In situ hybridization to detect bandicoot papillomatosis carcinomatosis virus type 1 in biopsies from endangered western barred bandicoots (*Perameles bougainville*)

Mark D. Bennett, Lucy Woolford, Amanda J. O’Hara, Kristin S. Warren and Philip K. Nicholls

School of Veterinary and Biomedical Sciences, Murdoch University, South Street, Murdoch, Western Australia 6150, Australia

Correspondence
Mark D. Bennett
m.bennett@murdoch.edu.au

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The western barred bandicoot (*Perameles bougainville*) is an endangered Australian marsupial species in which a papillomatosis and carcinomatosis syndrome occurs. Bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1) is associated with the lesions of this progressively debilitating syndrome. Five digoxigenin-labelled DNA probes were generated for in situ hybridization (ISH) and the technique was optimized and performed on formalin-fixed paraffin-embedded (FFPE) biopsies. Staining of keratinocyte and sebocyte nuclei within lesions was achieved with all five probes. The sensitivity of ISH (76.9 %) surpassed that of PCR (30.8 %) for FFPE samples. The sensitivity of ISH varied from 81 % (papillomas) and 70 % (carcinoma in situ) to 29 % (squamous cell carcinomas). The specificity of the test was confirmed using an irrelevant probe and papillomas from other species. These results strengthen the association between BPCV1 and the western barred bandicoot papillomatosis and carcinomatosis syndrome and give insight into the biology of the virus–host interaction.

The western barred bandicoot, *Perameles bougainville* Quoy and Gaimard, 1824, is an endangered Australian peramelid marsupial species (Friend & Burbidge, 2002; IUCN, 2007; Tyndale-Biscoe, 2005). Though once widely distributed across arid regions of southern mainland Australia, its range has contracted dramatically, due to predation by introduced carnivores, competition with introduced herbivores and habitat alterations (Friend & Burbidge, 2002). Believed extinct on the Australian mainland, natural populations of *P. bougainville* survive only on Dorre Island and Bernier Island, Western Australia (Friend & Burbidge, 2002; Tyndale-Biscoe, 2005). Captive breeding colonies have been established to bolster their dwindling numbers, but the discovery of a papillomatosis and carcinomatosis syndrome has hindered these efforts (Woolford et al., 2008).

A novel virus, tentatively named bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1), has been detected in association with the papillomatosis and carcinomatosis syndrome using PCR, multiply primed rolling circle amplification, transmission electron microscopy and immunohistochemistry (Woolford et al., 2007, 2008). Significantly, BPCV1 has a double-stranded circular DNA genome and is similar to members of the *Papillomaviridae* in genome size (~7.3 kb) and structural protein nucleotide and amino acid sequences, but has a genomic organization and putative transforming protein nucleotide and amino acid sequences most similar to members of the *Polyomaviridae* (Woolford et al., 2007). Four ORFs have been predicted after examination of the BPCV1 genome and named according to the proteins they are likely to encode: two putative capsid protein ORFs, *L1* and *L2*, and two putative transforming protein ORFs, large T antigen (*LTag*) and small t antigen (*stag*) (Woolford et al., 2007).

Mere detection of BPCV1 DNA was considered insufficient evidence to implicate it in the aetiology of the papillomatosis and carcinomatosis syndrome, because papillomavirus DNA can be detected in skin swabs taken from apparently healthy animals (Antonsson & Hansson, 2002). Indeed, several novel virus isolates attributed to the *Papillomaviridae* have been detected recently in Australian animals using PCR of swabs taken from non-lesional skin of koalas (*Phascolarctos cinereus*), eastern grey kangaroos (*Macropus giganteus*) and an echidna (*Tachyglossus aculeatus*) (Antonsson & McMillan, 2006). Furthermore, BPCV1 appears to resist degradation, as it can be detected in the environment and on fomites using PCR (F. Armin-Grimm, personal communication). Therefore, we attempted to detect BPCV1 DNA *in situ* within lesional biopsies to provide compelling evidence linking BPCV1 with the papillomatosis and carcinomatosis syndrome.
Biopsies were collected between 2000 and 2006 from *P. bougainville* expressing lesions typical of the papillomatosis and carcinomatosis syndrome. Samples were fixed in 10% neutral-buffered formalin (>24 h), processed with a Leica EG 1150C automated processor (Leica Microsystems) and embedded in paraffin. Sections (5 µm thickness) were cut using a Leica 2135 microtome (Leica Microsystems) and baked onto silanized glass microscope slides (ProSciTech). Haematoxylin and eosin-stained slides of lesions were examined by light microscopy and categorized as either papillomas, carcinomas *in situ* or squamous cell carcinomas according to the classification system of Goldschmidt *et al.* (1998).

