV strains has not yet been shown. Results of another study did reveal PV antigens in 44% of BSCs. Although the proportion of BSC samples with positive results for PV DNA in our study is lower (5 of 21 BSC samples), it should be kept in mind that the broad-range PCR assay may not be able to reveal all variants of FePVs. Furthermore, it is well-known that PCR detection of viral nucleic acids in formalin-fixed and paraffin-embedded tissues may be decreased in comparison to fresh tissue. Finally, the absence of PV DNA in SCC samples can also be explained by the so-called hit-and-run model, which postulates an initial transformation of the infected cell and a subsequent loss of PV DNA.

Full proof of the existence of the novel PVs that are predicted through the results of our study still needs to be provided. However, we suggest that a great diversity of FePVs may exist that is in need of detection and characterization. The future use of the technique applied here will help in identifying more affected cats with papilloma-associated diseases. Virologic studies can be initiated with the aim to better characterize these novel viruses. Cloning and sequencing of the entire genomes of these viruses will allow phylogenetic comparisons with HuPVs as well as discrimination between benign and high-risk variants. Such studies can eventually provide insights into the molecular pathways underlying the pathogenesis of these viruses in cats.

References
## Appendix

New papillomaviruslike sequences.

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Chapter 8

Clinical, histological and immunohistochemical study of feline viral plaques and Bowenoid in situ carcinomas

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Clinical, histological and immunohistochemical study of feline viral plaques and Bowenoid in situ carcinomas

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What is known about the topic of this paper
- Reports of papillomavirus-induced dermatitis in cats are rare.
- Lesions of feline viral plaques have been described as feline hyperpigmented plaques and are clinically indistinguishable from lesions of Bowenoid in situ carcinomas.
- Feline Bowenoid in situ carcinoma could be, like feline viral plaques, papillomavirus-induced.

What this paper adds to the field of veterinary dermatology
- Clinically, feline viral plaques and feline Bowenoid in situ carcinomas are indistinguishable.
- Feline viral plaques and feline Bowenoid in situ carcinomas might have the same viral cause.
- Feline viral plaques could be a precursory lesion of feline Bowenoid in situ carcinoma.

Abstract

Feline viral plaques (FVP) induced by papillomavirus (PV) are often hyperpigmented and flat warts. The fact that up to 47% of Bowenoid in situ carcinomas (BISC), which also usually occur in the form of hyperpigmented plaques, are positive for PV antigen in immunohistochemistry suggests that BISC could evolve from FVP.

The relationship between the presence of PV antigens and the clinical and histological features of 26 cases of feline dermatoses (clinically described as pigmented plaques and with histological diagnosis of FVP and/or BISC) was therefore determined. The cases were classified into one of the three following groups: FVP, FVP+BISC or BISC. Immunohistological detection of papillomavirus group-specific antigen was performed using a polyclonal rabbit antiovine papillomavirus antiserum.

Of the seven cases in the FVP group, six were deemed positive by immunohistology as were all 10 cats in the FVP+BISC group. On the other hand, only one of the nine BISC cats was positive. The presence of both FVP and BISC lesions in some cats and the high detection rate of PV antigens in the FVP and FVP+BISC groups suggest that both conditions might have the same viral cause and that some BISC may evolve from FVP. The low rate of viral antigen detection in the BISC group indicates another cause or a loss of viral replication during the carcinogenesis.

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Introduction

Papillomaviruses (PV) are highly diverse viruses that usually induce benign skin or mucous membrane proliferation in mammals and birds but can also cause squamous cell carcinomas.1 In humans, the PVs that induce benign hyperplasia and those that induce cancers are phylogenetically different.1 Benign hyperplasias (warts) usually regress after a few months, a regression associated with the development of cell-mediated immunity.2

In contrast with dogs, where PV infections are frequently observed, reports of PV-induced dermatoses are rare in cats.3–7 Lesions are usually flat and hyperpigmented, rather than exophytic and flesh colour warts, and spontaneous regression is rare.3–7 These lesions are usually, but not always, multiple and have been described as feline viral plaques (FVP).8

