**Bos grunniens** papillomavirus type 1: a novel deltapapillomavirus associated with fibropapilloma in yak

Wei Zhu,1,2 Jian-Bao Dong,1,2,3 Jun Zhang,4 Kazuyuki Uchida,5 Ken-ichi Watanabe,5 Yoshitaka Goto1 and Takeshi Haga6

1Department of Veterinary Microbiology, University of Miyazaki, Miyazaki, Japan
2Japan Society for the Promotion of Science, Tokyo, Japan
3The United Graduate School of Veterinary Science, Yamaguchi University, Yamaguchi, Japan
4Academy of Animal Science and Veterinary Medicine, Qinghai University, Xining, PR China
5Department of Veterinary Pathology, University of Tokyo, Tokyo, Japan
6Division of Infection Control and Disease Prevention, Department of Veterinary Medical Science, University of Tokyo, Tokyo, Japan

Papillomaviruses (PVs) have been widely identified among vertebrates, but have not yet been reported to infect yaks. We report, for the first time, a novel deltapapillomavirus that was associated with fibropapilloma in yak herds on the Qinghai–Tibetan Plateau. Six skin papilloma samples were collected and examined using histopathology, immunohistochemistry and PCR assays. The samples were identified as fibropapilloma and were found to contain PV antigens. Sequencing of diagnostic PCR products and the full-length genome revealed that all samples were infected with the same PV type. The whole virus genome was 7946 bp in length and possessed the common PV genomic organization. The virus was identified as a novel PV type and designated **Bos grunniens** papillomavirus type 1 (BgPV-1) based on the nucleotide sequence alignment of the L1 ORF. It is classified in the Delta-4 species of the genus **Deltapapillomavirus** based on phylogenetic analysis of the L1 ORF. Identification of this novel PV type provides further information about the pathology, development of diagnostic methods and evolutionary studies of the family **Papillomaviridae**.

**INTRODUCTION**

The papillomaviruses (PVs) are a heterogeneous group of non-enveloped DNA viruses associated with a wide spectrum of proliferative epithelial and fibroepithelial lesions, including benign skin and mucosal papillomas as well as cervical, oral and other epithelial cancers (Antonsson & Hansson, 2002; Parrish, 2011; zur Hausen, 2002). PVs are widespread in nature and have been detected not only in most mammals (de Villiers et al., 2004) but also in annamotes such as birds, snakes and turtles (Drury et al., 1998; Herbst et al., 2009; Lange et al., 2011). Over 100 different types have been identified in humans (Bernard et al., 2010). PVs are classified in the family **Papillomaviridae** and 30 genera have been designated so far according to nucleotide sequence diversity in the L1 ORF and their biological and pathological properties (Bernard et al., 2010; de Villiers et al., 2004). Since almost all PVs are highly specific to their natural hosts and cannot infect even closely related species (Bernard et al., 2010; Chow et al., 2010), they have often been designated according to their host species.

There are at least 12 PV types that infect members of the genus **Bos**, officially named **Bos taurus** papillomavirus 1 to 12 (BPV-1 to -12) and commonly called bovine papillomaviruses (Hatama et al., 2008, 2011; Parrish, 2011; Zhu et al., 2012). To date, all reported BPVs have been detected in **Bos taurus**, with no reports in other **Bos** species. Fifteen novel putative BPV types (BAA2 to 4, BAPV3 to 5, BAPV7 to 10, BAPV11MY and BPV/BR-UEL2 to 5) have been detected by PCR of healthy skin swabs or cutaneous warts from cattle kept in Sweden, Japan and Brazil (Antonsson & Hansson, 2002; Claus et al., 2008; Ogawa et al., 2004).

The yak, **Bos grunniens**, is herbivorous and predominantly inhabits the Qinghai–Tibetan Plateau, known colloquially as the ‘roof of the world’. Yaks are regarded as one of the world’s most remarkable domestic animals, as they thrive in extremely harsh, deprived conditions while providing a

[Supplementary material is available with the online version of this paper. The GenBank/EMBL/DDBJ accession numbers for the sequences of the whole BgPV-1 genome are JX174437–JX174442.]

