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Canine inverted papillomas associated with DNA of four different papillomaviruses

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Abstract

Inverted papillomas are uncommon papillomavirus (PV)-induced canine skin lesions. They consist of cup- to dome-shaped dermal nodules with a central pore filled with keratin. Histologically they are characterized by endophytic projections of the epidermis extending into dermis. Cytopathic effects of PVs infection include the presence of clumped keratohyalin granules, koilocytes and intranuclear inclusion bodies. Different DNA hybridization studies carried out with a canine oral papillomavirus (COPV) probe suggested that a different PV than COPV might cause these lesions. Canine papillomavirus 2 (CPV2) was discovered a few years ago in inverted papillomas of immunosuppressed beagles. Two other cases, presenting with distinct clinical and histological features have also been described. This study was carried out on four dogs with clinical and histological signs of inverted papillomas. Molecular biological analyses confirmed that PV DNA was present in all four lesions but demonstrated that the sequences in each case were different. One corresponded to COPV, the second to CPV2, and the third and fourth to unknown PVs. These findings suggest that inverted papillomas are not caused by one single PV type. Similar observations have also been made in human medicine.

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Introduction

In dogs papillomaviruses (PVs) induce several skin changes including exophytic papillomas, pigmented plaques and inverted papillomas. The last are rather uncommon PV-induced canine skin lesions that consist of cup- to dome-shaped nodules with a central pore filled with keratin.[1] Four different subtypes regarding body location, shape, size and colour have been reported. Classical inverted papillomas consist of large (1–2 cm) cup-shaped, greyish nodules with a large central pore and were initially described by Campbell *et al.*[2] Affected animals usually present with one or few lesions on the abdomen. The second subtype was described by Shimada *et al.* and consists of smaller (4 mm) dome-shaped flesh-coloured lesions disseminated all over the body.[3] Le Net *et al.* described a few years ago very small (2 mm) disseminated black papules with particular histological features (see below: intracytoplasmic eosinophilic inclusions) in a Boxer.[4] Finally, inverted papillomas were observed concomitantly with classical exophytic papillomas by Goldschmidt *et al.* in immunosuppressed beagles, most of them developing interdigitally.[5]

From the histological point of view, all inverted papillomas consist of endophytic, papillary projections of the epidermis extending into dermis. The cytopathic effects of PV infection include presence of clumped keratohyalin granules, koilocytes and less frequently, basophilic or eosinophilic intranuclear inclusions.[2–6] The papillomas described by Le Net stand out from the others because of their large, eosinophilic intracytoplasmic inclusions.[4]

Immunohistochemistry was used to confirm that PVs replicate in inverted papilloma lesions and, consequently, probably play a major role in their development. DNA hybridization studies carried out with a canine oral papillomavirus (COPV) probe suggested however that these lesions may be due to a different PV from that causing classical canine oral warts.[2] In fact, canine papillomavirus 2 (CPV2) was identified a few years ago in inverted papillomas from immunosuppressed Beagles.[5,7] All available clinical, histopathological and virological information suggest that inverted papillomas are probably not caused by one single virus.

This study was undertaken on four dogs with inverted papillomas, each representing one example of the four phenotypes described above. Clinical, histological and

virological features of these lesions are presented. Molecular biological analyses were carried out with combinations of PCR primers designed to amplify as many canine papillomavirus as possible. The goal of the study was to demonstrate the genetic diversity of canine inverted papilloma-associated PVs.

Materials and methods

Clinical and histological features

Four dogs with cutaneous nodules resembling inverted papillomas were included. Lesions were surgically removed and histological examination was carried out, using standard methods.

Molecular biological analyses

DNA was extracted from paraffin embedded tissues (cases 1, 2 and 4) or frozen tissue (case 3) using a DNeasy extraction kit (Qiagen, Basel, Switzerland). To identify PV DNA a combination of primers optimised for CPV detection was used, consisting of FAP64 and canPVf (CTT CCT GAW CCT AAY MAK TTT GC).[8] Amplification was undertaken in a PTC-200 thermo cycler (MJ Research, Watertown, MA, USA) under conditions of 94°C for 10 min followed by 45 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min. A final extension of 72°C for 10 min concluded the PCR. Electrophoresis in a 1% agarose gel containing ethidium bromide was used to detect the amplified fragments. The amplified sequences deriving from the L1 open reading frame were extracted from the gels with a QIAEX II (Qiagen) kit and were determined commercially (Microsynth) by cycle sequencing using an ABI 377 sequencer (Applied Biosystems). The obtained sequences of both strands were aligned and only the central double sequenced region excluding primer sequences and inconclusive end/start regions were used for further analysis (GQ204117–GQ204120). These sequences were compared with the NCBI gene bank using BlastX and BlastN (February 2009) analysis. Amino-acid alignments were performed in CLUSTAL_X version 1.83 with the default parameters; the output data were edited in Word (Microsoft).[9]

Results

Animals and clinical features

A 5-month-old male Flat coated Retriever (Dog 1) with an erythematous otitis externa and an 8 mm diameter greyish nodule on the abdomen (Figure 1) that was surgically removed and did not recur.