Feline multicentric in situ squamous cell carcinomas also usually occur as multiple hyperpigmented plaques that resemble those of human Bowen’s disease.9,10 Gross and coworkers, however, recently remarked that there are major differences between the human and the feline diseases, and have coined the term ‘Bowenoid in situ carcinoma’ (BISC) to describe the feline condition.9 As FVP clinically resembles BISC, it was suggested that both conditions may have the same cause, and one report mentions the association of both FVP and BISC on the same cat.11,12 Furthermore, it has been shown immunohistologically that up to 47% of feline BISC samples are positive for PV antigen, suggesting that BISC is virally induced and that FVP could be, at least in some instances, precursory lesions of feline BISC.11

Using records of the clinical, histological and immunohistological features of 26 cases of feline dermatoses clinically described as pigmented plaques and with an initial histological diagnosis of FVP and/or BISC, the hypotheses that both lesions are often associated in the same samples, and that PV antigens are present in the majority of these lesions, were tested.
Materials and methods

Animals

History and clinical information was obtained from 26 cats with hyperpigmented plaques. Cats were included, provided that a histological diagnosis of FVP and/or BISC had been made previously, and clinical data (including concurrent diseases, immunosuppressive therapy and evolution of the lesions, when available) were subsequently analysed for each of the three histological groups: FVP, FVP + BISC and BISC.

Statistical analysis

Data were analysed using nonparametric statistical methods (GraphPad PRISM® for Windows, version 4.0; GraphPad Software, Inc., San Diego, CA, USA). Kruskal-Wallis one-way ANOVA by ranks and the Dunn’s post-test for multiple comparisons were used to compare ages among the three histological groups.

Histological evaluation

Archival specimens of all 26 cats were compiled. These samples have been previously collected by biopsy from all 26 cats, fixed in formalin, and processed routinely to paraffin wax for histological assessment. Sections (5 µm) were cut, routinely processed and stained with hematoxylin and eosin. The following criteria were systematically assessed: severity and nature of the acanthosis, hypergranulosis and size of the keratohyalin granules, premature keratinization, involvement of the hair follicle in the pathological process, disorderly or abnormal maturation of the epidermis, atypia (pleomorphic or abnormally large nuclei, multinucleate cells), mitoses more than three cell layers above the basal cell layer, koilocytosis, clear cells and presence of intracytoplasmic pseudo-inclusions and intranuclear inclusions. Koliocytes were defined as keratinocytes with swollen cytoplasms and shrunken nuclei. Clear cells were defined as keratinocytes with swollen cytoplasms but rather enlarged, vesicular nuclei. These modified keratinocytes (clear cells and koilocytes) have been reported to be also regularly associated with human PV infection. When modified keratinocytes (clear cells and koilocytes) have been reported with swollen cytoplasm but rather enlarged, vesicular nuclei. These modifications in keratinocytes (clear cells and koilocytes) have been reported to be also regularly associated with human PV infection.

Immunohistochemical analysis

Papillomavirus antigen was detected (at the Immunology Laboratory of Prairie Diagnostic Services, Saskatoon, Saskatchewan, Canada) coated with 0.1% poly-D-lysine, from each tissue block were mounted on slides (Codon Slides, Fisher Scientific, Edmonton, AB, Canada) coated with 0.1% poly-D-lysine, digested with protease XIV (Sigma Chemical Co., St. Louis, MO, USA) and compact ones (present in the stratum granulosum) for 20 min at 42 °C and treated with a 1 : 2000 dilution of rabbit antiovine papillomavirus type-1 antibody (Dako Diagnostics Canada Inc., Mississauga, ON, Canada). A goat-antiiodinated antirabbit IgG (Vector Laboratories Inc., Burlington, ON, Canada) was used at a 1 : 400 dilution as the secondary antibody. Replicate sections were stained as above without protease digestion, and additional sections were stained with a normal rabbit antiserum as the primary antibody to provide negative control. A positive control tissue, canine cutaneous papilloma, was included in each assay run. Both diaminobenzidine (DAB) (Electron Microscopy Sciences, Fort Washington, PA, USA) and Nova Red (Vector Laboratories Inc., Burlington, ON, Canada) were used as chromogens on two different sections for each sample.

Results

Clinical information

The clinical data are summarized in Table 2. Differences between ages of cats in FVP, FVP + BISC and BISC groups (median 11.5, 12 and 13, respectively) were not statistically significant. The sizes of the groups did not allow a proper evaluation of potential breed or sex predispositions.