Total genomic DNA was isolated from 25 mg samples of 13 formalin-fixed paraffin-embedded (FFPE) lesional tissues using the DNeasy Tissue kit (Qiagen), according to the manufacturer’s protocol. PCR testing for the detection of BPCV1 DNA in total DNA extracted from FFPE lesional tissue was performed using a primer pair designed to amplify part of the putative BPCV1 L1 ORF region (5'-GAGGAGGGGATCCGTTGGC-3' and 5'-ATTGGTTTTGCCAGTTGAG-3'). PCR was performed in a total volume of 25 µl, containing 0.3 µM of each primer, 200 µM of each dNTP, 3.75 U *Taq* DNA polymerase ml⁻¹, 1.5 mM MgCl₂, pH 8.5, and 1 × DNA polymerase reaction buffer (Fisher Biotech Australia), with 2 µl of the 1:10-diluted extracted lesional DNA as template. An automated thermocycler (Perkin Elmer Gene Amp PCR System 2400) set for block temperatures was programmed as follows: 5 min at 94 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C, and finally 7 min at 72 °C. The PCR product was electrophoresed through an ethidium bromide-laced 1% agarose gel and visualized using UV transillumination.

PCR primers were designed to amplify DNA segments within four BPCV1 ORFs: *L1* (5'-AGATGGGCGTCCTC- AAGGTG-3' and 5'-TCATCATCCCTTTTGGC-3'; 250 bp), *L2* (5'-AAGGAAAATTTGAGGAAGACCA-3' and 5'-ACAGCATCACTGGGAGAT-3'; 236 bp), *stag* (5'-ATTCTGGATCCGATTGAGGAA-3' and 5'-CCCATTATACAGGAATTCATCAGTGA-3'; 308 bp) and *LTag* (5'-TGCAAATGTCAGCAGGATT-3' and 5'-TGTCGCGATCATATTGCCT-3'; 184 bp). PCRs were performed in a total volume of 40 µl with the following final concentrations of reagents: 200 µM each dNTP, 1 × PCR reaction buffer, 1.5 mM MgCl₂, pH 8.5, 200 nM each primer and 3.75 U *Taq* DNA polymerase ml⁻¹ with 2 µl of a 1:100 dilution of BPCV1 positive-control DNA as the template. This mixture was subjected to 95 °C for 3 min, followed by 25 cycles of 30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C, and finally 7 min at 72 °C in an automated thermocycler (Perkin Elmer Gene Amp PCR System 2400). The reaction was checked by visualizing an ampiclon of the expected size following electrophoresis through a 1% agarose gel laced with ethidium bromide, using a UV transilluminator. PCR products were purified using the QIAquick PCR purification kit (Qiagen) according to the manufacturer’s guidelines and DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

Plasmid DNA (1 µg) containing the BPCV1 genome cloned into pUC18 was incubated with 4 µl nick-translation digoxigenin-labeling kit mixture (Roche Diagnostics) in a total volume of 20 µl at 14 °C for 4 h. An extra 1 µl of the nick-translation digoxigenin-labeling kit mixture was added to the reaction tube and incubation at 14 °C was continued for a further 20 h. A 1 µl aliquot of the reaction mixture was electrophoresed through an ethidium bromide-laced 1% agarose gel and visualized under UV light to check that the genomic DNA had been digested to fragments shorter than ~500 bp. The reaction was stopped with 1.25 µl 0.5 M EDTA (pH 8) and the mixture was heated to 95 °C for 3 min; labelled DNA species were then purified using the QIAquick PCR purification kit (Qiagen) to remove labelled fragments shorter than ~40 bp.

