The clinically healthy skin of dogs is a potential reservoir for canine papillomaviruses

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Abstract

Papillomaviruses have been linked to several skin disorders in the dog. In order to have a suitable diagnostic tool for canine papillomavirus detection, eight PCRs with published primer combinations were evaluated. The most sensitive PCR was used to demonstrate that papillomavirus DNA can be detected on non-lesional skin of dogs.
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Running title: clinically healthy skin
Abstract

Papillomaviruses have been linked to several skin disorders in the dog. In order to have a suitable diagnostic tool for canine papillomavirus detection, eight PCRs with published primer combinations were evaluated. The most sensitive PCR was used to demonstrate that papillomavirus DNA can be detected on non-lesional skin of dogs.
Papillomaviruses (PVs) are predominantly species-specific pathogens of humans and animals that can induce benign as well as malignant neoplasias in the skin and mucous membranes (24). PV DNA has also been detected on the skin of clinically healthy humans and certain animal species (1-4, 6, 7, 16, 17). Likewise, clinically healthy dogs have been shown to carry antibodies against PVs (14). Nevertheless, previous attempts to detect PV DNA on the skin of healthy dogs have been unsuccessful (3).

To address this issue, eight PCR assays with previously published primer combinations targeting either the L1 or the E1 open reading frame were assessed (Table 1 and references therein). These PCR assays were evaluated for their ability to detect the DNA of the seven classified canine PVs (CPVs) (5, 9, 12, 20-22). Furthermore, the sensitivity and specificity of each assay was determined in the same context.

Eight different published primarily broad range primer pairs were tested (Table 1). Three of them are targeting conserved regions in the L1 open reading frame (ORF), namely canPVf/FAP64, FAP59/FAP64 and AR-L1F1/AR-L1R3 while five of the primer pairs target conserved stretches in the E1 ORF, namely CP4/CP5, PPF1/CP5, PapF/PapR, AR-E1F1/AR-E1R2, and AR-E1F2/AR-E1R9. Primers amplifying 585 bp of canine GAPDH were designed to test for host DNA dogGAPDH f (GGT GAT GCT GGT GCT GAG TA) and dogGAPDH r (GAC CAC CTG GTC CTC AGT GT). RedTaq (Sigma, Buchs, Switzerland) ready reaction mix was used according to the manufacturer’s recommendations. Three different protocols were used. In case of the primer combinations canPVf/FAP6410, AR-L1F1/AR-L1R3, AR-E1F1/AR-E1R2 and AR-E1F2/AR-R9 10 min of initial denaturation at 94°C were followed by 45 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C. The program concluded with a final elongation step of 72°C for 10 min. In case of FAP59/FAP64 and dogGAPDH f/dogGAPDH the protocol used started with 3 min at 94°C followed by 45 cycles of 30 sec at 94°C, 30 sec at 50°C and 30 sec at 72°C. The program used for the primer combinations
CP4/CP5, PPF1/CP5 and PapF/PapR started as well with 3 min at 94°C but was then followed by 40 cycles of 30 sec at 94°C, 30 sec at 42°C and 30 sec at 72°C.

To visualize PCR results 1% Agarose gels containing Ethidium bromide were used. Images were taken after a run of 35 minutes in an electric field of 5 V cm⁻¹ in TAE buffer.

Rolling circle amplification (RCA) was used to test for circular potentially papillomaviral DNA (Rector et al. 2004). DNA (1 µl) was used for RCA in a TempliPhi Amplification kit (General Electrics Biosciences, Glattbrugg, Switzerland). The protocol supplied by the manufacturer was used, with slight modifications. Namely, 1µl of 10 mM dNTPs were added and the reaction time was prolonged to 16 h at 30 °C. Two templates were used alternatively for the evaluation, one complete genomic clone of CPV1 in a pBluescript II KS+ vector (Stratagene, La Jolla, CA, USA) and one pET-DEST42 vector (Invitrogen, Basel, Switzerland) containing the entire L1 coding sequence of CPV1. The amplified DNA was digested with the restriction endonuclease EcoRI or EcoRV respectively. To visualize results 1% Agarose gels containing Ethidium bromide were used. Images were taken after 90 minutes in an electric field of 5 V cm⁻¹ in TAE buffer.