On clinical examination, FVP and BISC lesions were often indistinguishable and usually presented as solitary or multiple grey, tan to black papules or small flat plaques. Cats with both conditions usually presented lesions on more than one body area and all body regions could be affected. Very little follow-up information was available but cases of transformation of FVP into BISC after the initial histological diagnosis were not recorded. None of the affected cats had a known history of immunosuppressive drug administration or concurrent disease.

Histological examination

The results are summarized in Table 3. The diagnosis of FVP was made in seven cases (Table 3). Lesions consisted of well-demarcated epidermal hyperplasia with acanthosis, hyperpigmentation, hypergranulosis with clumped keratohyalin granules and numerous koliocytes.

Table 1. Histological features of feline viral plaque and bowenoid in situ carcinoma

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<tr>
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<th>Feline viral plaque</th>
<th>Bowenoid in situ carcinoma</th>
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<tr>
<td>Acanthosis</td>
<td>Mild to moderate</td>
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<td>Follicular involvement</td>
<td>Sometimes</td>
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</tr>
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<td>Differentiation</td>
<td>Normal</td>
<td>Dysplastic epidermis, loss of polarity</td>
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<tr>
<td>Keratohyalin granules</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Koliocytes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Intracytoplasmic pseudo-inclusions</td>
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<td>Yes</td>
</tr>
<tr>
<td>Atypia</td>
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<td>Yes</td>
</tr>
<tr>
<td>Mitotic activity</td>
<td>No</td>
<td>Moderate</td>
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FVP and BISC in cats with hyperpigmented plaques

The diagnosis of FVP and BISC in cats with hyperpigmented plaques...
seemed to result from the condensation of fibrillar ones (more prevalent in the stratum spinosum) (Fig. 4). Intranuclear inclusions were not observed.

**FVP + BISC**

Interestingly, both BISC and FVP changes were present in 10 cats, sometimes in the same, sometimes in different, skin samples (Fig. 5a,b). Transition lesions exhibiting both FVP and BISC features were also sometimes observed.

**BISC**
The diagnosis of BISC was made on nine cases. These lesions consisted of sharply demarcated expansion of the epidermis with irregular acanthosis and broad rete ridges. Irregular acanthosis frequently descended around hair follicles. The epidermis was disorganized with a marked loss of cellular polarity and loss of normal stratification of the stratum basale and spinosum in all cases (wind-blown appearance). Keratinocytes with a hyperchromatic nucleus were present throughout the whole epidermis. Atypia was variable in nature and intensity (anisocytosis, anisocryosis and rare binucleated keratinocytes). Rare mitotic figures were present in all samples. Scattered apoptotic keratinocytes were present in four BISC samples. Koilocytes were present in all of them (Fig. 6). Other clear cells with rather enlarged vesicular nuclei were also observed. The cells (koilocytes and clear cells) contained sometimes intracytoplasmic additional blue-grey fibrillar pseudo-inclusions (three of nine cases). Clumped keratohyalin granules were seen in one of nine BISC cases. Erosions or ulcerations were present in five of nine cases.

**Immunohistochemical examination**

Results are summarized in Table 3. Of the seven cases of the FVP group, six were positive for PV antigen. Interestingly, all of the 10 samples with BISC and FVP lesion types were positive (Fig. 7). Only one of the nine BISC cases was deemed positive (11%). PV antigens were always visualized in the nucleus of the koilocytes; intracytoplasmic pseudo-inclusions remained unstained (Fig. 4).

**Discussion**

The clinical resemblance between BISC and FVP and the presence of both lesions in some cats suggest that some BISC evolve from FVP. Furthermore, despite the absence
FVP and BISC in cats with hyperpigmented plaques

Figure 2. Cat no. 26. Pigmented plaques at the base of the ear and the pinna diagnosed as feline bowenoid in situ carcinoma. Note the slightly raised and ulcerated lesions partially covered by crusts.

Figure 3. Cat no. 10. Histology of a feline viral plaque. Note the presence of clear cells (ballooned cytoplasm, rather swollen nucleus (black arrow) with intracytoplasmic pseudo-inclusions (red arrow). Haematoxylin and eosin. Magnification ×10. Bar = 200 μm.

