JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2011, p. 707–709 Vol. 49, No. 2
0095-1137/11/$12.00 doi:10.1128/JCM.02047-10

Clinically Healthy Skin of Dogs Is a Potential Reservoir for Canine Papillomaviruses▽#

Christian E. Lange,[1,2]* Sabrina Zollinger,[2] Kurt Tobler,[2] Mathias Ackermann,[2] and Claude Favrot[1]

Dermatology Department, Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland,[1] and Institute of Virology, Vetsuisse Faculty, Winterthurerstrasse 266a, CH-8057 Zurich, Switzerland[2]

Received 11 October 2010/Returned for modification 31 October 2010/Accepted 7 December 2010

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 nonlesional 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 (ORF) were assessed (Table 1 and see 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 were determined in the same context.

Eight different published, primarily broad-range primer pairs were tested (Table 1). Three of them target conserved regions in the L1 open reading frame, 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 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed to test for host DNA (dogGAPDHf, GGT GAT GCT GGT GCT GAG TA; dogGAPDHr, 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 the cases of the primer combinations canPVf/FAP64, AR-L1F1/AR-L1R3, AR-E1F1/AR-E1R2, and AR-E1F2/AR-E1R9, 10 min of initial denaturation at 94°C was followed by 45 cycles of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The protocol concluded with a final elongation step of 72°C for 10 min. In the case of FAP59/FAP64 and dogGAPDHf/dogGAPDHr, the protocol used started with 3 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. The protocol used for the primer combinations CP4/CP5, PPF1/CP5, and PapF/PapR also started with 3 min at 94°C but was then followed by 40 cycles of 30 s at 94°C, 30 s at 42°C, and 30 s at 72°C.

To visualize PCR results, 1% agarose gels containing ethidium bromide were used. Images were taken after a run of 35 min in an electric field of 5 V cm^{-1} in Tris-acetate-EDTA (TAE) buffer.

Rolling circle amplification (RCA) was used to test for circular, potentially papillomaviral DNA (18). 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, 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs) was 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) 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 min 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) were used in the cases of CPV1 (EcoRI), CPV3 (SacI), CPV5 (ClaI), CPV6 (EagI), and CPV7 (HinDIII); a partial genomic clone was used in the case of CPV4 (KpnI). PCR products of E1 or L1 target regions were used in the cases of CPV2 and CPV4 (E1 CPV2 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 CCA GT; E1 CPV4 reverse, CCC TCG TCC TCT TGA TCA CT). For the evaluation of the dogGAPDH primers, a PCR product of the whole dogGAPDH (forward, ATG GTG AAG GTC GGA GTC AA; reverse, TTA CTC CTT GGA

* Corresponding author. Mailing address: Dermatology Department, Clinic for Small Animal Internal Medicine, Vetsuisse Faculty, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland. Phone: 41 44 6358387. Fax: 41 44 6358930. E-mail: clange@vetclinics.uzh.ch.

Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 15 December 2010.

TABLE 1. Primers and detection levels indicating the minimum concentration of molecules required for detection of CPVs

Target gene	Primer names and sequences[a]	No. of molecules/reaction required to detect[b]:						
		CPV1	CPV2	CPV3	CPV4	CPV5	CPV6	CPV7
L1	canPVf/FAP64 (13); CTTCCTGAWCCTAAYMAKTTTGC/CCWATATCWVHCATNTCNCCATC	10^1	10^4	10^1	10^1	10^2	10^1	10^4
	FAP59/FAP64 (10); TAACWGTNGGNCAYCCWTATT/CCWATATCWVHCATNTCNCCATC	10^4	10^6	10^9	10^8	10^8	ND	10^8
	AR-L1F1/AR-L1R3 (19); TTDCAGATGGCNGTNTGGCT/CATRTCHCCATCYTCWAT	10^4	10^6	10^5	10^6	10^6	10^5	10^6
E1	CP4/CP5 (11); ATGGTACARTGGGCATWTGA/GAGGYTGCAACCAAAAMTGRCT	10^1	10^4	10^5	10^1	10^2	10^1	10^3
	PPF1/CP5 (11); AACAATGTGTAGACATTATAAACGAGC/GAGGYTGCAACCAAAAMTGRCT	10^4	10^8	ND	10^7	ND	10^4	ND
	PapF/PapR (23); ATGGCGGATAAAAAAGGTA/AACAGCTGTTTTTTAGCTTTTTT	10^5	ND	ND	ND	ND	10^6	ND
	AR-E1F1/AR-E1R2 (19); CAGGGVMWTTCCCTGBARYTGTTYC/TCATANGCCCACTGNACCAT	10^1	10^5	10^6	10^7	10^6	10^4	10^7
	AR-E1F2/AR-E1R9 (15); ATGGTNCAGTGGGCNTATGA/CATTWGTDGTDAYMAGSAKRGGVGGGCA	10^1	10^3	10^2	10^7	10^6	10^3	10^6

[a] Nomenclature for nucleotide symbols is according to Cornish-Bowden (8).
[b] ND, not detected.

GGC CAT GTA) served as the 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 number of molecules per microliter was calculated. DNA from a keratinocyte cell culture (Bex), being free of any known PV DNA, served as a negative control for PV primers (see Fig. S1 in the supplemental material). Thus, 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 at 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 afterward placed in a 1.5-ml Eppendorf tube containing 1 ml of sterile 0.9% NaCl. The samples were kept at 4°C for no longer than 24 h 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 centrifuged at 15,000 × *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 afterward extracted using the DNeasy extraction kit (Qiagen, Hombrechtikon, Switzerland) by following the manufacturer's instructions, finally eluting DNA in 100 μl sterile water. Extraction and handling of this sample DNA were performed entirely separately from any other PV research.

