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A novel bovine papillomavirus type in the genus *Dyokappapapillomavirus*

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Abstract Papillomaviruses are a diverse group of viruses that are known to infect a wide range of animal species. Bovine papillomaviruses (BPVs) are divided into at least 21 genotypes (BPV1 to BPV21), with most BPV isolates/strains described to date belonging to one of four genera, including *Deltapapillomavirus*, *Xipapillomavirus*, *Epsilonpapillomavirus* and *Dyoxipapillomavirus*. Here, we describe the identification and genetic characterization of a new BPV type in the genus *Dyokappapapillomavirus*. A farm in the state of New York, USA, reported chronic cases of vulvovaginitis in Holstein cows in 2016. Biopsies and/or swab samples collected from the vaginal mucosa were subjected to diagnostic investigation. Conventional diagnostic assays yielded negative results, and vaginal swab samples were subjected to viral metagenomic sequencing. Notably, BLAST searches revealed a papillomavirus genome with 7480 bp in length (67% nt sequence identity to BPV16). Additionally, phylogenetic analysis of the L1 gene of the papillomavirus identified here (tentatively named BPV22) revealed that it clusters with members of the genus *Dyokappapapillomavirus*. Interestingly, the recently identified BPV16, which was detected in fibropapilloma lesions in cattle also clusters within the *Dyokappapapillomavirus* group. Each virus, however, forms a separate branch in the phylogenetic tree. These results indicate that the putative BPV22 represents the second BPV within the genus *Dyokappapapillomavirus*.

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Introduction

The family *Papillomaviridae* comprise a diverse group of viruses that have been detected in a wide range of animal species [1]. Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses containing a genome that is approximately 8 kb in length [2]. Although PVs have frequently been associated with proliferative lesions or cancers affecting the stratified squamous epithelium of the skin and/or mucous membranes in vertebrates, there are several reports demonstrating the detection of PVs in healthy skin and/or mucous membranes [1–4].

The bovine papillomaviruses (BPVs) are divided into at least 21 genotypes (BPV1 to BPV21) and have been characterized based on analysis of the L1 gene and other biological properties [2, 5]. Most BPV isolates/strains described to date belong to one of four genera, including *Deltapapillomavirus*, *Xipapillomavirus*, *Epsilonpapillomavirus*, and *Dyoxipapillomavirus* [1, 2, 5]. Recently BPV16 was classified as a member of the genus *Dyokappapapillomavirus*, and two new genera containing three BPV types were proposed [5]. BPV16 was detected in fibropapilloma lesions in cattle in Brazil [5]. Other dyokappa PVs known to infect ruminants are ovine (*Ovis aries*) papillomavirus 3 (OaPV3) and alpine chamois (*Rupicapra r. rupicapra*) papillomavirus 1 (RrPV1) [6, 7]. OaPV3 was first identified in Sardinia, Italy, being initially associated with squamous cell carcinoma in sheep, and it was later found in healthy skin of sheep from two different flocks, in Italy [6]. RrPV1 was found in the northwestern

region of the Italian peninsula, in a nasal neoplasia in a free-range alpine chamois [7]. Here, we describe the identification and genetic characterization of a new BPV type within the genus *Dyokappapapillomavirus*.

Materials and methods

A farm in the state of New York, USA, reported chronic cases of vulvovaginitis in Holstein cows in 2016. Biopsies and/or swab samples collected from the vaginal mucosa were subjected to virological, bacteriological and histopathological examination. Additionally, vaginal swabs from four animals were subjected to viral metagenomic sequencing. For this, samples were subjected to treatment with DNAse (10 U; Turbo DNAse, Ambion, Thermo Fisher Scientific, Waltham, MA) and 5'-phosphate-dependent exonuclease (Terminator^{TM} 5'-Phosphate-Dependent Exonuclease, Epicentre, Illumina, San Diego, CA) for 30 min at 37 °C, and viral nucleic acid was purified using a GeneJET Viral DNA and RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA). DNA libraries were prepared using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA), and sequencing was performed using a MiSeq Reagent Nano Kit v2 and the Illumina MiSeq sequencing platform (Illumina, San Diego, CA). Pair read sequences were assembled using Ray v2.3.1 [8], and the resultant contigs were subjected to Blastn search.

