

PAPILLOMAVIRUS-ASSOCIATED FOCAL ORAL HYPERPLASIA IN WILD AND CAPTIVE ASIAN LIONS (*PANTHERA LEO PERSICA*)

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Abstract: Four Asian lions (*Panthera leo persica*), two wild and two captive, were diagnosed with focal oral hyperplasia affecting the ventral surface of their tongues. Focal, flat, sessile lesions consisted of hyperplastic, stratified squamous epithelium. Koilocytotic atypia was evident in the upper layers of cells, some of which contained characteristic intranuclear papillomavirus particles visible by electron microscopy. In addition, large amphophilic cytoplasmic inclusions were evident in the koilocytes and were considered to be a product of the viral E4 gene. Papillomavirus group-specific antigens were detected by immunohistochemistry in the atypical cell nuclei. Conserved papillomavirus antigenic epitopes differed from epitopes found in cutaneous papillomavirus-induced lesions from domestic cats. An 8,000-base pair DNA fragment, linearized by *Bam HI* digestion, was detected by Southern blot hybridization probed with a mixed human papillomavirus genomic probe. Limited restriction endonuclease studies of DNA prepared using an oral hyperplastic lesion from an Asian lion indicate that this is a novel feline papillomavirus different from the domestic cat cutaneous papillomavirus. This new virus has been designated "PIPV."

Key words: Papillomavirus, Asian lion, *Panthera leo persica*, felidae, PIPV.

INTRODUCTION

The success of captive breeding programs for endangered species depends on careful evaluation of wild-caught animals to prevent introduction of serious infectious diseases into the captive population. Furthermore, introduction of subclinical diseases into taxonomic families can be devastating. Such has been the case in Felidae, with outbreaks of feline infectious peritonitis¹⁵ and rhinotracheitis^{8,18} in captive cheetahs. Careful physical examination together with serologic testing during quarantine must be practiced in order to detect novel

or unsuspected diseases that could be devastating for other species.

The Asian lion (*Panthera leo persica*) is an endangered species for which a captive breeding program was established in the United States and India. During routine physical examinations of seven wild-born, captive lions at the Szekkarbang Zoo in Gujarat, India, and four wild lions at the Gir Forest Sanctuary in India, focal oral hyperplasia was observed on the ventral surfaces of the tongues of four animals. This lesion in felids and similar lesions in other vertebrate species are caused by papillomaviruses,²¹ herpesviruses,⁷ poxviruses,¹² chemical carcinogens,²² diet,¹³ and genetic defects.²⁶ Several of these infectious agents can limit the value of the animal in the breeding program.³² Furthermore, since poxviruses¹¹ and papillomaviruses^{16,31} can infect related species, there is concern about possible spread of infection to other felids within the captive collection and perhaps to domestic cats that enter zoo grounds.

MATERIALS AND METHODS

Over a 10-day period, the eleven adult Asian lions (seven males, four females)

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Figure 1. Ventral surface of tongue of Asian lion showing papillomavirus-induced focal epithelial hyperplasia.

were anesthetized with tiletamine HCl and zolazepam HCl (Telazol, Aveco, Fort Dodge, Iowa 50501, USA) delivered by blow dart at a dose of 400–500 mg for a male and 350–450 mg for a female. The lions were clinically evaluated for reproductive and genetic studies. Two of the wild and two of the captive lions (three males, one female) had small, soft, light pink, oval, slightly raised, flat sessile lesions ranging from 4–8 mm in diameter on the ventral surfaces of their tongues (Fig. 1). Representative tongue lesions from a wild and a captive lion were bisected, fixed in 10% neutral buffered formalin and cacodylate-buffered glutaraldehyde, and frozen in tissue culture media in liquid nitrogen for further testing. Formalin-fixed tissues were embedded routinely in paraffin, serially sectioned at 6 μm , and stained with hematoxylin and eosin. Thin sections of the glutaraldehyde-fixed specimens were prepared for electron microscopy utilizing standard embedding techniques. One-micron-thick sections, stained with toluidine blue, were

examined by light microscopy to select sites for electron microscopic evaluation. Ultrathin sections were stained with uranyl acetate and lead citrate and examined as previously described.²⁵

