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

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Summary

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 labeled DNA probes were generated for *in situ* hybridization (ISH), the technique was optimized and performed on formalin fixed paraffin embedded (FFPE) biopsies. Staining of keratinocyte and sebocytes nuclei within lesions was achieved with all 5 probes. The sensitivity of ISH (76.9%) surpassed that of polymerase chain reaction (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 test's specificity 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 and 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 and Burbidge, 2002). Believed extinct on the Australian mainland, natural populations of *P. bougainville* survive only on Dorre Island and Bernier Island, Western Australia (Friend and Burbidge, 2002; Tyndale-Biscoe, 2005). Captive breeding colonies were established to bolster their dwindling numbers, but the discovery of a papillomatosis and carcinomatosis syndrome hindered these efforts (Woolford *et al.*, in press a).

A novel virus, tentatively named bandicoot papillomatosis carcinomatosis virus type 1 (BPCV1), was detected in association with the papillomatosis and carcinomatosis syndrome using polymerase chain reaction (PCR), multiply primed rolling circle amplification, transmission electron microscopy and immunohistochemistry (Woolford *et al.*, in press a, b). Significantly, BPCV1 had a double-stranded circular DNA genome and was similar to members of the *Papillomaviridae* in genomic size (~7.3 kb) and structural protein nucleotide and amino acid sequences, but had genomic organization and putative transforming protein nucleotide and amino acid sequences most similar to members of the *Polyomaviridae* (Woolford *et al.*, in press b). Four open reading frames (ORFs) were predicted after examination of the BPCV1 genome and named according to the proteins they were likely to encode: 2 putative capsid protein ORFs, *L1* and *L2*, and 2 putative transforming protein ORFs, large T antigen (*LTag*) and small t antigen (*stag*) (Woolford *et al.*, in press b).

Mere detection of BPCV1 DNA was considered insufficient evidence to implicate it in the etiology of the papillomatosis and carcinomatosis syndrome, because papillomavirus DNA can be detected in skin swabs taken from apparently healthy animals (Antonsson and Hansson, 2002). Indeed, several novel

virus isolates attributed to *Papillomaviridae* have been recently detected 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 and McMillan, 2006). Furthermore, BPCV1 appears to resist degradation as it can be detected in the environment and on fomites using PCR (F. Armin-Grimm, pers. comm.). Therefore, we attempted to demonstrate 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 hours), processed with a Leica EG 1150C automated processor (Leica Microsystems) and embedded in paraffin. Sections were cut (5 µm thickness) using a Leica 2135 microtome (Leica Microsystems) and baked onto silanised 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 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'-GAGGAGGGACATCAGGTGAC-3' and 5'-ATTGTTTTGCCCAGTTGCTC-3'). PCR was performed in a total volume of 25 µl, containing 0.3 µM of each primer, 200 µM of each deoxyribonucleic acid triphosphate (dNTP), 3.75 $U.mL^{-1}$ *Taq* DNA polymerase, 1.5 mM $MgCl_2$ pH 8.5 and 1x 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 minutes at 94 °C, then 25 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C and 30 seconds at 72 °C, then 7 minutes at 72 °C. The PCR product was electrophoresed through an ethidium bromide laced 2% agarose gel and examined for the expected 172 bp amplicon using UV transillumination.

PCR primers were designed to amplify DNA segments within four BPCV1 ORFs: *L1* (5'-AGATTGGCGTTCCTAAGGTG-3' and 5'-TCATCATCCCCTTCTTTTGC-3'; 250 bp), *L2* (5'-AAGGACAAAATTGAAGGAACCA-3' and 5'-ACAGCATCAACTGGGAGGAT-3'; 236 bp), *stag* (5'-ATTCTGGATCCAGTGAGGGAA-3' and 5'-CCCATAATTAACAGAATTCATCAGTGA-3'; 308 bp) and *LTag* (5'-TGCAAAGTCCGCTAAGGATT-3' and 5'-TGTGGCGAATCATCTTTGTC-3'; 184 bp). PCR reactions were performed in a total volume of 40 μL with the following final concentrations of reagents: 200 μM each dNTP, 1x PCR reaction buffer, 1.5 mM $MgCl_2$ pH 8.5, 200 nM of each primer and 3.75 $U.mL^{-1}$ *Taq* DNA polymerase with 2 μL of 1:100 dilution of BPCV1 positive control DNA as template. This mixture was subjected to 95 °C for 3 minutes, then 25 cycles of 30 seconds at 95 °C, 30 seconds at 58 °C and 45 seconds at 72 °C, then 7 minutes at 72 °C in an automated thermocycler (Perkin Elmer Gene Amp PCR System 2400). The reaction was checked by visualizing an amplicon of 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 concentration determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc.).