Table 3. Histopathological findings

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<th>Case</th>
<th>Margins?</th>
<th>Hyperpig.</th>
<th>Koilocytes/clear cells</th>
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Margins?, presence of lesional margins; Hyperpig., hyperpigmentation; Dyskerat., dyskeratosis; Comp. ps. incl., compact pseudo-inclusions; Fibr. ps. incl., fibrillar pseudo-inclusions; KH Gran., clumped keratohyalin granules; PV-Ag, papillomavirus antigen. Pos, positive; Neg, negative.
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of statistically significant difference, cats affected by FVP tended to be younger than those affected by BISC: this could imply that FVP are precursor lesions of BISC. However, while BISC affected the face, neck or the limbs in most cases, FVP lesions were more often present on the trunk even if other areas, including neck and face, were affected. This finding does not seem to support the hypothesis that BISC evolve from FVP but the discrepancy could be explained by a higher cancerization rate of lesions located on the face and neck, for example as a result of increased ultraviolet radiations exposure, compared to those in other regions of the body.

Figure 4. Cat no. 9. Immunohistochemical analysis of a feline viral plaque. Note the presence of positive nuclei (black arrow). The fibrillar (red arrow) and the solid (green arrow) intracytoplasmic inclusions remained unstained. Diaminobenzidine. Magnification x40. Bar = 50 µm.

Figure 5. Cat no. 16. Histology of two lesions present on the same biopsy sample. Haematoxylin and eosin. Magnification x40. Bar = 50 µm. (a) Feline viral plaque. Note the moderate acanthosis. The stratification and the differentiation of the epidermis are conserved. Koilocytes and clumped keratohyalin granules are the most obvious papillomaviruses’ cytopathic characteristics on this lesion. (b). Early Bowenoid in situ carcinoma. Note the acanthosis, the obvious disorganization of the epidermis and the abnormal differentiation of most keratinocytes. Clumped keratohyalin granules and one single koilocyte are the only papillomavirus cytopathic effects noticed on this lesion.

Figure 6. Cat no. 21. Histology of a feline Bowenoid in situ carcinoma. Note the marked acanthosis (black stars: acanthotic epidermis), the follicular involvement (black points), the loss of polarity and the presence of numerous koilocytes (arrow). Haematoxylin and eosin. Magnification x10. Bar = 200 µm.
FVP usually conserved the general organization of the epidermis and atypia was absent, whereas BISC lesions were disorganized and abnormal keratinocytes were present throughout the epidermis. However, both conditions share numerous histological features: irregular acanthosis with rete ridges formations, presence of clumped keratohyalin granules, koilocytes and clear cells. The presence of koilocytes or clear cells in all BISC lesions (including IHC-negative ones) might be regarded as a proof of presence of the virus. These cells with vacuolated cytoplasm and shrunken, pycnotic nuclei are usually considered highly suggestive of PV infections. All the authors who have studied feline BISC have recognized these cells, but two of three have not used the term ‘koilocyte’ to describe them. In situ hybridization studies could be helpful to determine if these cells actually harbour PV nucleic acids and if the term ‘koilocyte’ is appropriate.

In both FVP and BISC samples, fibrillar and compact pseudo-inclusions were seen. In one case both were present in the same sample, and compact ones (more present in the stratum granulosum) seemed to result from the condensation of fibrillar ones (more prevalent in the stratum spinosum) (Fig. 4). This condensation has already been described by Carney and coworkers.

Our study demonstrates that the association between FVP and BISC is frequent and occurs sometimes on the same skin lesion. Additionally, cases of overlapping BISC and FVP lesions have been detected. This association was already described before. These similarities support the hypothesis that FVP could be precursory lesions of BISC.

All except one FVP and FVP + BISC cases were positive for PV antigen by immunohistochemistry (IHC). As pseudo-inclusions were present in the negative case, it can be considered that all these samples were infected by PV. Furthermore, as IHC detects capsid antigens, it can be concluded that productive infection occurred in all positive samples (all FVP lesions and positive BISC). These findings support the hypothesis that PVs play an active role in the development of such lesions. It must, however, be borne in mind that PVs are sometimes commensal, and nucleic acids are often uncovered in normal mammalian skin. However, genome copy number is usually very low and productive infection rarely occurs in such cases. Establishing causality between the presence of viruses in skin lesions and oncogenesis remains problematic, and the presence of replicating viruses cannot be regarded as a sufficient proof. In vitro studies are mandatory to establish such causality.

