Papillomavirus in healthy skin of Australian animals

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Papillomaviruses are a group of ubiquitous viruses that are often found in normal skin of humans, as well as a range of different vertebrates. In this study, swab samples collected from the healthy skin of 225 Australian animals from 54 species were analysed for the presence of papillomavirus DNA with the general skin papillomavirus primer pair FAP59/FAP64. A total of five putative and potential new animal papillomavirus types were identified from three different animal species. The papillomaviruses were detected in one monotreme and two marsupial species: three from koalas, and one each from an Eastern grey kangaroo and an echidna. The papillomavirus prevalence in the three species was 14% (10/72) in koalas, 20% (1/5) in echidnas and 4% (1/23) in Eastern grey kangaroos. Phylogenetic analysis was performed on the putative koala papillomavirus type that could be cloned and it appears in the phylogenic tree as a novel putative papillomavirus genus. The data extend the range of species infected by papillomaviruses to the most primitive mammals: the monotremes and the marsupials.

INTRODUCTION

Papillomaviruses (PVs) are small, epitheliotropic, DNA viruses that cause epithelial proliferations in skin and mucosa. They have been found in a large number of vertebrate species, including man, and are assumed to have evolved alongside their hosts (Bernard, 1994; Sundberg, 1987). Hitherto, 96 human papillomavirus (HPV) types have been fully characterized, and another 100 have been isolated and partially sequenced (de Villiers et al., 2004). Many papillomaviruses have also been described from a number of other vertebrate species, e.g. 6 bovine papillomavirus types isolated from cattle (BPV-1 to -6) (Sundberg, 1987) and 12 putative papillomavirus types have been detected in rhesus monkeys (RhPV-a to -k and RhPV-m) (Chan et al., 1997). Papillomavirus infections are highly host specific and no papillomavirus type has been shown to have both humans and an animal species as its natural hosts (Chan et al., 1997; Shadan & Villarreal, 1993).

Previous reports (Antonsson et al., 2000, 2003a, b) have shown that HPV is commonly present in the healthy skin of healthy humans as a presumed commensal agent. Recently, we have also shown that skin papillomviruses can readily be detected in healthy skin from many different animal species (Antonsson & Hansson, 2002). Furthermore, these animal papillomavirus types were sufficiently genetically related to their human counterparts to be identifiable by a human skin papillomavirus primer set (FAP59 and FAP64) (Forslund et al., 1999). Given that papillomaviruses appear to be so widespread, we wished to investigate the distribution of papillomaviruses in Australian fauna. Here we analysed skin swab samples from 54 different Australian animal species that have never before been reported to harbour papillomavirus in healthy skin.

METHODS

Animals. Specimens were collected from the Currumbin Wildlife Sanctuary, Underwater World, Lone Pine Koala Sanctuary and Australia Zoo, all in Queensland, Australia. Skin swabs from 225 animals were collected from the following 54 species: 72 Northern koalas (Phascolarctos cinereus), 23 Eastern grey kangaroos (Macropus giganteus), 12 red kangaroos (Macropus rufus), 3 red-neck wallabies (Macropus rufogriseus), 3 swamp wallabies (Wallabia bicolor), 5 whip-tail wallabies (Macropus parryi), 1 greater bilby (Macrotis lagotis), 2 red-legged pademelons (Thylogale stigmatica), 1 ring-tail possum (Pseudochirus peregrinus), 4 brush-tail possums (Trichosurus vulpecular), 1 common wombat (Vombatus ursinus), 3 coastal carpet pythons (Morelia spilota mcdowelli), 1 Darwin carpet python (Morelia viridis), 4 shingleback lizards (Tachyglossus aculeatus), 3 emus (Dromaius novaehollandiae), 1 Northern quoll (Dasyurus hallucatus), 1 great glider (Schoinobates volans), 1 black flying fox (Pteropus alecto), 3 grey-headed flying foxes (Pteropus poliocephalus), 3 bearded dragons (Pogona barbata), 4 shingleback lizards (Trachydosaurus rugosus), 2 carpet pythons (Morelia spilota), 2 olive pythons (Liasis olivaceus), 1 water python (Liasis fuscus), 1 Children’s python (Liasis childrei), 3 coastal carpet pythons (Morelia spilota mcdowelli), 1 Darwin carpet python (Morelia viridis), 1 jungle python (Morelia spilota cheynei), 1 scrub python (Morelia amethistina), 1 spotted python (Antaresia maculosa), 3 green tree frogs (Litoria caerulea), 1 Peron’s tree frog (Litoria peronii), 1 splendid tree frog (Litoria splendida), 5 kookaburras (Ducula australis), 2 sulphur-crested cockatoos (Cacatua galerita), 4 tawny frogmouths (Podargus strigoides), 1 pigeon (Phapu chalcoptrera), 1 electus parrot (Electus roratus), 2 purple-crowned lorikeets (Glossopsitta porphyrophthalma), 1 superb fruit dove (Ptilinopus superbus), 1 brown falcon (Falco berigora), 1 crested hawk (Aviceda subcristata), 1 Australian sealion (Neophoca cinerea), 1 salt-water crocodile (Crocodylus porosus), 1 superb fruit dove (Ptilinopus superbus), 1 brown falcon (Falco berigora), 1 crested hawk (Aviceda subcristata), 1 Australian sealion (Neophoca cinerea), 1 salt-water crocodile (Crocodylus porosus),