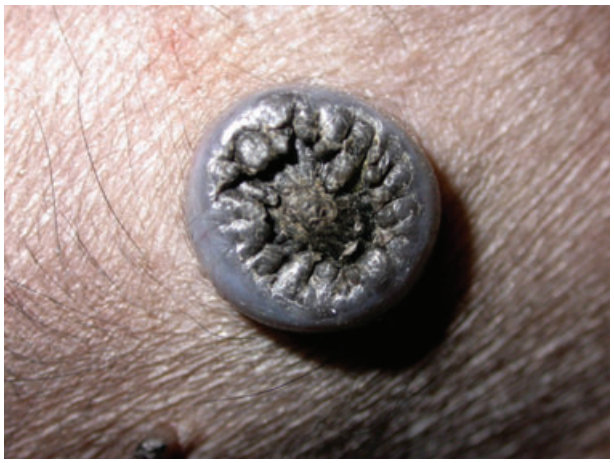

Figure 1. Case 1: Large classical 'Campbell-type' inverted papilloma on the abdomen. Note the typical dome-shape aspect and the central pore.

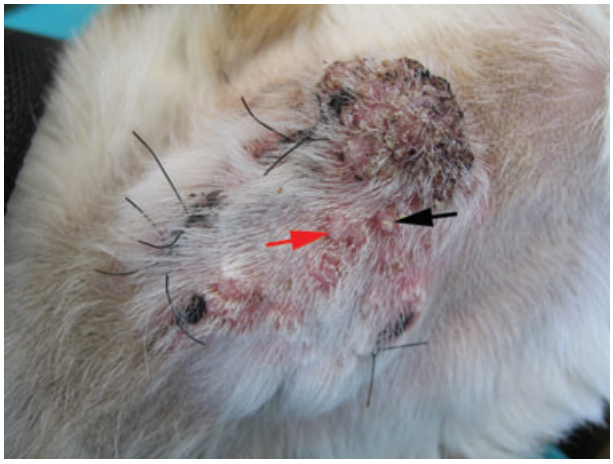

Figure 2. Case 2: Smaller 'Shimada-type' inverted papillomas. Note the dome-shaped aspect (red arrow) and the keratin plugs (black arrow).

A 11-year-old intact female Beagle (Dog 2) with an 8 mm diameter greyish nodule on the paw pad that never recurred following surgical removal.

A 4-year-old female Beagle (Dog 3) with a pruritic plaque on the ventral neck, evolving concomitantly with a pyometra. The skin lesion consisted of numerous small (1–2 mm diameter) dome-shaped white papular lesions (Figure 2). The dog was spayed and skin samples were taken by biopsy for histopathological and virological examination. The lesions regressed spontaneously within 30 days.

The fourth case was a 5-year-old female Rhodesian Ridgeback (Dog 4) with four black small nodules (2 mm diameter).

Lesions were surgically removed: three were melanocytomas and one (concave face of the pinna) an inverted papilloma. Lesions did not recur after excision.

Histpathological features

All the papillomas shared a sharply demarcated cup-shaped aspect characterized by centripetal papillary projections of hyperplastic squamous epithelium with a central core of keratin layers including parakeratotic cells (Figure 3) although the lesions in dogs 3 and 4 were smaller. The basal layers were hyperplastic with a moderate number of mitotic figures. Multifocally, the subcorneal epithelium showed a variable number of koilocytes occasionally with basophilic intranuclear inclusions and a few large keratohyalin granules (Figure 4). Additionally, in one case (case 4), eosinophilic intracytoplasmic pseudoinclusions were observed (Figure 5) while papillary projections were moderate. Based on these observations a diagnosis of inverted papilloma was made.