Serial sections of paraffin-embedded tissues were stained for papillomavirus group-specific antigens using a rabbit polyclonal antibody (DAKO Corp., Carpinteria, California 93013, USA) that broadly cross-reacts with mammalian and avian papillomaviruses.^{10,25} A panel of mouse monoclonal antibodies directed against linear epitopes of phylogenetically conserved bovine papillomavirus type 1 (BPV-1) L1 and L2 gene products was also used.¹⁰ The reaction was developed using a modification of the avidin–biotin complex technique.¹⁰

Frozen hyperplastic lesions were finely minced, and genomic DNA was prepared as previously described.³³ Briefly, the tissue was digested with proteinase K (Boehringer Mannheim, Indianapolis, Indiana 46250, USA), treated with DNase-free RNase A (Boehringer Mannheim), extract-



Figure 2. Focal sessile plaque of proliferating stratified squamous epithelium is supported by thin fibrovascular stalks. H&E, $\times 40$.

ed with phenol and chloroform, and precipitated with 70% ethanol. Total cellular DNA (5 μ g) was digested with *Bam* *HI* restriction endonuclease, and electrophoretically separated on a 1% agarose gel. The DNA on the gel was depurinated, denatured, and transferred under alkaline conditions to a charge-modified nylon membrane (Genescreen Plus, DuPont NEN Research Products, Boston, Massachusetts 02118, USA).²⁰ The membrane was hybridized under low-stringency conditions with a radiolabeled cocktail probe consisting of equal amounts (50 ng each) of human papillomavirus (HPV) DNA (HPV-1, -11, -16, and -18). After posthybridization washing of the membranes under low-stringency conditions, the membrane was exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, New York 14650, USA) for 72 hr at -70° C using intensifying screens (Cronex Lightning-Plus, DuPont de Nemours, Wilmington, Delaware 19801, USA).

RESULTS

Microscopically, the sessile tongue lesions consisted of marked squamous cell proliferation with short, broad, rete ridge formation. Thin dermal papillae containing capillaries separated the short rete ridges (Fig. 2). Foci of degenerating cells were evident in the upper stratum spinosum and stratum granulosum (Fig. 3). These cells displayed atypical nuclei surrounded by clear cytoplasm. Single large, amphophilic, inclusion-like structures were eccentrically located in the cytoplasm adjacent to the nucleus; a prominent round, vesiculated nucleus was centrally or eccentrically located within affected cells (Fig. 3). These degenerating cells had features consistent with those described for koilocytes, cells exhibiting the cytopathic effects of papillomavirus infection.²¹ By electron microscopy the inclusions in the koilocytes appeared as finely granular to fibrillar, electron-dense, cytoplasmic aggregates that often molded around nuclei (Fig. 4). Within the nucleus



Figure 3. Higher magnification of a field from Figure 2 showing a cluster of degenerated cells (koilocytes) in the upper stratum spinosum with swollen, clear cytoplasm, cytoplasmic inclusion bodies, and vesicular nuclei. H&E, $\times 500$.

of some of these cells were 50–52-nm, viruslike particles that were ultrastructurally compatible with papillomaviruses (Fig. 5).

Papillomavirus cross-reactive antigens, several of which are recognized by carefully characterized monoclonal antibodies, were identified within the nuclei of koilocytotic cells (Table 1, Fig. 6). The cytoplasmic, inclusion-like structures did not stain for structural viral proteins. The BPV-1 epitopes conserved by the Asian lion oral papillomavirus were different from those of the domestic cat cutaneous papillomavirus (Table 1). However, the canine oral papillomavirus had similar epitope conservation.

A low-stringency Southern blot hybrid-

ization of a *Bam* *HI* restriction endonuclease digest on the DNA extract of the oral lesion with a cocktail probe of HPV-1, -11, -16, and -18 DNA revealed a single, linear, 8,000-bp hybridizing band (Fig. 7).