All sampling was carried out in accordance with 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/reaction or less, while CPV2 and CPV7 DNA was still detected at a target concentration of 10^4 molecules/reaction. 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; see also Fig. S1 in the supplemental material). The reason may be that some primers aligned well with certain CPV sequences but poorly with others (see Fig. S2 in the supplemental material). 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 favored for the detection of CPV DNA on healthy skin.

Based on the primer evaluation, the canPVf/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 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 (see Table S3 in the supplemental material). CPV DNA was detected in more than 50% of the dogs (see Table S3 in the supplemental material), i.e., in 23% of oral samples whose complementary skin samples were negative, 14% of skin samples (oral samples negative), and 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 canPVf/FAP64 although the dogGAPDH PCR assay had been negative. A number of randomly selected products arising from canPVf/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, highly sensitive primer pair suggests that the clinically unaffected skin and oral cavity of dogs might be a reservoir for canine papillomaviruses.

We 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

1. **Antonsson, A., et al.** 2003. Prevalence and type spectrum of human papillomaviruses in healthy skin samples collected in three continents. J. Gen. Virol. **84:**1881–1886.
2. **Antonsson, A., O. Forslund, H. Ekberg, G. Sterner, and B. G. Hansson.** 2000. The ubiquity and impressive genomic diversity of human skin papillomaviruses suggest a commensalic nature of these viruses. J. Virol. **74:**11636–11641.
3. **Antonsson, A., and B. G. Hansson.** 2002. Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. J. Virol. **76:**12537–12542.
4. **Antonsson, A., and N. McMillan.** 2006. Papillomavirus in healthy skin of Australian animals. J. Gen. Virol. **87:**3195–3200.
5. **Bernard, H., et al.** 2010. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology **401:**70–79.
6. **Bosch, F., A. Lorincz, N. Muñoz, C. Meijer, and K. Shah.** 2002. The causal relation between human papillomavirus and cervical cancer. J. Clin. Pathol. **55:**244–265.
7. **Boxman, I., et al.** 1997. Detection of human papillomavirus DNA in plucked hairs from renal transplant recipients and healthy volunteers. J. Invest. Dermatol. **108:**712–715.
8. **Cornish-Bowden, A.** 1985. Nomenclature for incompletely specified bases in nucleic acid sequences: recommendations 1984. Nucleic Acids Res. **13:**3021–3030.
9. **Delius, H., M. Van Ranst, A. Jenson, H. zur Hausen, and J. Sundberg.** 1994. Canine oral papillomavirus genomic sequence: a unique 1.5-kb intervening sequence between the E2 and L2 open reading frames. Virology **204:**447–452.
10. **Forslund, O., A. Antonsson, P. Nordin, B. Stenquist, and B. Hansson.** 1999. A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. J. Gen. Virol. **80**(Pt 9)**:**2437–2443.
11. **Iftner, A., et al.** 2003. The prevalence of human papillomavirus genotypes in nonmelanoma skin cancers of nonimmunosuppressed individuals identifies high-risk genital types as possible risk factors. Cancer Res. **63:**7515–7519.
12. **Lange, C. E., et al.** 2009. Three novel canine papillomaviruses support taxonomic clade formation. J. Gen. Virol. **90:**2615–2621.
13. **Lange, C. E., et al.** 2010. Canine inverted papillomas associated with DNA of four different papillomaviruses. Vet. Dermatol. **21:**287–291.
14. **Lange, C. E., et al.** 2009. Detection of antibodies against epidermodysplasia verruciformis-associated canine papillomavirus 3 in sera of dogs from Europe and Africa by enzyme-linked immunosorbent assay. Clin. Vaccine Immunol. **16:**66–72.
15. **Manire, C., et al.** 2008. Proliferative dermatitis in a loggerhead turtle, Caretta caretta, and a green turtle, Chelonia mydas, associated with novel papillomaviruses. Vet. Microbiol. **130:**227–237.
16. **Munday, J., and A. Witham.** 2010. Frequent detection of papillomavirus DNA in clinically normal skin of cats infected and noninfected with feline immunodeficiency virus. Vet. Dermatol. **21:**307–310.
17. **Ogawa, T., et al.** 2004. Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. J. Gen. Virol. **85:**2191–2197.
18. **Rector, A., R. Tachezy, and M. Van Ranst.** 2004. A sequence-independent strategy for detection and cloning of circular DNA virus genomes by using multiply primed rolling-circle amplification. J. Virol. **78:**4993–4998.
19. **Rector, A., et al.** 2005. Isolation and cloning of the raccoon (Procyon lotor) papillomavirus type 1 by using degenerate papillomavirus-specific primers. J. Gen. Virol. **86:**2029–2033.
20. **Tobler, K., C. Favrot, G. Nespeca, and M. Ackermann.** 2006. Detection of the prototype of a potential novel genus in the family Papillomaviridae in association with canine epidermodysplasia verruciformis. J. Gen. Virol. **87:**3551–3557.
21. **Tobler, K., C. Lange, D. Carlotti, M. Ackermann, and C. Favrot.** 2008. Detection of a novel papillomavirus in pigmented plaques of four pugs. Vet. Dermatol. **19:**21–25.
22. **Yuan, H., et al.** 2007. An epidermotropic canine papillomavirus with malignant potential contains an E5 gene and establishes a unique genus. Virology **359:**28–36.
23. **Zaugg, N., G. Nespeca, B. Hauser, M. Ackermann, and C. Favrot.** 2005. Detection of novel papillomaviruses in canine mucosal, cutaneous and in situ squamous cell carcinomas. Vet. Dermatol. **16:**290–298.
24. **zur Hausen, H.** 2000. Papillomaviruses causing cancer: evasion from host-cell control in early events in carcinogenesis. J. Natl. Cancer Inst. **92:**690–698.