Molecular biological analyses

The histological findings of koilocytes and intranuclear inclusions suggest the replication of PVs in the four cases. The PV specific DNA amplification and PCR sequencing in each of the four samples revealed in case 1 that the sequence obtained (GQ204117) corresponded at the amino acid level (BlastX) exactly to that of COPV (97/97). In case 2 the sequence obtained (GQ204118)

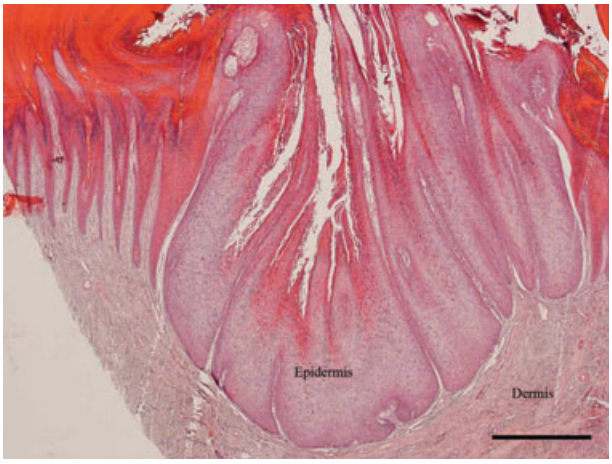

Figure 3. Case 2: Microscopic appearance of an inverted papilloma. Note the papillary projections in the dermis of the squamous epithelium. H&E. Bar = 0.5 mm.

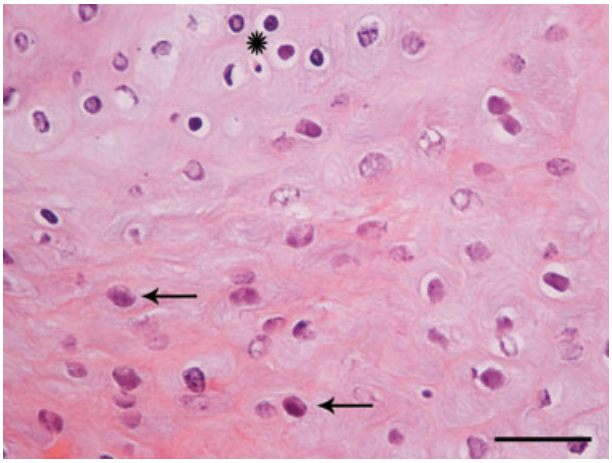

Figure 4. Inverted papilloma (case 2). Note the presence of koilocytes (star) and intranuclear inclusions (arrows) in the lesional epidermis. H&E. Bar = 50 μm.

coded for exactly the same amino acids as CPV2 (94/94).[5,7] The analysis of the third sample revealed a sequence (GQ204119) not identical with any published PV sequence. The highest similarities with this sequence were found among members of the genus Lambda PV. According to the translated sequence COPV was identified as the closest canine PV sharing 70% identity (68/96). Identity was highest among the Lambda PVs of the cat (FdPV1), the lynx (LrPV1), the puma (PcPV1) with 78% each (75/96) and the snow leopard (UuPV1) with

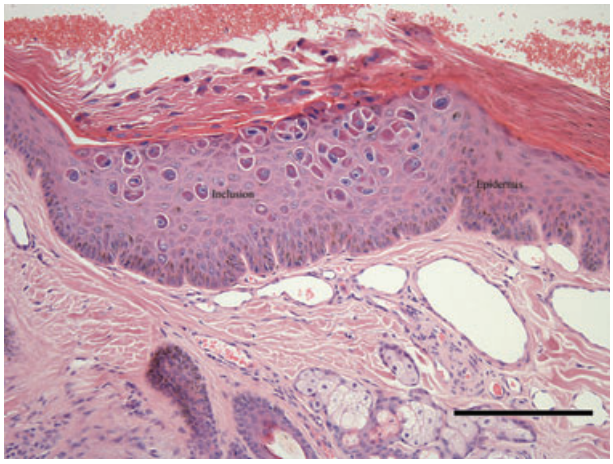

Figure 5. 'LeNet-type' papilloma. Note the large intracytoplasmic eosinophilic inclusions (I) in the epidermis (E). H&E. Bar = 50 μm.

75% (72/96). In case 4 another thus far unknown PV DNA (GQ204120) was uncovered. The BlastX comparison revealed again similarities with Lambda PVs and COPV being the closest canine PV with 70% identity (67/95). Also PcPV1 with 71% (69/96), UuPV1 (67/95) and LrPV1 (68/96) with 70% each displayed similarity to the novel sequence. The homologies and differences of the four sequences were also analysed based on an alignment of an 84 amino-acid stretch covered by all four sequences (Figure 6). It points out, that aligned amino acid sequences of cases 1 and 2 are identical to references sequences of COPV and CPV2, respectively. In contrast, cases 3 and 4 sequences do not match any known, PV sequence. The two DNAs amplified in samples three and four were additionally analysed at the nucleotide level (BlastN), and were found to share 74% identities (212/284).