This novel papillomavirus was abbreviated “PIPV” following the nomenclature guidelines for nonhuman papillomaviruses.³⁴

DISCUSSION

Until recently, felids were thought to be one of the few taxa of mammals that did not become infected by their own papillomavirus(es). Although reports indicated that domestic cats were afflicted by cutaneous

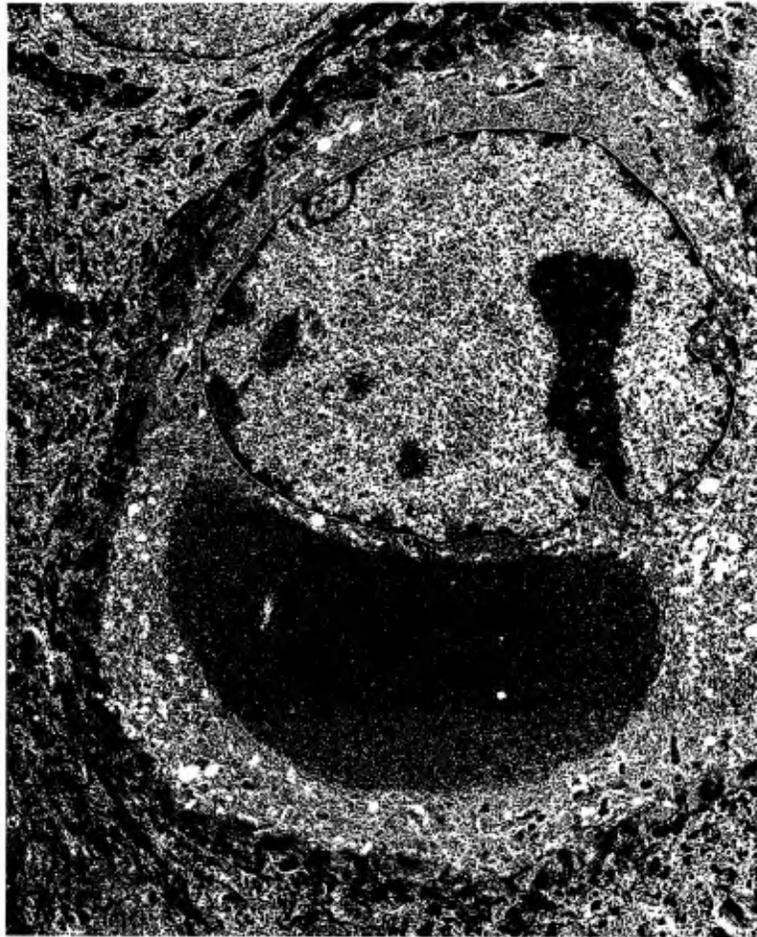


Figure 4. Ultrastructural appearance of koilocyte with granular intracytoplasmic inclusion body (non-viral) from Asian lion with focal oral hyperplasia. $\times 5,550$.

and oral papillomas,¹⁹ no viral etiology had been demonstrated.²⁵ Recently, a unique papillomavirus was identified and partially characterized in focal epidermal and follicular hyperplasia in aged Persian cats.² A subsequent report identified a papillomavirus infection in an immunosuppressed domestic cat.⁴ Referenced in the first article² is an unpublished case of papillomavirus-positive, focal oral hyperplasia in a clouded leopard (*Neofelis nebulosa*), and we have identified papillomavirus-positive, hyperplastic tongue lesions (unpubl. data) in an Asian lion from the London Zoo (provided by Dr. A. Cunningham) and in three captive

snow leopards (*Panthera uncia*), a clouded leopard, and an Asian desert cat (*Felis bieti*) from zoos in the former U.S.S.R. Focal oral hyperplasia has also been associated with papillomavirus infection in Florida panthers (*Felis concolor coryi*) and bobcats (*Felis rufus*).²⁷ These observations indicate that domestic and exotic felids (both wild and captive) are infected by papillomaviruses.