Purified subORF PCR product DNA (1 µg) was incubated with 4 µl nick-translation digoxigenin-labeling kit mixture (Roche Diagnostics) in a total volume of 20 µl at 14 °C for 2 h. A 1 µl volume of 0.5 M EDTA (pH 8) was added to stop the reaction and the mixture was heated to 95 °C for 3 min and stored at 4 °C.

Biopsies on silanized slides were deparaffinized in two changes of xylene and then rehydrated through 100, 95 and 70% ethanol and tap water and finally immersed in PBS with 0.05% Tween 20 (pH 7.5) (PBST). Up to 50 µl DNA probe cocktail mixture (consisting of 50% formamide, 10% dextran sulphate, 2 × SSC buffer and >7.5 ng digoxigenin-labelled DNA) was pipetted onto each tissue section, covered with a glass coverslip and incubated in an oven at 95 °C for 15 min. Each slide was then allowed to cool for at least 30 min. The coverslip was removed and the slide washed in 2 × SSC buffer with 0.05% Tween 20 (pH 7). Blocking solution [4 µg BSA ml⁻¹ in Tris-buffered saline with 0.05% Tween 20 (pH 7) (TBST)] was added to the section for 5 min and alkaline-phosphatase-conjugated anti-digoxigenin mAb (Roche Diagnostics) diluted 1:600 in blocking solution was then applied to cover the tissue completely and left at room temperature for 1 h. Slides were thoroughly washed with TBST and antibody binding was demonstrated by covering the section with precipitating BM Purple AP substrate (Roche Diagnostics) laced with levamisole (300 µg ml⁻¹) to block endogenous alkaline phosphatase activity. The slides were left to develop in a dark humid chamber for 4–16 h, washed thoroughly in tap water, counterstained with Brazilin haematoxylin, washed again in tap water and wet-mounted with Apathy’s solution.

A DNA probe created to detect DNA from an oyster parasite (*Haplosporidium* sp.) was kindly provided by Douglas Bearham (School of Veterinary and Biomedical Sciences, Murdoch University) as an irrelevant control.
probe and was tested on a section of positive-control tissue. FFPE tissue sections from papillomas from dogs and southern brown bandicoots (*Isoodon obesulus*) were tested using BPCV1 DNA probes. Also, immediately following the rehydration of tissue sections, some slides were treated with DNase-free RNase (Sigma) for 30 min at room temperature to remove RNA from tissue sections.

There was no staining of positive-control tissue using the irrelevant *Haplosporidium* sp. DNA probe, nor were tissues from canine or southern brown bandicoot papillomas stained by our ISH technique (Fig. 1b, c). In positive-control tissue sections tested with our ISH probes, the positive staining was restricted to the nuclei of keratinocytes and sebocytes (Figs 2 and 3). There was no staining of the cytoplasm or any part of the dermis, sweat glands or hypodermis, and RNase pretreatment had no demonstrable effect on staining intensity or staining pattern (Fig. 1a).

Of 13 lesions histologically consistent with the papillomatosis and carcinomatosis syndrome tested for BPCV1 DNA using ISH and PCR, five results were in agreement: three (23.1%) were positive by both methods and two (15.4%) were negative by both methods. Conflicting results were obtained in eight cases: seven (53.8%) were ISH-positive but PCR-negative, while only one (7.7%) was PCR-positive and ISH-negative. Therefore, when performed on FFPE biopsies of lesions histologically consistent with the papillomatosis and carcinomatosis syndrome and assuming that all such lesions were BPCV1-associated, the sensitivity of ISH was 76.9% and the sensitivity of PCR was 30.8%. Thirteen of 16 (81%) lesions categorized histologically as papillomas were positive using ISH, compared with seven of 10 (70%) carcinomas *in situ* and just five of 17 (29%) squamous cell carcinomas.

Many protocols for ISH recorded in the literature are remarkably complex and call for unusual reagents (Lamar-Jones, 2002; Rolighed & Lindeberg, 1996). Our ISH protocol utilized a simplified and rapid protocol to yield excellent, reproducible results. Steps such as digestion in pepsin/HCl or proteinase K, post-fixation, pre-hybridization and lengthy incubations following the DNA denaturation step were all entirely dispensable. We found that immersing sections in alkaline phosphatase substrate buffer (pH 9.0–9.5) significantly inhibited the staining reaction. Trace amounts of levamisole added to BM Purple solution were highly effective at suppressing endogenous alkaline phosphatase activity (such as that seen in *P. bougainville* sweat glands); however, at concentrations above approximately 1.5 mg ml$^{-1}$, levamisole significantly slowed the colour development reaction.