Genomic DNA (1 μg) of plasmid DNA 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 hours. 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 hours. 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 μL of 0.5 M EDTA pH 8 and the mixture was heated to 95 °C for 3 minutes, then labeled DNA species were purified using the QIAquick® PCR purification kit (Qiagen) to remove labeled fragments shorter than ~40 bp. Purified sub-ORF 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 hours. 1.25 μL of 0.5 M EDTA pH 8 was added to stop the reaction and the mixture was heated to 95 °C for 3 minutes and stored at 4 °C.

Biopsies on silanised slides were deparaffinized in two changes of xylene, then rehydrated through 100%, 95%, 70% ethanol and tap water, then immersed in phosphate buffered saline with 0.05% Tween-20 pH 7.5 (PBST). Up to 50 μL DNA probe cocktail mixture (consisting of 50% formamide, 10% dextran sulphate, 2x SSC buffer, >7.5 ng digoxigenin-labeled DNA) was pipetted onto each tissue section, covered with a glass coverslip and incubated in an oven at 95 °C for 15 minutes. Each slide was allowed to cool for at least 30 minutes. The cover slip was removed and the slide washed in 2x SSC buffer with 0.05% Tween-20 pH 7. Blocking solution (4 $\mu g.mL^{-1}$ bovine serum albumin in tris buffered saline with 0.05% Tween-20 pH 7 (TBST)) was added to the section for 5 minutes, then alkaline phosphatase conjugated anti-digoxigenin monoclonal antibody (Roche Diagnostics) diluted 1:600 in the blocking solution was applied to completely cover the tissue and left at room temperature for 1 hour. 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 hours, 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 as an irrelevant control probe, and tested on a section of positive control tissue. FFPE tissue sections from dog and southern brown bandicoot (*Isoodon obesulus*) papillomas 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 minutes 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. In positive control tissue sections tested with our ISH probes, the positive staining was restricted to the nuclei of keratinocytes and sebocytes. 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.

Of 13 lesions histologically consistent with the papillomatosis and carcinomatosis syndrome tested for BPCV1 DNA using ISH and PCR, 5 results were in agreement: 3 (23.1%) were positive by both methods and 2 (15.4%) were negative by both methods. Conflicting results were obtained in 8 cases: 7 (53.8%) were ISH-positive but PCR negative, while only 1 (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 histologically categorized as papillomas were positive using ISH, compared to 7 of 10 (70%) carcinoma *in situ* and just 5 of 17 (29%) for squamous cell carcinomas.

Many protocols for ISH recorded in the literature are remarkably complex and call for unusual reagents (Jones, 2002; Rolighed and 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 immersing sections in alkaline phosphatase substrate buffer (pH 9 - 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. Furthermore, the viral DNA sequences were found most prominently in the nuclei of basal keratinocytes of the epidermis and external root sheath, followed by 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 and Lowy, 2007; Nicholls *et al.*, 2001; Peh *et al.*, 2002). Interestingly, nuclei of sebaceous cells also stained positively using this ISH technique. 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 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 isolates genome (Bennett *et al.,* in preparation). 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 muco-cutaneous lesions of western barred bandicoot papillomatosis and carcinomatosis syndrome and suggested that ISH was a more sensitive test than PCR for FFPE tissue samples at detecting BPCV1 in papillomatous lesions from *P. bougainville*. ISH demonstrated BPCV1 DNA in 7 of 9 (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 reliably diagnose BPCV1 infection in properly collected and processed pre-neoplastic biopsies from *P. bougainville*.

Acknowledgments

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Figures Captions

Figure 1. *In situ* hybridization performed on papillomas from *P. bougainville*, demonstrating strong positive staining restricted to the nuclei of external root sheath keratinocytes. The dermis and sweat glands are unstained. Brazilin haematoxylin counter-stain; bar = 50 μm. (A) Genomic probe. (B) L1 probe. (C) L2 probe. (D) LTag probe. (E) stag probe.

Figure 2. *In situ* hybridization performed on papillomas from *P. bougainville*, demonstrating positive staining of sebocyte nuclei. Brazilin haematoxylin counter-stain; bar = 20 μm. (A) Genomic probe. (B) L1 probe. (C) L2 probe. (D) LTag probe. (E) stag probe.