Although originally considered to be species- and anatomic-site-specific, we now know that some papillomaviruses cross-infect closely related species, usually within the same genus; for example, canine papillomaviruses infect both dogs and coyotes,

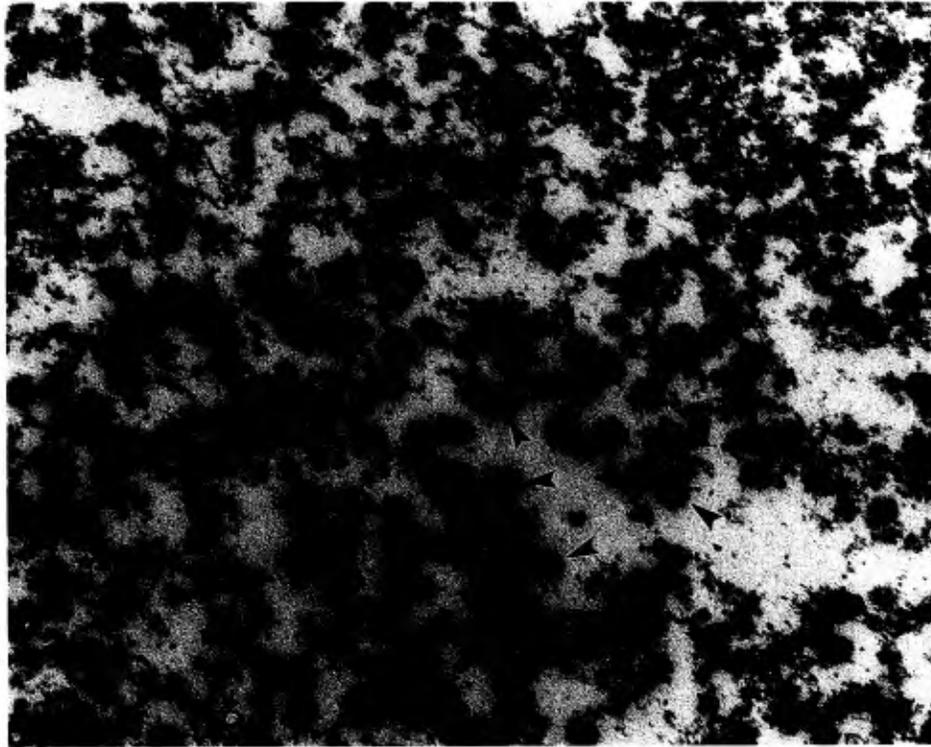


Figure 5. Ultrastructural appearance of koilocyte containing numerous round-uniformly sized (50–52 nm), viruslike structures compatible with papillomaviruses within the karyoplasm (arrows). $\times 86,625$.

and domestic rabbit (*Oryctolagus cuniculus*) oral papillomaviruses infect wild cottontail rabbits (*Sylvilagus floridanus*).^{17,31} Papillomaviruses also induce fibromatous lesions that do not produce infectious virions (i.e., are nonproductive lesions) in a wider range of hosts (equine sarcoid, hamster cutaneous fibroma), for example, BPV-

1.¹⁶ Our findings of papillomavirus cross-reactive antigens, homologous papillomavirus DNA sequences, and papillomavirus-like particles 50–55 nm in diameter confirm that the focal oral hyperplasia in the Asian lions was associated with a papillomavirus infection. These findings also indicate that the virus is unique. The single *Bam HI* site

Table 1. Papillomavirus antigenic epitopes are conserved to various degrees between different papillomaviruses, providing a means to differentiate new viruses.