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livelihood for local people (Gerald et al., 2003). Although PVs have been widely reported in vertebrates, they have not yet been reported in yaks. We describe a novel deltapapillomavirus that was associated with fibropapilloma in yaks. The virus was designated *Bos grunniens* papillomavirus type 1 (BgPV-1) according to the criteria of the Papillomavirus Study Group for the International Committee on Taxonomy of Viruses (ICTV).

**RESULTS**

**PCR diagnostic assay**

DNA was extracted from papilloma samples collected from infected domestic yaks. PV DNA was detected by PCR using primer pairs FAP59/FAP64 (Forslund et al., 1999) and MY09/MY11 (Manos et al., 1989). All six samples were found to be positive with FAP59/FAP64 but negative with MY09/MY11. FAP59/FAP64 PCR products were cloned and sequenced and confirmed to be 431 bp. Sequence alignment of all six samples revealed 100% nucleotide sequence identity. Sequence similarity analysis with the **BLAST** tool of the National Center for Biotechnology Information (NCBI) showed that subgenomic fragments of the putative PVs were related to BPVs.

**Histopathology and immunohistochemistry**

Histopathology and immunohistochemistry were performed on six samples, Qh-1 to -6 (one or two samples from each farm), to identify the papilloma type and detect PV antigens. Morphological lesions of neoplastic tissues from six cases were almost identical, and the neoplastic masses consisted of a mixed proliferation of epithelial and mesenchymal components (Fig. 1a, b). The epidermis showed moderate to severe irregular papillary proliferation of the prickle cell layer (Fig. 1b) accompanied by vacuolar changes and orthokeratotic hyperkeratosis. The proliferating epithelium did not invade into the dermis. In five cases, pale intranuclear inclusion bodies were occasionally observed in the prickle cells. PV antigens were detected by immunohistochemistry and were present as intranuclear inclusions (Fig. 1d). In the mesenchymal tissues, there was diffuse irregular proliferation of fibroblasts with moderate

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*Fig. 1. Histopathological features and immunohistochemical staining of fibropapillomas excised from yaks. (a) Haematoxylin & eosin stain. (b) Masson’s trichrome. (c) Cytokeratin immunostaining. (d) PV immunostaining. Bars, 2 mm (a–c) and 100 μm (d). The neoplastic mass consists of a mixed proliferation of papillary epidermis (a, c) and fibrous connective tissues (b). In the prickle cell layer, there are intranuclear inclusion bodies (arrows) that are immunopositive for PV antigen (d).*
collagen production. There was little cellular atypia or polymorphism in the proliferating cells, including prickle cells and fibroblasts. Based on these histopathological characteristics and the presence of PV antigens, the neoplastic masses examined were diagnosed as fibropapillomas associated with papilloma virus infections.

**Whole-genome organization and sequence similarity**

Full-length genome sequences were amplified and sequenced from samples Qh-1 to -6. Each of the samples contained a unique complete PV genome sequence, which was confirmed by rolling-circle amplification (RCA) and long PCR. Full-length genomes of all six strains were 7946 bp, with a G+C content of 44.4 mol%. Sequence alignment revealed 99.9–100% nucleotide sequence identity among them, and there was no specific variation that caused significant differences in ORFs, motifs, etc. They possessed a typical PV genome organization, consisting of the long control region (LCR) and early and late regions.

Because the genome is circular, the first nucleotide of the E6 ORF was assigned position 1. One strain (Qh-3) was selected as a representative for genomic analysis in this study, because of the extremely high sequence similarity between the six strains. The LCR was 924 bp, located at positions 7023–7946 (Fig. S1, available in JGV Online), and has only one non-coding region. The early region contains ORFs for E6 (414 bp), E7 (381 bp), E1 (1821 bp), E2 (1239 bp), E4 (342 bp) and E5 (135 bp), in that order. The late region consists of two ORFs, L2 (1404 bp) and L1 (1494 bp), which respectively encode the minor and major capsid proteins. These cooperate to package the viral DNA into the virion.