Almost all cats affected by BISC were deemed negative by IHC. These findings might suggest that BISC has two distinct causes and that only a subgroup of BISC is virally induced. A loss of viral replication during the cancerization process could also explain these findings. In fact latent PV infection or infection with minimal replication may remain undetected by IHC, because of the relatively low sensitivity of such techniques. The ‘hit and run’ model, which postulates an initial cellular transformation by the virus and a subsequent loss of viral genome, could account for the negative IHC in some BISC lesions. Furthermore, it was recently demonstrated that PVs maintained productive infections in precursory lesions of cervical cancer but that capsid antigens were no longer produced in late cervical cancers. In conclusion, a loss of viral protein expression in advanced cases of BISC seems likely.

Feline BISC has long been considered the counterpart of human Bowen’s disease (BD) – an in situ squamous cell carcinoma that presents as solitary, well-circumscribed, erythematous plaques and occurs on the face, extremities and genitalia. Koilocytes are usually not present in such lesions. Human bowenoid papulosis is characterized by genital pigmented verrucous papules or plaques. This condition is also histologically characterized by in situ SCC lesions but, in contrast to BD, Bowenoid papulosis lacks full-thickness epidermal atypia. PV DNA is uncovered in virtually all samples of bowenoid papulosis but data concerning the presence of PV in human BD remain contradictory. Furthermore, PVs that infect human bowenoid papulosis and BD are usually to mucosal and not to cutaneous strains. These data show that feline BISC lesions display substantial differences from both human conditions and justify the use of a specific denomination, as emphasized by Gross and coworkers.

The results of the present study support the hypothesis that some BISC evolve from FVP lesions and the causative role of PV. However, evidence that these PVs are able to induce cancerization in mammalian skin is lacking and further studies are warranted. Nucleic acids amplification techniques could establish which PVs are present in FVP and BISC lesions and whether BISC samples without FVP are really sterile or infected by dormant PV. As well, in vitro studies addressing the transforming potential of feline PV are required to better understand the role that these viruses play in this condition.

References

3. Carney HC, England JJ, Hodgin EC et al. Papillomavirus infection...
Résumé  Les plaques virales du chat (FVP) induites par le virus papiloma (PV) se présentent souvent comme des plaques hyperpigmentées. Le fait que jusqu'à 47% des carcinomes in situ bowenoides (BISC), qui se présentent aussi sous la forme de plaques hyperpigmentées, sont positifs pour l'antigène de PV par immunohistochimie suggère que les BISC pourraient provenir de FVP. La relation entre la présence d'antigènes de PV et les données cliniques et histologiques de 26 cas de dermatoses félines cliniquement répertoriées comme des plaques hyperpigmentées avec un diagnostic histologique de FVP et/ou de BISC a été recherchée. Les cas ont été classés en trois groupes : FVP, FVP + BISC ou BISC. La recherche immunohistochimique de papillomavirus a été réalisée en utilisant un antisérum polyclonal de lapin anti-bovin. Sur les sept cas du groupe FVP, six étaient positifs à l'immunohistochimie, un seul des neuf cas du groupe BISC a été considéré positif. La présence de nombreuses plaques hyperpigmentées, qui se présentent aussi sous la forme de plaques hétérogènes et de BISC, a été observée. On pourrait donc penser que les BISC peuvent provenir de FVP. Le faible taux de détection d'antigène viral dans le groupe BISC indique une autre cause, ou la perturbation de la réplication virale pendant la cancérogénèse.