Figure 3. Selected controls used to confirm the specificity of the *in situ* hybridization technique. Brazilin haematoxylin counter-stain; bar = 50 μm. (A) DNase-free RNase pretreatment did not affect positive nuclear staining. (B) A digoxigenin-labeled DNA probe designed to demonstrate *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-labeled DNA probes designed to detect BPCV1.

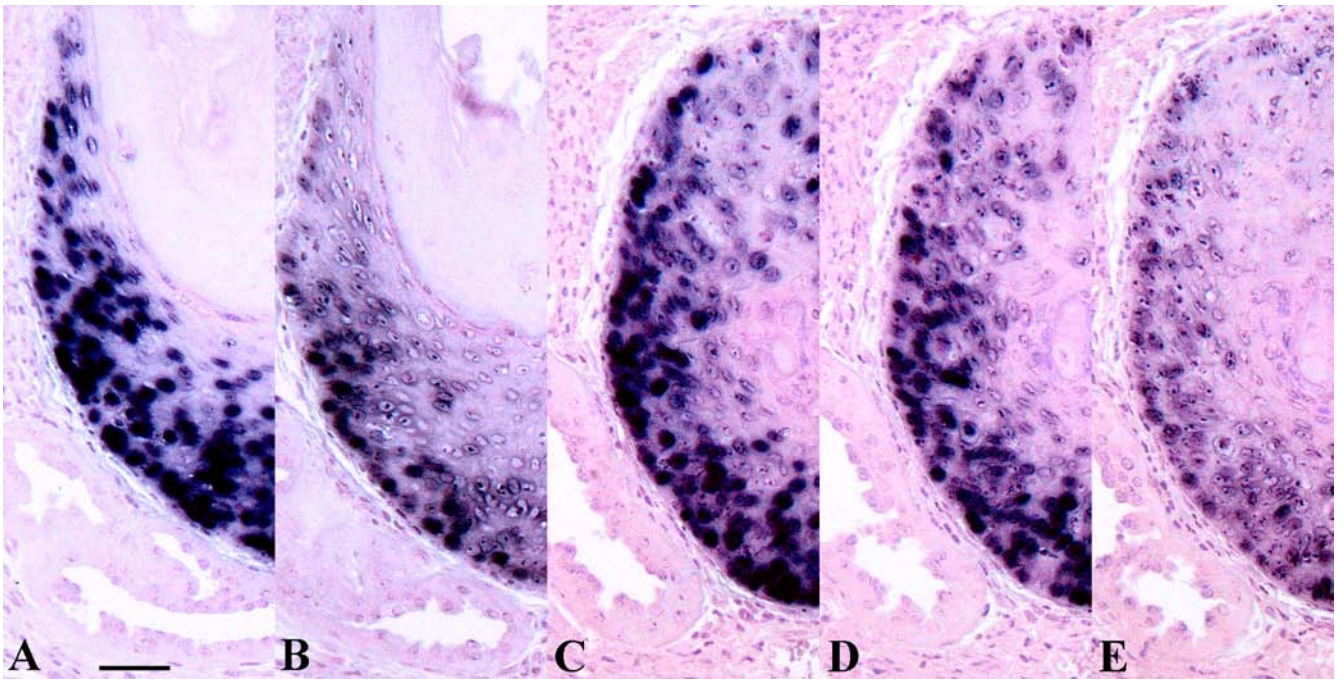

Fig. 1.