To evaluate the spectrum of the primers, clones or PCR products of the target regions from the seven PVs were used as templates. Whole genomes cloned into pBluescript II KS+ (Stratagene, La Jolla, CA, USA) were used in case of CPV1 (EcoRI), CPV3 (SacI), CPV5 (ClaI), CPV6 (EagI) and CPV7 (HinDIII), a partial genomic clone was used in case of CPV4 (KpnI). PCR products of E1 or L1 target regions were used in case of CPV2 and CPV4 (E1 forward GTG GTT TGT TGT GCA TGA GG, E1 CPV2 reverse CCA AAG TCC ATG GTT CAT CC, L1 CPV2 forward TGA TAC ACA GGA AGC GCA AA, L1 CPV2 reverse TGC CTT CCT TCT TTT CTT TGA, E1 CPV4 forward ACC CAG GAG AGG GTA ATG GTG AAG GTC GGA GTC AA, reverse TTA CTC CTT GGA GGC CAT GTA) served as template. To
determine the approximate detection levels of the PCRs serial dilutions were made from $10^9$
down to $10^1$ molecules of every template. For that purpose DNA concentrations of the
template stock solutions were measured using a Spectrophotometer (ND-1000, Thermo
Scientific, Zurich, Switzerland). According to the size of the individual templates the amount
of molecules per microliter was calculated. DNA from a keratinocyte cell culture (Bex) being
free of any known PV DNA served as negative control for PV primers (Figure S2). Therefore
DNA was extracted from $10^6$ cells with a DNeasy extraction kit (Qiagen, Hombrechtikon,
Switzerland) resulting in 83 ng/µl. Eight serial dilutions 1:10 were made.
The test population of 95 dogs was recruited from patients of the small animal hospital of the
University of Zurich displaying no clinical signs of any described kind of papillomatosis. To
obtain skin samples from these dogs, fresh cytobrush sticks were used in the oral cavity and
on the interdigital skin. The cytobrushes were rubbed six turns on the target regions and
afterwards placed in an 1.5ml Eppendorf tube containing 1ml of sterile 0.9% NaCl. The
samples were kept at 4°C for no longer than 24 hours and were then stored below -18°C until
extraction. Before DNA extraction, each cytobrush was flushed repeatedly with the 0.9%
NaCl it had been kept in. To concentrate all cells and cell debris at the bottom of the tube,
tubes (still containing the cytobrush tip) were centrifuge at 15000 g for 10 min. The cytobrush
tip and the 0.9% NaCl were then carefully removed except for about the last 25 µl. DNA was
afterwards extracted using the DNeasy extraction kit (Qiagen, Hombrechtikon, Switzerland)
following the manufacturer’s instructions, eluting DNA finally in 100µl sterile water.
Extraction and handling of this sample DNA was performed entirely separated from any other
PV research.
All sampling was carried out with respect to the Swiss regulations about research on animals.
As shown in Table 1, PCR with the L1 primer combination canPVf/FAP64 detected five of
the seven CPVs at a target concentration of $10^2$ molecules or less, while CPV2- and CPV7-
DNA was still detected at a target concentration of $10^4$ molecules. Assays with E1 primers
CP4/CP5 performed similarly but needed more target to detect CPV3. All tested PCRs with other primer combinations showed less sensitivity and/or less specificity (Table 1, S1). The reason may be that some primers aligned well with certain CPV sequences but poorly with others (S2). RCA is a frequently used alternative method to amplify PV DNA (18). Evaluation of its sensitivity revealed that it required a minimum of $10^6$ molecules to successfully amplify PV DNA (data not shown), thus RCA was not favoured for the detection of CPV DNA on the healthy skin.

Based on the primer evaluation, the canPVI/FAP64 PCR was chosen to assess cytobrush samples from the skin and oral mucosa of 95 asymptomatic dogs. A dogGAPDH PCR assay (requiring $10^4$ molecules for a positive detection) was used as an amplification control. This PCR provided a positive signal with all samples taken from the oral cavity and with 81% of samples taken from the interdigital skin (S3). CPV DNA was detected in more than 50% of the dogs (S3), i.e. in 23% of oral samples whose complementary skin samples were negative; 14% of skin samples (oral samples negative); 15% of samples from both locations. In 48% of the dogs PV DNA was detected neither in skin nor in oral samples. Interestingly, in two cases putative PV DNA was amplified from the skin with canPVI/FAP64 although the dogGAPDH PCR assay had been negative. A number of randomly selected products arising from canPVI/FAP64-mediated PCR were sequenced. The sequences obtained from two oral and three skin samples were clearly identified as CPV1. In contrast, the amplification products of five oral and two skin samples did not yield meaningful sequencing results, most probably due to the presence of DNA from more than one PV type in the sample.

In conclusion, the sensitivity and specificity for each of eight previously described PV primer pairs was assessed in the context of PCR for the detection of CPV DNA. Application of a broad-range, high sensitive primer pair suggests that the clinically unaffected skin and oral cavity of dogs might be a reservoir for canine papillomaviruses.
The authors thank the colleagues of the division of dermatology, especially Stefan Hobi, Sabrina Meury and Sylvia Wilhelm for support with the sample acquisition. This study was funded by the Krebsliga Switzerland.
References


Table 1: Primers and detection levels indicating the minimum of molecules required for detection. Nomenclature for nucleotide symbols according to Cornish-Bowden (8).

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