Resumen  Las placas virales felinas (FVP) inducidas por el virus papiloma son a menudo verrugas hiperpigmentadas y planas. El hecho de que hasta un 47% de los carcinomas in situ bovenoides (BISC), que también ocurren como placas hiperpigmentadas, sean positivos al antígeno del virus papiloma mediante inmunohistochimia sugiere que las BISC pueden provenir de FVP. La relación entre la presencia de antígenos del virus del papiloma y las características clínicas e histológicas de 26 casos de dermatosis félines clínicamente reportadas como placas hiperpigmentadas con diagnóstico histológico de FVP y/o de BISC se investigó. Los casos se categorizaron en tres grupos: FVP, FVP + BISC o BISC. La investigación inmunohistochimica de papilomavirus se realizó en seis de los set de casos de FVP (6/7) y solo uno de los nueve casos de BISC. La presencia de numerosas placas hiperpigmentadas, que también ocurren como placas planas, son positivos al antígeno del virus papiloma mediante inmunohistochimia sugiere que las BISC pueden provenir de FVP. El bajo porcentaje de detección de antígeno viral en el grupo BISC indica una otra causa, o la alteración de la replicación viral durante la cáncerogénesis.
FVP and BISC in cats with hyperpigmented plaques

progresar desde FVP. El bajo porcentaje de detección de antígeno vírico en el grupo BISC sugiere otra causa o una pérdida de replicación viral durante el proceso de carcinogénesis.

Chapter 9

Summarizing discussion and further studies
Viruses replicate inside cells by synthesizing their own proteins and assembling them into virions. This replication is associated with various cytopathic effects, which are, usually, typical or pathognomonic of one specific virus. Poxviruses infections are associated with large intracytoplasmic inclusions and herpesvirus infections with intranuclear inclusions, for example. Aside from these cytopathic effects, viruses induce macroscopic changes which are sometimes, easily recognizable. Papillomaviruses induce cauliflower-like lesions, the so-called warts, which are virtually pathognomonic. Poxviruses and herpesviruses induce pock lesions and vesicles, respectively, which are very typical of these infections. These virus-associated changes have long been described in dogs and cats [1]. Viruses may also induce some less obvious changes, which are described in humans but remained often undescribed in canine and feline. These changes may be due to various pathogenic states like minimal viral replication, latency or non-productive infections.

This thesis aims to describe some of these undescribed virus-induced skin changes in dogs and cats and, especially, papillomavirus-induced ones.

We first described a case of canine erythema multiforme presumably associated with parvovirus infection [2]. We hypothesized that an infection of stem cells and primary amplifying keratinocytes occurred following hematogenic dissemination of the parvovirus. Viral antigens could have been presented by class I major histocompatibility complex molecules at the surface of the keratinocytes. Recognition of the viral antigens by T-lymphocytes, possibly sensitised by a previous parvovirus vaccination would have triggered these cytotoxic T-cells to induce apoptosis of infected keratinocytes. In this case, clinical and pathological lesions are not due to the cytopathic effect of the virus itself but to the T lymphocyte-induced cytolysis. Interestingly, virus infections (especially herpes simplex infection but also B19 parvovirus infections) are the most frequent causes of erythema multiforme in humans [3, 4]. This case was the first report of virus-associated erythema multiforme in dogs. This report leaves however some moot questions that warrant some further studies. The most important question is to know whether canine parvovirus usually replicates in the skin of affected dogs without causing any cytopathic effects or if the skin contamination reported in this study was incidental or due to a specific parvovirus strains. Second, as parvovirus antigens have been uncovered in the affected skin, one cannot exclude a direct effect of the virus infection associated to a secondary lymphocytic reaction.
The second article of this thesis aimed to describe some previously unknown cutaneous consequences of FeLV infection.

FeLV is a member of the oncornavirus subfamily of retroviruses, which replicates in many tissues like bone marrow, salivary glands and respiratory epithelium. Its replication in the feline skin was already described by Gross and coworkers and associated with the so-called giant cell (multinucleated keratinocytes) dermatosis and horn formation [1, 5]. Multinucleated keratinocytes are sometimes observed in humans in association with neoplastic conditions, infectious diseases like herpesvirus infection and immunologic disorders [6]. Retroviruses also possess fusion proteins, which are able to induce syncytium formation in infected tissues [7, 8]. However, although FeLV infection is a frequent disease, syncytium are rarely observed in the affected skin of infected cats [5]. We described another case of FeLV-associated giant cell dermatosis with an ulcerative phenotype and demonstrated the presence of both FeLV antigens and proviral sequences in the lesional skin. Gross and coworkers suggested that these cytopathic effects were not the direct consequence of FeLV infection but the early stage of carcinomatous transformation [5]. The presence of FeLV antigens in the affected skin of the cat we observed, suggested an active replication of the virus and supported the hypothesis of a direct cytopathic effect. Furthermore, Rohn and coworkers demonstrated that FeLV variants do possess various pathogenic and cytopathic effects[9]. All in all, we considered more likely that these changes are the direct consequence of infection with a specific and rare variant of FeLV. This hypothesis however warrants further investigation.