Given the novelty of the BPCV1 genome, it was important to verify that both papillomavirus-like and polyomavirus-like DNA sequences were detectable *in situ* within the lesions of the western barred bandicoot papillomatosis and carcinomatosis syndrome. The identical staining patterns of keratinocytes and sebocytes obtained using DNA probes constructed to anneal with $L1$, $L2$, $LTag$ and $stag$ ORFs as well as the BPCV1 genomic probe confirmed the presence of the novel virus genotype, including both papillomavirus and polyomavirus-like ORFs within lesions (Figs 2 and 3). Furthermore, the viral DNA sequences were found most prominently in the nuclei of basal keratinocytes of the epidermis and external root sheath, followed by

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**Fig. 1.** Selected controls used to confirm the specificity of the ISH technique. Brazilin haematoxylin counterstain; bar, 50 μm. (a) DNase-free RNase pretreatment did not affect positive nuclear staining. (b) A digoxigenin-labelled DNA probe designed to detect *Haplosporidium* sp. DNA sequences failed to stain papillomas from *P. bougainville*. (c) Papillomas from a related bandicoot species, *Isoodon obesulus*, did not stain positively using any digoxigenin-labelled DNA probes designed to detect BPCV1.
keratinocytes of the stratum spinosum and, to a lesser extent, stratum granulosum. This pattern of distribution is largely consistent with the expected biology of papillomavirus infections (Howley & Lowy, 2007; Nicholls et al., 2001; Peh et al., 2002). Interestingly, nuclei of sebaceous cells also stained positively using this ISH technique (Fig. 3). Detection of virus DNA in sebocytes is unusual but not unprecedented: human papillomavirus DNA has been demonstrated by ISH in sebaceous carcinomas (Hayashi et al., 1994) and Micromys minutus papillomavirus has been associated with sebaceous carcinoma in that species (Sundberg et al., 1988). It is conceivable that P. bougainville sebocytes could be permissive for BPCV1 growth. If that is the case, BPCV1 virions may be shed in sebum as well as in exfoliated epithelial cells. Despite sebaceous cells and hair follicle keratinocytes staining positively, sweat gland nuclei showed no positive staining, indicating that the sweat gland cells may be refractory to infection with BPCV1. One severely affected P. bougainville had multifocal pulmonary metastases of a squamous cell carcinoma. While the hyperplastic cutaneous lesions from this individual were strongly ISH-positive, the pulmonary metastases were only very weakly ISH-positive. It remains unclear whether this is related to a genuine ‘hit and run’
transforming effect of the virus. If it is possible for BPCV1 DNA to integrate into the host’s DNA, perhaps such integrated components are less easily detected with this ISH technique.

Southern brown bandicoot papillomatosis is also associated with viral DNA, and the BPCV1 stag DNA probe has a nucleotide sequence similarity of ~91% to the corresponding region of the southern brown bandicoot virus isolate genome (M. D. Bennett, L. Woolford, T. Oldfield, K. S. Warren, P. K. Nicholls and A. J. O’Hara, unpublished results). The lack of cross-reactivity of this probe with lesions from a related species, the southern brown bandicoot (Isoodon obesulus), indicated a high level of specificity for the DNA probes employed in this ISH method.

These ISH results confirmed that BPCV1 DNA was present within the cutaneous and mucocutaneous lesions of western barred bandicoot papillomatosis and carcinomatosis syndrome and suggested that ISH was a more sensitive test than PCR for FFPE tissue samples for detecting BPCV1 in papillomatous lesions from P. bougainville. ISH demonstrated BPCV1 DNA in seven of nine (77.8%) cases that tested negative using PCR. The single case for which ISH failed to detect BPCV1 DNA but PCR succeeded was a squamous cell carcinoma. The sensitivity of this ISH technique diminished as lesions progressed from benign to malignant histological grades. This optimized ISH technique can diagnose BPCV1 infection reliably in properly collected and processed preneoplastic biopsies from P. bougainville.

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