Whole-genome sequence alignments revealed that the closest related PVs were BPV-1 (82.3% similarity to Qh-3) and BPV-2 (82.1%). When each ORF was compared with other PVs, the nucleotide identities were ≤83.8% (E6), ≤87.9% (E7), ≤88.0% (E1), ≤91.7% (E2), ≤90.1% (E4), ≤91.1% (E5), ≤73.6% (L2) and ≤80.8% (L1). In 1995, the definition of novel PV types was released from the International...
Papillomavirus Workshop held in Quebec. A PV strain can be recognized as a novel type if the complete genome has been cloned and the DNA sequence of the L1 ORF shares less than 90% similarity with the closest known PV type (Bernard et al., 2010; de Villiers et al., 2004). Based on this criterion, the PV strain identified in this study should be designated a novel PV type. Because it was isolated from Bos grumniens, it was designated BgPV-1.

The L1 ORF is the most conserved region in PVs and, according to the current genus classification system, most types within a PV genus share more than 60% nucleotide identity in this region (Bernard et al., 2010; de Villiers et al., 2004). Sequence alignments revealed that the L1 ORF of BgPV-1 shares 62.0–81.3% nucleotide identity with other members of genus Deltapapillomavirus and 62.4–64.0% identity with members of the genus Epsilonpapillomavirus (Table S1). Therefore, the genus for BgPV-1 cannot be defined based solely on the nucleotide identity of the L1 ORF; instead, a phylogenetic tree was reconstructed with optimized alignments based on the nucleotide sequence of the L1 ORF.

**Phylogenetic analysis of BgPV-1**

The L1 ORF has been used to identify new PV types and new genera have been defined by phylogenetic analysis over the past 20 years (Bernard et al., 2010; de Villiers et al., 2004). In the present study, we identified a new PV type, BgPV-1, based on the fact that the L1 ORF shared less than 90% identity with other PVs. The phylogenetic relationship of BgPV-1 was established based on multiple alignments of the L1 ORF with representative types of each PV genus, and the virus was clearly classified into the genus Deltapapillomavirus (Fig. 2). There are six Deltapapillomavirus species, according to previous reports: Delta-1 (labelled δ1 in Fig. 2; AaPV-1 and RtPV-1), Delta-2 (OvPV1), Delta-3 (OaPV1 and OaPV2), Delta-4 (BPV-1 and BPV-2), Delta-5 (CcaPV-1) and Delta-6 (CdPV-1 and CdPV-2) (Bernard et al., 2010; Ure et al., 2011). BgPV-1 was grouped into the Delta-4 branch, which also contains BPV-1 and BPV-2 (Fig. 2).

**DISCUSSION**

The yak predominantly inhabits the Qinghai–Tibetan Plateau, providing a livelihood for the local people. Almost everything from the yak is either used directly or sold to provide an income to sustain the lives of the herdsmen and their families. The yak hide and pelt in particular have great importance in the local economy (Gerald et al., 2003). The occurrence of papillomatosis...
destroys or reduces the quality of the hide and pelt, causing economic losses. Although papillomatosis has been causing problems in yaks, there has so far not been any genomic information relating to yak PVs. Here, we report for the first time the characterization of a novel deltapapillomavirus, BgPV-1, that is associated with fibropapilloma in yaks.

In the present study, six skin papillomas were detected with the PCR assay, and whole genomes from each of them were sequenced. Based on the high nucleotide sequence identities, it is suggested that the infection was caused by the same PV strain. We assume that BgPV-1 is the major cause of skin fibropapillomatosis in yaks on the Qinghai–Tibetan Plateau because the samples were collected from different areas more than 50 km apart and the infected yaks were born and grew up on their own farms without interchanged with other farms. In addition, almost all PVs are reported to be highly specific to their natural hosts and cannot infect even closely related species (Bernard et al., 2010; Chow et al., 2010).

To date, ten PV types belonging to the genus Deltapapillomavirus have been identified: AaPV-1, BPV-1, BPV-2, CcaPV-1, CdPV-1, CdPV-2, OaPV-1, OaPV-2, OvPV-1 and RvPV-1. All of them were reported to be associated with fibropapilloma in animals, including elk, cattle, deer, camel and sheep (Ahola et al., 1986; Chen et al., 1982; Erdélyi et al., 2008; Groff & Lancaster, 1985; Moreno-Lopez et al., 1987; Ure et al., 2011). In this study, we report a novel deltapapillomavirus, BgPV-1, that was also associated with fibropapilloma. This result provides further information for the characterization of the genus Deltapapillomavirus.