Feline internal lymphomas are often the consequence of FeLV infection. Cutaneous lymphomas, however, usually occur on FeLV-negative cats [10]. FeLV genomic sequences have however already been sometimes amplified from cutaneous lymphomas [11]. The originality of the case we reported lies on the fact that FeLV antigens have been demonstrated in the affected skin of a serologically negative cat. These findings suggest that productive FeLV infection may in some instances occur and may be restricted to the skin. Further studies are needed to demonstrate the existence of multiple FeLV strains with various physiologic and pathologic properties.

Papillomaviruses (PV) are host-specific epitheliotropic viruses that infect the skin and mucous membranes. As these viruses do not possess the enzymatic machinery required for replication, they depend upon host-cell machinery to achieve this process and upon host-cell
differentiation for completion of their life cycle [12]. As more than 150 different PV have been isolated from the human lesional or healthy skin, only a few PV have been identified in carnivores [13, 14]. In this thesis, we have demonstrated the existence of new papillomavirus-like sequences in various canine and feline lesions, including cyclosporine A-associated exophytic lesions, in situ and invasive carcinomas [15, 16]. We have used two sets of primers designed for the amplification of a sequence of the E1 gene of PV. The narrow-range set of primers was supposed to amplify canine and feline PV and their close relatives [16]. The broad range PCR system was designed to amplify up to 64 human PVs and several animal PV such as canine and feline PV[16, 17]. These studies have shown the existence of at least six feline and five canine unknown papillomavirus-like sequences. As the classification of papillomaviruses is based upon L1 gene, the amplification of sequences of the E1 gene does not allow proper evaluation of these sequences and classification of the newly uncovered PVs but these results suggested however that canine and feline lesional skin can be infected by PV of great genetic diversity. It would be of great interest to amplify and clone these novel canine and feline PVs. Fortunately, a new technique, the rolling-circle amplification (RCA) technique, was recently introduced to amplify and isolate circular DNA and, especially human and animal PVs [18, 19]. RCA is a multiple random primed, sequence-independent amplification of circular DNA. Furthermore, the amplification is as effective as PCR. Therefore, only minute amount of crudely isolated DNA from tissue can be used for amplification of papillomavirus genomic DNA.

RCA analysis of canine and feline skin samples will permit to determine whether healthy skin harbors PVs and to sequence PVs that are present in lesional skin. This descriptive study is the mandatory initial step for a better understanding of the role that play PV in the development of skin lesion in dogs and cats, and, especially, in the development of skin cancers. We have already applied this new technique to the isolation and cloning of a new canine PV (CPV3)[20].

In mammals and birds, PV induce a wide range of cutaneous and mucous changes such as exophytic and flat warts, precancerous and cancerous lesions. They are considered important carcinogens in humans and some high-risk PVs are directly responsible for the development of cervical cancers in women[21-23]. Even though the link between cervical carcinoma and human PV is clear, the role of PVs in the development of cutaneous squamous cell carcinoma (SCC) is not as definite [24]. There is however emerging epidemiological evidence to suggest
that PV might play an important role in skin cancerogenesis, especially in epidermodysplasia (EV) associated-one. Establishing causality between the presence of PV in a skin lesion and the development of the lesion is nevertheless problematic [25]. Criteria have been proposed to establish this relationship but difficulties in culturing PVs have made their fulfillment often impossible [22, 25]. Additionally the use of extremely sensitive nested polymerase chain reaction (PCR) makes possible the detection of minute amounts of viral DNA (even 0.05 viral genome per cell). The presence of such an amount of PV nucleic acid does not indicate a productive infection and can also be found in healthy skin [26, 27]. Evidence also suggests that ultraviolet (UV) radiation contributes to the cancerization of some PV-associated skin cancers [25, 28]. All in all, the role of PV in the development of skin cancer in humans remains questionable.