Antibodies host	Lesion	DAKO ^a	AU-1 ^b	AU-2 ^b	AU-3 ^b	AU-4 ^b	AU-5 ^b	AU-6 ^b	1H8 ^b
Asian lion	Focal oral hyperplasia	pos	pos	neg	neg	neg	neg	neg	pos
Domestic cat	Cutaneous papilloma	pos	pos	neg	neg	neg	neg	pos	pos
Domestic dog	Oral papilloma	pos	pos	neg	neg	neg	neg	neg	pos
Cow (BPV-1)	Cutaneous fibropapilloma	pos	pos						
Cow (BPV-2)	Cutaneous fibropapilloma	pos	pos	neg	pos	pos	pos	pos	pos

^a Rabbit polyclonal antibody.

^b Mouse monoclonal antibody.

BPV = bovine papillomavirus; neg = negative by immunohistochemistry; pos = positive by immunohistochemistry.

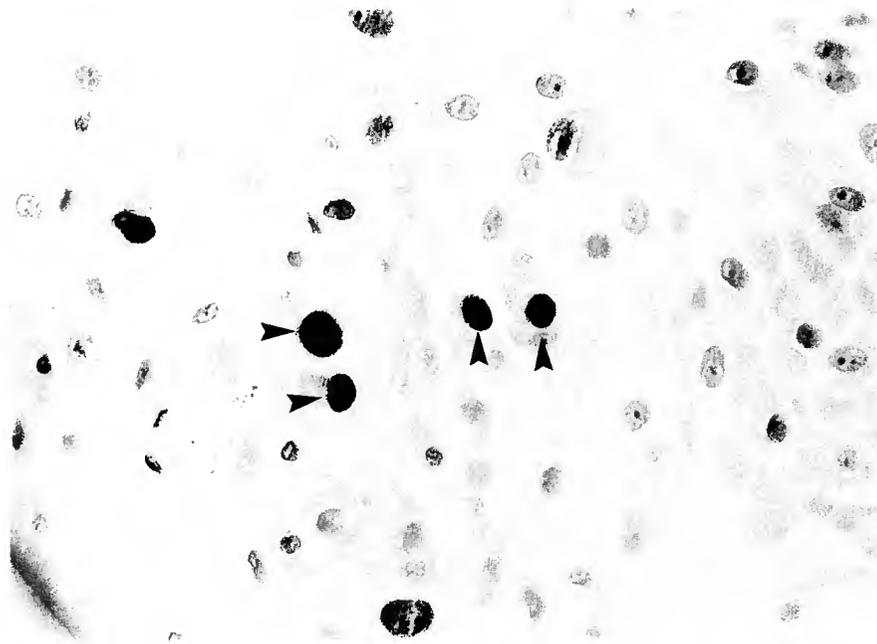


Figure 6. Cells with swollen cytoplasm and cytoplasmic inclusions have a black-staining nucleus (arrows) containing papillomavirus antigen. Immunoperoxidase, hematoxylin counterstain, $\times 500$.

of the Asian lion papillomavirus is not present in the domestic cat papillomavirus (M. Van Ranst *et al.*, unpubl. data), confirming that these are two novel and distinct feline papillomaviruses. The canine oral papillomavirus is also different since its genome is larger and yields three fragments when digested with *Bam HI*.²⁹ Detailed molecular studies are in progress utilizing the frozen tissues from these cases to confirm these preliminary observations. Cloning, characterization, and sequencing of the DNA of the Asian lion papillomavirus will reveal its phylogenetic relationship to papillomaviruses associated with focal oral hyperplasia in other species and to the other feline papillomaviruses.

Papillomaviruses typically induce a variety of benign proliferative lesions in most species, that have been classified as focal hyperplasia, papillomas, fibropapillomas, keratoacanthomas, fibromas, or related tumor types.^{2,19,21,30,37} Focal oral hyperplasia is a papillomavirus-induced disease in humans and chimpanzees (*Pan Spp.*) in which

the lesions are broad, flat, and unstaked. These features differentiate them from papillomas,^{32,35,37} and are similar to mild forms of oral papillomatosis that occur in dogs (*Canis spp.*)³¹ rabbits (*Lagomorpha Spp.*)^{14,23} and characterize the sublingual forms observed in our Asian lions. Some human and animal papillomaviruses are also capable of inducing malignant neoplasms, primarily squamous cell carcinomas.²⁸