BgPV-1 possesses a typical PV genome organization, consisting of three regions known as the LCR, early and late regions. Some PVs, such as BPV-4, possess two non-coding regions: the first is located upstream of the early region and the second is between the L2 and L1 ORFs (Patel et al., 1987). In the BgPV-1 genome, the LCR forms only one region, located between ORFs L1 and E6 (Fig. S1). The early region of PVs encodes non-structural viral proteins involved in viral DNA replication, transcription, and cell transformation. Depending on the individual virus, the early region can encode up to eight proteins (E1–E8) (Howley & Lowy, 2007). BgPV-1 has ORFs for E6, E7, E1, E2, E4 and E5, but an E8 was not predicted, which is similar to other deltapapillomaviruses. Most regulatory element motifs for virus replication and transcription were found in BgPV-1 when compared with BPVs and other PVs. The pRbBD in E7 was reported to work with ZnBD to perform functions in viral replication and transcription. Therefore, the result of this study contribute another piece of evidence that supports the above conclusion.

METHODS

Sample collection and diagnosis. Six skin papilloma samples were collected from six infected domestic yaks (Fig. 3) at three farms (two samples from each farm) that were more than 50 km apart. Whole papilloma biopsies from infected yaks were excised surgically using a local anaesthetic. Half of each sample was fixed with formalin for histopathology and immunohistochemistry, and the other half was stored at −20 °C for DNA extraction and genomic analysis. DNA was extracted with a Qiagen blood and tissue kit. Diagnostic PCR assays were performed with primer pairs FAP59/FAP64 (Forlund et al., 1999) and MY09/11 (Manos et al., 1989). PCR products were cloned into the pMD20-T vector (Takara) and sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Sequences were aligned with Seqman DNASTAR software (Lasergene). Similarity analysis was performed using the BLAST tool (http://blast.ncbi.nlm.nih.gov).

Histopathology and immunohistochemistry. Tissue samples were fixed with 10% formalin and embedded in paraffin using a routine procedure. All sections were stained with haematoxylin and eosin and Masson’s trichrome. Immunohistochemistry for detection of PV antigens and identification of the epithelial and mesenchymal components of neoplastic tissues was performed by the Envision polymer method (Dako-Japan) using standard reagents. Rabbit antisera against BPV (Quartett) and mouse mAbs against cytokeratin (AE1/AE3; Dako-Japan) and vimentin (V9; Dako-Japan) were used as primary antibodies and visualized using 3,3′-diaminobenzidine. As a BPV positive control, a papilloma sample from cattle was used. For the negative control, immunostaining was conducted without primary antigen. Mayer’s haematoxylin was used as a counterstain.

Whole-genome cloning and sequencing. An RCA protocol was used to enrich circular PV DNA with the TemplPhi 100 Amplification kit (Amersham Biosciences) according to the manufacturer’s instructions. Primer-walking sequencing was carried out using the RCA products as template. Sequence information obtained from the 431 bp FAP59/FAP64 fragment was used to design a pair of specific primers, BgPV1-1F (5′-CACAAACACAGATGCACGGGAA-3′) and BgPV1-1R (5′-CTGTCCCCGAGATACCTGAATA-3′). These primers were designed to amplify the remaining part of the genome and PCR was performed with the LA Taq kit (Takara) according to the manufacturer’s protocol. Subsequently, PCR products were gel-purified using a QIAquick gel extraction kit (Qiagen). Purified products were sequenced using the primer-walking method. In order to confirm the correct sequence, the whole genome was sequenced at least three times as outlined above. To reconstitute the full-length genome sequence, overlapping sequences were assembled into contiguous sequences using Seqman DNASTAR software (Lasergene).

Genome organization and phylogenetic analysis. For genome organization analysis, putative ORFs and their corresponding amino acids were predicted using the ORF finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), and similarity analysis was performed using BLAST. Peptide motif analysis was performed with the TSEARCH tool of the Parallel Protein Information Analysis system (http://www.cbrc.}

http://vir.sgmjournals.org
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, 2437–2443.