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is DQ464069.
2 blotched fantail rays (Taeniura meyneni), 6 brown estuary stingrays (Dasyatis fluviorum), 1 blue tang fish (Acanthurus leucosternon), 4 brown-banded bamboo sharks (Chiloscyllium punctatum), 1 epaullette shark (Hemisphyllum freycineti), 3 wobegong sharks (Orectolobus maculatus), 1 broad-shell turtle (Chelodina expansa), 1 green turtle (Chelonia mydas), 1 Keff’s river turtle (Emydura krefftii), 4 loggerhead turtles (Caretta caretta), 5 saw-shell turtles (Elseya latisternum), plus 1 octopus and 6 starfish (unidentified species). Swabs were also collected from seven wart-like lesions from four different koalas. Furthermore, six wart-like lesions from the chest of a 2-year-old male koala were removed under general anaesthesia.

This project was approved by the Animal Ethics Committee, University of Queensland (CICR 158/05 and CICR 297/06).

**Samples.** Swab samples were collected with moist (0-9% NaCl solution) cotton-tipped swabs (Swarstedt), which were drawn back and forth five times over the forehead skin or fur within an area of 4 by 8 cm, and then suspended in 1 ml 0-9% NaCl solution. No DNA extraction was performed prior to PCR. Samples were kept at 4°C for a maximum of 72 h before being analysed.

Three of the wart-like lesions were stored at −20°C (without 0-9% NaCl) until analysed. DNA was then extracted using the DNeasy tissue kit (Qiagen). The other three wart-like lesions were put in formalin and were used for histopathology analysis.

**PCR and HPV type determination.** A PCR test with the primer pair FAP59/FAP64 was used for detection of skin HPV DNA, as previously described (Forslund et al., 1999). The protocol was followed as described except for the MgCl₂ concentration, which was modified to 3-5 mM. HPV 11 (plasmid) was used as a positive control. The PCR products were cloned into the pCR-script SK(+) cloning vector (Stratagene). Between two and four clones per sample were sequenced with both forward and reverse primers (BigDye Terminator v3.1 cycle sequencing kit; Applied Biosystems), and the sequences obtained were compared with available sequences in the GenBank database using the BLAST server (http://www.ncbi.nlm.nih.gov/blast/).

The ability of the FAP59/FAP64 PCR to detect skin papillomavirus from the animal swab samples was compared with that of an animal papillomavirus PCR described by Rector et al. (2005b). Briefly, a 5 µl sample was used in the PCR with the AR-L1FI/AR-L1R3 primer pair, which yielded a PCR product of 600 bp. The COPV plasmid was used as a positive control and H₂O without template as a negative control. The bands yielded from the AR-L1 PCR were cut out from the gel and purified on a spin column (Perfectprep gel cleanup kit; Eppendorf), eluted in 30 µl H₂O, then cloned and sequenced as described above.

A new type-specific primer pair were designed for the new putative koala PV type detected. The new primers were designed using the FAP59/FAP64 primers and the new putative koala papillomavirus type as templates, which gave the following primer sequences: KoPVFAP59 (forward primer) 5’-TATCGTGTTGGGGCATTGGG-3’ and KoPVFAP64 (reverse primer) 5’-GATGCCGACATGTCTGGGTTAGG-3’. The same protocol as described above for FAP59/FAP64 was followed.

Since full-length L1 sequences were not obtained, the new PV isolate detected in this study, which was cloned and submitted to GenBank (accession no. DQ464069), was designated a putative PV type. The guidelines from the Papillomavirus Nomenclature Committee 1995 (14th International Papillomavirus Conference, Quebec City, Quebec, Canada) for defining a new putative HPV type (de Villiers, 2001) were followed. This new putative animal PV type has been designated by an initial letter combination for the species it was found in, i.e. Ko for koala, followed by AA and a unique number (KoAA1), as for previously identified putative animal papillomavirus types (Antonsson & Hansson, 2002). The papillomavirus isolates identified in this paper by direct sequencing were designated potential PV types.