According to these results each of the four unique cases was associated with a different PV, two of which are as yet unknown.

Discussion

Canine endophytic (inverted) papillomas have been reported only infrequently.[1–6,10] PV-specific cytopathic changes were observed in all reported cases. The latter changes, as well as immunostaining studies support the causative role of PV(s).[2,3,6] Our results demonstrating the presence of PV DNA suggest that these viruses may be the causative agent of these lesions and the phenotypic

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          98                                                                  180
COPV      RLVWGLRGLEIGRGQPLGISVTGHPTFDRYNDVENPNKNLAGHGGG-TDSRVNMGLDPKQTQMFMIGCKPALGEHWSLTRWCTG
Case 1    RLVWGLRGLEIGRGQPLGISVTGHPTFDRYNDVENPNKNLAGHGGG-TDSRVNMGLDPKQTQMFMIGCKPALGEHWSLTRWCTG
Case 3    RLVWALRGLEIDRGQPLGVSVTGNTTFDRYSDVENPNKNPTDHDKENTDPRVNVALDPKQTQLFLVGCKPAIGEHWIQARWCVG
Case 4    RLVWGLRGLEIDRGQPLGISVTGNPTFDKFSDVENSNKVQTDHDKD-ADTRVNIGLDPKQTQLFLIGCKPAIGEHWVQARWCVG
Case 2    RLVWRLTGIEIGRGGPLGFGTTGNFLFDRLQDTENPNNTKVAT----TDDRQNVSMDPKQTQLFVVGCTPCKGEHWDQAPRCDN
CPV2      RLVWRLTGIEIGRGGPLGFGTTGNFLFDRLQDTENPNNTKVAT----TDDRQNVSMDPKQTQLFVVGCTPCKGEHWDQAPRCDN
          96                                                                  175
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Figure 6. Alignment of the overlapping putative amino-acid (AA) sequences encoded by the PCR fragments. Reference sequences of COPV (NC_001619) and CPV2 (NC_006564) in bold. Conserved AAs are underlaid in gray. Numbers indicate first and last amino acid of the reference L1 proteins. Note that the sequence corresponding to case 1 is identical to COPV and the sequence corresponding to case 2 identical to CPV2, while the sequences associated with cases 3 and 4 exhibit various differences to the presented sequences.

differences support reports in the literature that suggest that more than one PV may be involved. The cases in this study illustrated this phenotypic variability: In fact, case 1 was very similar to the description made by Campbell, while cases 2, 3 and 4 likely correspond to the Goldschmidt-, Shimada- and Le Net-types, respectively.[2–5] The PV DNA sequences demonstrated clearly the presence of four different viruses but the finding of these PV DNAs in the lesions does not prove causality as PV DNA has been demonstrated on unaffected skin in several other species.[11–13] However, the histological findings clearly indicate viral replication and causality is consequently likely. Interestingly the four papillomas were harbouring the DNA of one unique PV each. Hints for the presence of more than one PV DNA in each lesion were not present, but cannot entirely be ruled out due to the limited knowledge about the genetic diversity of canine PVs. The DNA found in case 1 matched exactly with COPV, which is known to induce oral papillomas in canids but has also been found in other papillomas. This result contrasts with DNA hybridization studies carried out by Campbell *et al.*, which excluded the presence of COPV in similar lesions.[2] In contrast, CPV2 DNA was amplified from case 2, which shares many similarities with cases described by Goldschmidt *et al.*[5,7] The viral DNA found in cases 3 and 4 appears to belong to two novel and distinct canine PVs but the sequences suggest, that these two viruses may belong to the same genus as COPV (Lambda PVs).

Inverted papillomas are infrequently observed in humans.[14] Most of them occur in the nasal cavity, although cutaneous involvement has been described.[15] Several PVs have been associated with the development of these lesions and neoplastic transformation often occurs when high-risk human PVs are involved.[14,16,17]

From the four cases presented here it can be speculated, that in dogs as in humans certain PVs might be associated with distinct forms of endophytic, inverted papillomas. Further studies are required to confirm the genetic diversity of inverted papillomas-associated PVs and to better characterized the novel PVs.