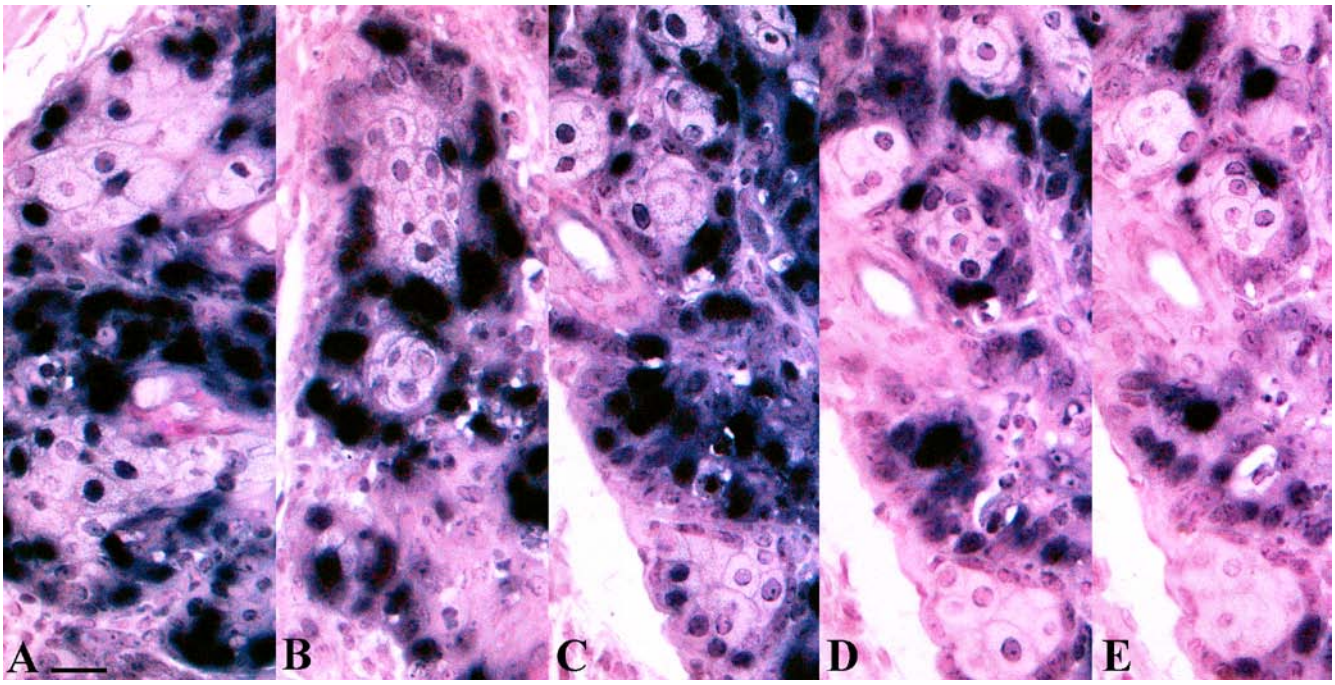

Fig. 2.

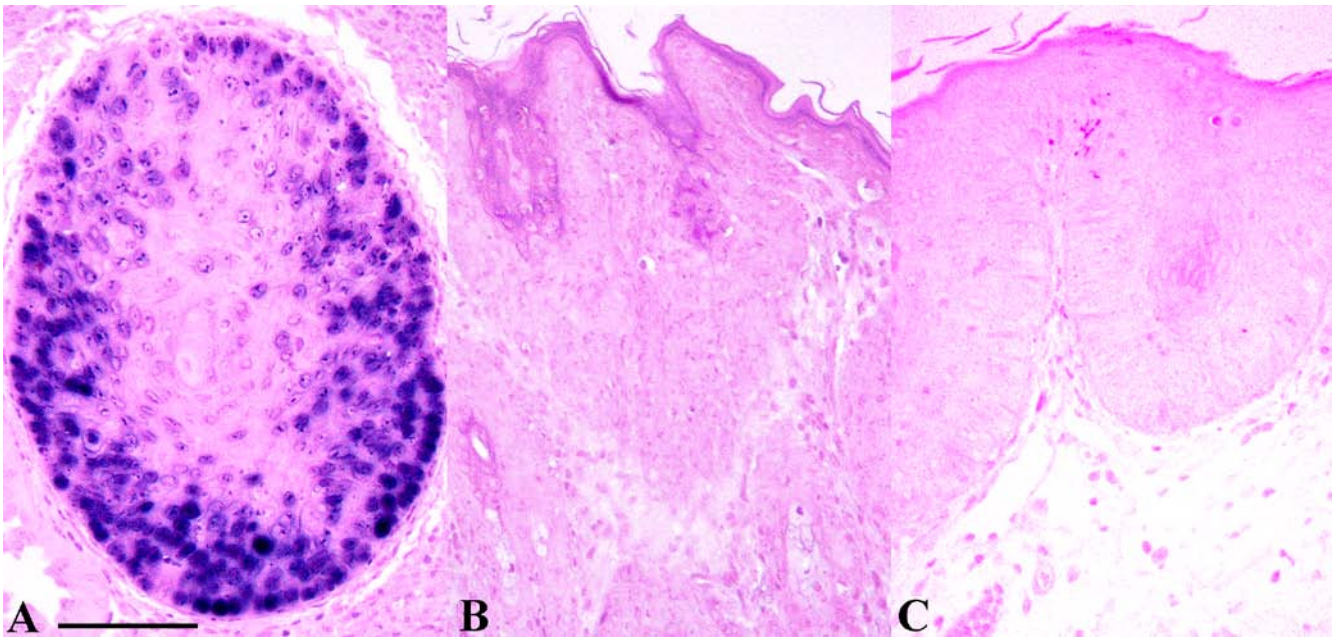

Fig. 3.