Some animals models support the causative role of PV in the induction of skin SCC: A few decades ago, it was demonstrated that cottontail-rabbit PV (CRPV) are able to induce skin cancers in rabbit [29-30]. Other studies have also established that attenuated life canine oral PV (COPV) vaccine induce SCC in Beagles [32]. Additionally, canine and feline can be affected by skin conditions that share some similarities with human EV and cancerization has been reported in some patients [33]. Epidemiologic studies have demonstrated association between carnivores SCC and PV but causality has never been established [16, 33-40]. This thesis has confirmed the epidemiologic association between some groups of feline and canine skin cancers and PV infections and the genetic diversity of carnivore PVs [16, 20, 34, 40]. Aside from the identification and cloning of these new PVs, the most important studies to carry out would be to determine the relative prevalence of each new carnivore PV and to demonstrate in vitro that, at least some of them, are able to induce keratinocyte transformation and immortalization.

We have, for example, detected, cloned, and sequenced a novel PV (CPV3), which was associated with a case of canine epidermodysplasia verruciformis [20]. The affected dog developed multiple plaques and one single interdigital lesion of in situ SCC [20]. DNA of CPV3 was uncovered in each lesion tested (including SCC) but was not present in intact skin of the same dog. Sequence-independent, multiply primed rolling-circle amplification was used to amplify, clone, and sequence the entire genome of CPV3. Indeed, analysis of the cloned and sequenced canine papilloma genome allowed its classification as a member of a new papillomavirus genus (GenBank accession DQ295066). Additionally, mRNA for the putative transforming protein E6 was discovered in each tested lesion: These findings
demonstrated that CPV3 was transcriptionally active in the mentioned skin lesions and supported the hypothesis of a causative role of CPV3 in the pathogenesis of canine EV [41]. Complementary DNA of the p53 transcript of the affected dog was cloned from blood, intact skin as well as skin lesions and its nucleotide sequence was found not to differ from the wild type canine p53 sequence. This finding suggested that the development of malignancy could not be attributed to UV-dependent mutagenesis. Therefore, the hypothesis was supported that transforming proteins of CPV3 induced cancer development by different mechanism than human EV-associated PVs [41].

As well, we are currently studying the relative prevalence of CPV3 in canine sera. We have cloned CP3-L1 gene (codes for major CPV3 capsid protein) and generated antisera against this protein. Our goal would be to establish an ELISA test and to evaluate 500 already collected canine sera.

All in all, the findings in this thesis, the subsequent and future studies open new avenues to study PV-induced skin cancerogenesis. As at least some carnivore conditions bear major resemblances with human ones, these breakthroughs will reveal helpful for both veterinary and human oncology.


Chapter 10

Zusammenfassung und weitere Studien


Das Ziel des zweiten Teils dieser Arbeit war es, einige vorgängig unbekannte kutane Folgen der FeLV-Infektion zu beschreiben.

einer Infektion mit spezifischen und seltenen Varianten von FeLV sind. Diese Auffassung bedarf jedoch weiterer Untersuchungen.


Wir haben zum Beispiel ein neuartiges Papillomavirus (CPV3) entdeckt, geklont und sequenziert, das mit einem Fall von Caniner Epidermodysplasia verruciformis assoziiert war [20]. Der befallene Hund entwickelte multiple Plaques und eine einzige interdigitale Läsion von in-situ SCC [20]. In jeder untersuchten Läsion (einschliesslich SCC) wurde

Wir untersuchen gegenwärtig auch die relative Prävalenz von CPV3 in caninen Seren. Wir haben die CP3-L1 Gene geklont (Kodes für CPV3 Haupt-Kapsidprotein) und Antiseren gegen dieses Protein generiert. Unser Ziel ist es, einen ELISA Test zu etablieren und 500 vorgängig gesammelte canine Seren zu evaluieren.

Schliesslich öffnen die Resultate dieser Arbeit and hoffentlich zukünftiger Studien neue Zugänge zur Erforschung der Papillomavirus-induzierten Hautkanzerogenese. Das sollte sich sowohl für die veterinär- als auch die human-mediizinische Onkologie als hilfreich erweisen, wenn man bedenkt, dass es mehrere ähnliche Hautkonditionen bei Karnivoren und Menschen gibt.