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Résumé Les papillomes inversés sont des lésions cutanées canines rares, liées à un papillomavirus (PV). Ils consistent en des nodules dermiques convexes ou en dôme avec un pore central rempli de kératine. Histologiquement, ils sont caractérisés par des projections endophytiques de l'épiderme jusque dans le derme. Les effets cytopathogènes d'une infection à PV incluent la présence de granules agglomérées de kératohyaline, de koïlocytes et de corps d'inclusion intranucléaires. Différentes études d'hybridation d'ADN effectuées à partir de papillomavirus canin oral (COPV) ont montré qu'un PV différent de COPV pourrait être à l'origine de ces lésions. Le papillomavirus canin 2 (CPV2) a été découvert il y a quelques années dans les papillomes inversés de beagles immunodéprimés. Deux autres cas, présentant des caractéristiques cliniques et histologiques distinctes ont également été décrits. Quatre chiens présentant des signes cliniques et histologiques de papillomes inversés ont été inclus dans cette étude. Les analyses biologiques moléculaires ont confirmées que l'ADN de papillomavirus était présent dans les quatre lésions mais les séquences étaient à chaque fois différentes. L'une correspondait à COPV, la seconde à CPV2, la troisième et la quatrième à des PV non décrits. Ces résultats suggèrent que les papillomes inversés ne

sont pas dus à un seul type de PV. Des observations identiques ont également été faites en médecine humaine.

Resumen Los papilomas invertidos con lesiones de la piel poco comunes inducidas por el virus papiloma (PV). Consisten en nódulos dérmicos en forma de copa o cúpula con un poro central relleno de queratina. Histológicamente se caracterizan por proyecciones endofíticas de la epidermis que se extienden en la dermis. Los efectos citopáticos de la infección con PV incluyen la presencia de gránulos de queratohialina agrupados, koilocitos y cuerpos de inclusión intranucleares. Estudios de hibridación de DNA llevados a cabo con un segmento de papilomavirus canino oral (COPV) indican que un virus diferente puede ser el causante de la lesiones. Hace unos años se caracterizó el papilomavirus canino tipo 2 en papilomas invertidos de perros Beagle inmunosuprimidos. Otros dos casos con características clínicas e histológicas diferentes también han sido descritos. Este estudio se desarrollo en cuatro perros con signos clínicos de papilomas invertidos confirmado por histopatología. Un análisis moléculas confirmó la presencia de DNA de PV en las cuatro lesiones pero indicó que la secuencia era diferente en cada lesión. Uno correspondía a COPV, el segundo a CPV2 y el tercero y el cuarto a PV desconocidos. Estos hallazgos sugieren que los papilomas invertidos no están causados por un solo tipo de PV. Observaciones similares se han realizado en medicina humana.

Zusammenfassung Invertierte Papillome sind unübliche Papillomavirus (PV)-induzierte canine Hautveränderungen. Sie bestehen aus becher- bis kuppelförmigen dermalen Knoten mit einer zentralen Pore, die mit Keratin gefüllt ist. Histologisch werden sie charakterisiert durch endophytische Projektionen der Epidermis, die sich bis in die Dermis erstrecken. Zu den zytopathischen Auswirkungen der PV-Infektion zählen das Auftreten von geklumpten Keratohyalingranula, Koilozyten und intranukleäre Einschlusskörperchen. Verschiedene DNA Hybridisierungsstudien, die mit einer canines oralen Papillomavirus (COPV) Sonde durchgeführt wurden, zeigten Hinweise darauf, dass ein anderer PV als das COPV die Ursache für diese Läsionen sein könnte. Der canine Papillomavirus 2 (CPV2) wurde vor einigen Jahren in invertierten Papillomen von immunsupprimierten Beagles entdeckt. Zwei weitere Fälle, die sich mit eindeutigen klinischen und histologischen Charakteristika präsentierten, sind ebenfalls beschrieben. Diese Studie wurde an vier Hunden mit klinischen und histologischen Anzeichen von invertierten Papillomen durchgeführt. Eine molekularbiologische Analyse bestätigte, dass PV DNA in allen vier Veränderungen vorhanden war, zeigte allerdings, dass die Sequenzen für jeden Fall verschieden waren. Eine stimmte mit der von COPV überein, die zweite mit CPV2 und die dritte und vierte mit unbekannten PVs. Diese Ergebnisse sind ein Hinweis darauf, dass invertierte Papillome nicht von einem einzigen PV Typ verursacht werden. Ähnliche Beobachtungen wurden auch in der Humanmedizin gemacht.