**Phylogenetic analysis.** Phylogenetic analysis was based on multiple alignment with ClustalX (version 1.8) (Jeanmougin et al., 1998), and the alignments were edited with Genedoc (version 2.4.000) (Nicholas et al., 1997). PHYLIP (version 3.5) (Felsenstein, 1982, 1985) was used for neighbour-joining and maximum-likelihood analyses. These programs were obtained from the website http://evolution.genetics.washington.edu/phylip/software.html.

The taxonomic system with papillomavirus genus groups α to σ was used (de Villiers et al., 2004). This system was applied to demonstrate relatedness between the PV type candidates and previously established PV types.

The region of the L1 gene amplified by the primer pair FAP59/FAP64 was used for the phylogenetic analysis, extending from nucleotide 6044 to 6480 relative to the HPV20 sequence. The following papillomaviruses were included in the phylogenetic analysis (GenBank accession nos, in parentheses): HPV 1 (V01116), HPV 4 (X70827), HPV 5 (M17463), HPV 8 (M12737), HPV 9 (X74464), HPV 12 (X74466), HPV 14 (X74467), HPV 15 (X74468), HPV 17 (X74469), HPV 19 (X74470), HPV 20 (U31778), HPV 21 (U31779), HPV 22 (U31780), HPV 23 (U31781), HPV 24 (NC_001683), HPV 25 (X74471), HPV 36 (U31785), HPV 37 (U31786), HPV 38 (U31787), HPV 41 (X56147), HPV 48 (U31789), HPV 49 (X74480), HPV 50 (U31790), HPV 60 (U31792), HPV 63 (X70828), HPV 65 (X70829), HPV 75 (Y15173), HPV 76 (Y15174), HPV 80 (Y15176), HPV 92 (AF353140), HPV 93 (AY382778), HPV 95 (AJ620210) and HPV 96 (AY382779), colobus monkey PV type 2 (CgPV 2; U72630), rhesus monkey PV type 1 (RhpV 1; M60184), chimpanzee PV type 1 (CPV 1; AF020905), pygmy chimpanzee PV type 1 (PCPV 1; X62844), bovine (cattle) PV type 1 (BPV 1; X02346), BPV 2 (M20219), BPV 3 (AF486184), BPV 4 (X05817), BPV 5 (AF457465), BPV 6 (AJ620208), ovine (sheep) PV type 1 (OvPV 1; U85594), OvPV 2 (U85585), European elk PV (EEPV; M15953), deer PV (DVP; M19190), canine (dog) oral PV (COPV; L22695), canine PV type 2 (CPV 2; AJ722648), rabit oral PV (ROPV; AF227420), Felis domesticus (cat) PV (FdPV; AF480454), Equis caballus (horse) PV (EcPV; AF498323), Trichoglossus manatus latreillii (Florida manatee) PV (TmPV; NC_005663), Eretthizon dorsatum (North American porcupine) PV (EdPV; NC_006951), Procyon lotor (raccoon) PV (PjPV; NC_001750), Psittacus erithacus timneh (African grey parrot) PV (PePV; NC_003973), Fringilla coelebs (chaffinch) PV (FcPV; AY057109), hamster oral PV (HOPV; E15110), Reindeer PV (RPV; AF443292), Phocoena spinipinnis (Burmeister’s porpoise) PV (PpPV; AJ238373) and Mastomys natalensis (South African mouse) PV (MnPV; U01834). Also included in the analysis was the putative new koala PV type KoAA1 (DQ464069). The DNA sequences from previously characterized PV types were obtained from GenBank (http://www.ncbi.nlm.nih.gov/).

The potential PV types described in this paper were not included in the phylogenetic analysis as they were only sequenced once with either FAP59 or FAP64.

**RESULTS**

**Papillomavirus findings**

Altogether swab samples from 225 Australian animals, belonging to 54 different species, plus 3 wart-like biopsies from a koala, were analysed and the PV type determined. We report here that a total of five putative and potential new
animal PV types were identified from three different animal species (Table 1) based on DNA sequencing of FAP PCR amplicons.