Productive papillomavirus infections result in specific cytopathology of infected cells. The cytopathology varies with specific virus type³; however, the general changes consist of cytoplasmic swelling, clearing of the cytoplasm (failure to take up stain), and formation of bizarre, keratohyalin-like granules or cytoplasmic, inclusion-like structures. The cytoplasmic, inclusion-like structures are considered to be a product of the early viral gene, E4.¹ These distinct morphologic features create a cell that is referred to as a clear cell,⁵ pale cell,⁶ or koilocyte.⁹ This general cytopathologic pat-

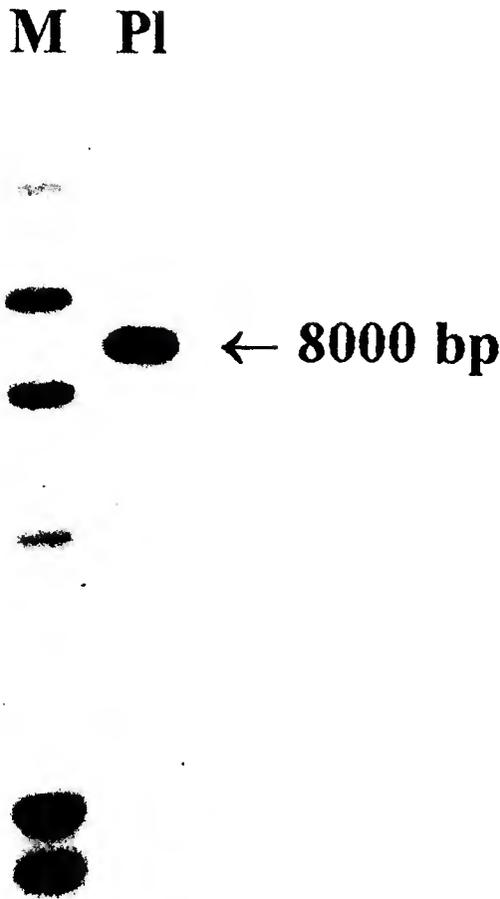


Figure 7. Southern blot. **Lane M:** Molecular weight marker consisting of HindIII-generated lambda phage DNA fragments (23, 130 base pairs [bp]; 9, 146 bp; 6,557 bp; 4,361 bp; 2,322 bp; 2,027 bp). **Lane PI:** *Bam*HI restriction endonuclease digest on total cellular DNA of the Asian lion oral hyperplasia results in linear 8,000-bp papillomavirus band (arrow).

tern for productive papillomavirus infections was evident in the focal oral hyperplasia cases biopsied from Asian lions in this study. The structure of the large, cytoplasmic, amphophilic inclusions in koilocytes was very similar to that reported in domestic cat cutaneous papillomavirus infections² and may represent abnormal assembly of keratin filaments. It is not considered part of the assembly process of vi-

rions since viral antigens were not identified by immunohistochemistry, nor were complete virions visualized by transmission electron microscopy.

The close molecular homology between the pygmy chimpanzee (*Pan paniscus*) oral papillomavirus and several of the human papillomaviruses suggests that the nonhuman primate papillomaviruses may be a public health concern.^{32,36,37} Therefore, although not yet tested, it is likely that the Asian lion oral papillomavirus would infect other domestic and wild felids upon contact. Accordingly, there is concern that if the Asian lion papillomavirus is inducible in these potentially susceptible felids, severe lesions could result. Therefore, caution should be exercised during any contact between exotic felids with focal oral hyperplasia and other exotic or domestic felids. Further comparative studies are required to characterize this new virus.

CONCLUSIONS

Focal oral hyperplasia associated with a papillomavirus infection is a newly recognized infectious disease of wild and captive exotic felids. Although easily overlooked because lesions are subtle and found on the ventral surface of the tongue, this disease may spread to closely related species maintained in confinement. The significance to the health and management of Asian lions in captivity has yet to be determined.

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