Papillomavirus was isolated from 10 out of 72 (14%) samples from koalas (Table 2), with 3 putative and potential new koala PV types identified, each from individual koalas. However, only one of the three koala papillomavirus types, KoAA1, could be cloned for sequencing. BLAST analysis of the 440 bp cloned fragment from the FAP PCR revealed that KoAA1 showed closest similarity to HPV 1a (71%) when compared with all sequences in GenBank. The other two potential new types were identified by direct sequencing and both showed closest similarity to KoAA1. Once the sequence of the KoAA1 type was obtained we designed more specific primers for this type and using this KoAA1-specific primer pair we were able to identify KoAA1 from the same koalas as virus has been previously detected with the FAP59/FAP64 primers. This is the first description of papillomavirus sequences in koala species.

One out of twenty-three (4%) Eastern grey kangaroos were PV positive with the FAP59/FAP64 primer pair and one potential papillomavirus type was detected by direct sequencing from the FAP59/FAP64 PCR product. BLAST analysis revealed the kangaroo PV sequence showed closest similarity to HPV-1a. Five echidnas were sampled and one of these animals was found to be PV positive (20%). One potential new PV type was identified by direct sequencing and BLAST analysis showed its closest similarity was to HPV-41. This is believed to be the first description of PV in kangaroo and echidna.

PCR with the primer pair FAP59/FAP64 did not identify any PV DNA in the samples from the following animal species: red kangaroo, wallaby, possum, wombat, dingo, emu, quoll, great glider, black flying fox, grey-headed flying fox, saltwater crocodile, bearded dragon, carpet python, olive python, coastal carpet python, Darwin carpet python, scrub python, spotted python, Children’s python, water python, frog, kookaburra, cockatoo, froghmouth, pigeon, purple-crowned lorikeet, eclectus parrot, superb fruit dove, brown falcon, crested hawk, sealion, ray, blue tang fish, shark, turtle, octopus or starfish.

An alternative animal papillomavirus PCR pair, AR-L1F1/AR-L1R3, as described by Rector et al. (2005b), was tested but no papillomavirus DNA was detected in any of the 225 swab samples despite our positive control (COPV plasmid) yielding an amplicon of the correct size.

**Wart-like lesions from a koala**

During sampling a number of wart-like lesions were noted on the chest of a 2-year-old male koala and three of these were removed for analysis. One of the three lesions tested positive for PV DNA and was identified as KoAA1. After histopathology analysis, the lesion was confirmed to be a sebaceous gland hyperplasia rather than a wart. These scent/sebaceous glands are present on the chest and are used for marking territory.

**Phylogenetic analysis**

Phylogenetic trees obtained by the neighbour-joining, maximum-parsimony and maximum-likelihood algorithms were compared. The tree in Fig. 1 is divided into the genera $\alpha$ to $\sigma$ and shows 34 fully characterized human skin PV types, 28 fully sequenced animal PV types, and the putative new potential KoAA2 and KoAA3 (pKoAA2 and pKoAA3). This sample was a biopsy of a sebaceous gland hyperplasia.

Table 1. Species in which new putative and potential papillomavirus types were detected with the general primer pair FAP59/FAP64

<table>
<thead>
<tr>
<th>Animal species</th>
<th>No. tested</th>
<th>No. PV positive</th>
<th>No. of putative/potential PV types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koala</td>
<td>72</td>
<td>10*</td>
<td>3†</td>
</tr>
<tr>
<td>Eastern grey kangaroo</td>
<td>23</td>
<td>1</td>
<td>1‡</td>
</tr>
<tr>
<td>Echidna</td>
<td>5</td>
<td>1</td>
<td>1‡</td>
</tr>
</tbody>
</table>

*Nine swab samples from normal skin plus one specimen from a biopsy of a sebaceous gland hyperplasia.

†One of the sequences was cloned and sequenced, while the other two were obtained by direct sequencing.

‡Sequence for the putative new PV type was obtained by direct sequencing of the FAP PCR product.

Table 2. Putative and potential PV types found with the two different primer pairs FAP59/FAP64 and KoPV FAP59/ FAP64 in koalas

<table>
<thead>
<tr>
<th>Koala</th>
<th>PV types detected*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4</td>
<td>KoAA1</td>
</tr>
<tr>
<td>A14</td>
<td>pKoAA2</td>
</tr>
<tr>
<td>A16</td>
<td>KoAA1</td>
</tr>
<tr>
<td>A18</td>
<td>KoAA1</td>
</tr>
<tr>
<td>A39†</td>
<td>KoAA1</td>
</tr>
<tr>
<td>L1</td>
<td>pKoAA3</td>
</tr>
<tr>
<td>L2</td>
<td>KoAA1</td>
</tr>
<tr>
<td>L12</td>
<td>pKoAA3</td>
</tr>
<tr>
<td>L16</td>
<td>KoAA1</td>
</tr>
<tr>
<td>L19</td>
<td>KoAA1</td>
</tr>
</tbody>
</table>

*The two sequences obtained by direct sequencing are named potential KoAA2 and KoAA3 (pKoAA2 and pKoAA3).

†This sample was a biopsy of a sebaceous gland hyperplasia.
Fig. 1. Phylogenetic analysis of the fully characterized human skin papillomavirus types and the previously described animal papillomaviruses (both indicated in bold), together with the putative koala papillomavirus type (KoAA1) found in this study. The tree is divided into the genera of α to π, together with one new tentative group comprising KoAA1. The analysis was based on neighbour-joining evaluation of a segment of the L1 gene. Thirty-four fully characterized cutaneous HPV types were included in the analysis together with twenty-eight animal papillomavirus (PV) types. OvPV, ovine (sheep) PV; ROPV, rabbit oral PV; MnPV, Mastomys natalensis (South African mouse) PV; COPV, canine (dog) oral PV; PCPV-1, pygmy chimpanzee PV type 1; CgPV-2, Colobus monkey PV type 2; DPV, deer PV; FdPV, Felis domesticus (cat) PV; EcPV, Equus caballus (horse) PV; TmPV, Trichechus manatus latirostris (Florida manatee) PV; EdPV, Erethizon dorsatum (North American porcupine) PV; PIPV, Procyon lotor (raccoon) PV; PePV, Psittacus erithacus timneh (African grey parrot) PV; FcPV, Fringilla coelebs (chaffinch) PV; CPV-2, canine PV type 2; HOPV, hamster oral PV; RPV, reindeer PV; PsPV, Phocoena spinipinnis (Burmeister’s porpoise) PV and BPV, Bovine (cattle) PV.
koala PV type (KoAA1) described in the present study. The phylogenetic analysis places KoAA1 in its own branch in the phylogenetic tree. Furthermore, a phylogenetic analysis based on amino acid alignment was performed and the resulting tree was very much the same as that based on nucleic acid alignment.

**DISCUSSION**

Epidemiology of papillomaviruses in normal skin of Australian animals as far as we know has never been studied before. In this study we found an overall prevalence of 5% (12/225), compared to 36% (40/111) in our previous animal study (Antonsson & Hansson, 2002). In our previous study we analysed samples from several monkey species that are closely related to humans (the primer pair used was designed for human papillomaviruses), while in this study we have been examining several distantly related species, such as sharks, turtles and starfish, which could explain the lower prevalence. Alternatively, the long-time isolation of the Australian continent from the rest of the world could also mean that the papillomaviruses of these species have been more isolated. Such isolation would suggest any potential Australian PVs are more distantly related to other animal papillomaviruses and therefore our PCR primer pair may be under-reporting PV prevalence.

Since Australia separated from the other continents about 95 million years, its wildlife has been able to evolve independently. Many of the animals of Australia are distinctly primitive, such as the monotremes that lay eggs and suckle their young, and the marsupials. We found putative and potential papillomavirus types in both a monotreme species (echidna) and marsupials (koala and Eastern grey kangaroo). Only one of the four papillomavirus types could be cloned and sequenced, and hence included in a phylogenetic analysis. The other three sequences were obtained by direct sequencing and were not analysed phylogenetically. Interestingly, the new putative koala PV type, KoAA1, forms a new branch or even a putative new genus, close to the root in the phylogenetic tree. Only one putative papillomavirus type has been described in marsupials before and it was isolated from a skin lesion on the tail of a brush-tail possum (Perrott et al., 2000). However, this PV type was detected with the MY09/MY11 primer pair, which does not overlap the PCR product of the FAP59/FAP64 primers (used here), meaning we could not include it in our phylogenetic analysis.

The alternative PCR did not detect any papillomavirus in our specimens. However, this primer pair was designed to detect PV DNA in dermal proliferations of racoons with high copy numbers of PV virions (Rector et al., 2005b) so it is not surprising that PV could not be detected in our swab samples with presumably low number of virus particles.

This study shows that even the healthy skin of primitive mammals like monotremes and marsupials harbour sub-clinical PV infections, and further shows the ubiquity of skin PVs. Our findings suggest that the phylogenetic tree of the family *Papillomaviridae* is far from complete and this contention is supported by the recent characterization of a large number of new animal PVs that make up new genera of the growing phylogenetic tree (Ghim et al., 2004; Rector et al., 2004, 2005a, b; Tachezy et al., 2002a, b; Van Doorslaer et al., 2006).

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