The classification of a papillomavirus genome identified in the sequence dataset was assessed using the Pavic L1 Taxonomy Tool (https://pave.niaid.nih.gov/#analyze/l1_taxonomy_tool). ClustalW was used for the alignment of select L1 nucleotide sequences, including sequences of BPV representing the four known BPV genera and all other members of the genus *Dyokappapapillomavirus*. Phylogenetic analysis was performed using the neighbor-joining method in MEGA 6 [9]. To assess whether the identified papillomavirus was present in other animals from the same farm, eight additional samples not subjected to metagenomics sequencing were tested by PCR. For this, a set of primers targeting the L1 gene was designed using the PrimerQuest tool (http://www.idtdna.com/Primerquest/Home/Index). The primers BPV-F (5'-ATAGAGGCG GTAATGTCAAAGAA-3') and BPV-R (5'-TCACAC TGTCAGGGCATTTAG-3') were used for PCR screening. Samples were tested by PCR using the 2X Q5 High-Fidelity PCR Kit (New England Biolabs) following the manufacturer's recommendations. Briefly, initial denaturation was performed at 98 °C for 1 min followed by 35 cycles at 98 °C for 30 s, 52 °C for 20 s, and 72 °C for 30 s, with a final elongation step at 72 °C for 2 min. PCR amplicons were analyzed in 1% agarose gel stained with GelRed (Biotium, Inc., Fremont, CA) and visualized under UV light. PCR amplicons were purified from the agarose gel using the GeneJET Gel Extraction Kit (Thermo Fisher Scientific, Waltham, MA) and subjected to DNA sequencing using the ABI 3500Xl Genetic Analyzer (ABI, Foster City, CA).

Results and discussion

In the present study, we describe the identification of a novel bovine papillomavirus tentatively named BPV22. Following a complete diagnostic investigation of a chronic case of vulvovaginitis in Holstein cows, no viral or bacterial agents were isolated from affected animals using conventional virus isolation or aerobic bacterial culture techniques. The histological changes observed in biopsy sections consisted of lymphoplasmacytic inflammatory infiltrate with variable epithelial involvement. These changes were nonspecific but suggestive of chronic antigen stimulation. Some degree of epithelial degeneration was also observed. Given the inconclusive results of the conventional diagnostic assays, vaginal swab samples were subjected to viral metagenomic sequencing. Notably, while most sequences present in the dataset mapped to bacterial genomes, a 7480-bp papillomavirus genome was identified in one sample (GenBank accession no. KY705374). Approximately 880 reads mapped to the papillomavirus genome, resulting in a uniform 13X coverage of the genome.

The complete genome sequence of the newly identified papillomavirus was analyzed. Blastn analysis showed that the BPV22 complete genome shares 67% nt identity with BPV16 (KU519391; coverage of 65%) and 66% nt identity with OaPV3 (FJ796965; coverage of 50%). These results indicate that BPV22 represents a new BPV type within the genus *Dyokappapapillomavirus,* which includes three other ruminant PVs [5–7]. Similar results were obtained when the amino acid sequences of E1-E2-L2-L1 were subjected to phylogenetic analysis (data not shown). Interestingly, BPV16, which was detected in fibropapilloma lesions in cattle, also clusters within the Dyokappa group [5]. Each virus, however, forms a separate branch in the phylogenetic three (Fig. 1).

The 7480-nt genome of BPV22 contains six open reading frames (ORFs) including E6, E7, E1, E2, L2, L1 and a long control region (LCR) with 484 nt located between the stop codon of L1 and the start codon of E6 (Fig. 2). Analysis of the BPV22 genes did not reveal the existence of an E1-E4 splicing event, thus the E4 gene was not annotated in BPV22. Notably, the E5 ORF is also absent in the BPV22 genome. Similar to other PVs, the BPV22 genome contains nine polyadenylation sites (AATAAA) at positions 3, 1165, 3008, 4506, 5208, 6212, 6368, 6626, and 6899. All positions are annotated based on

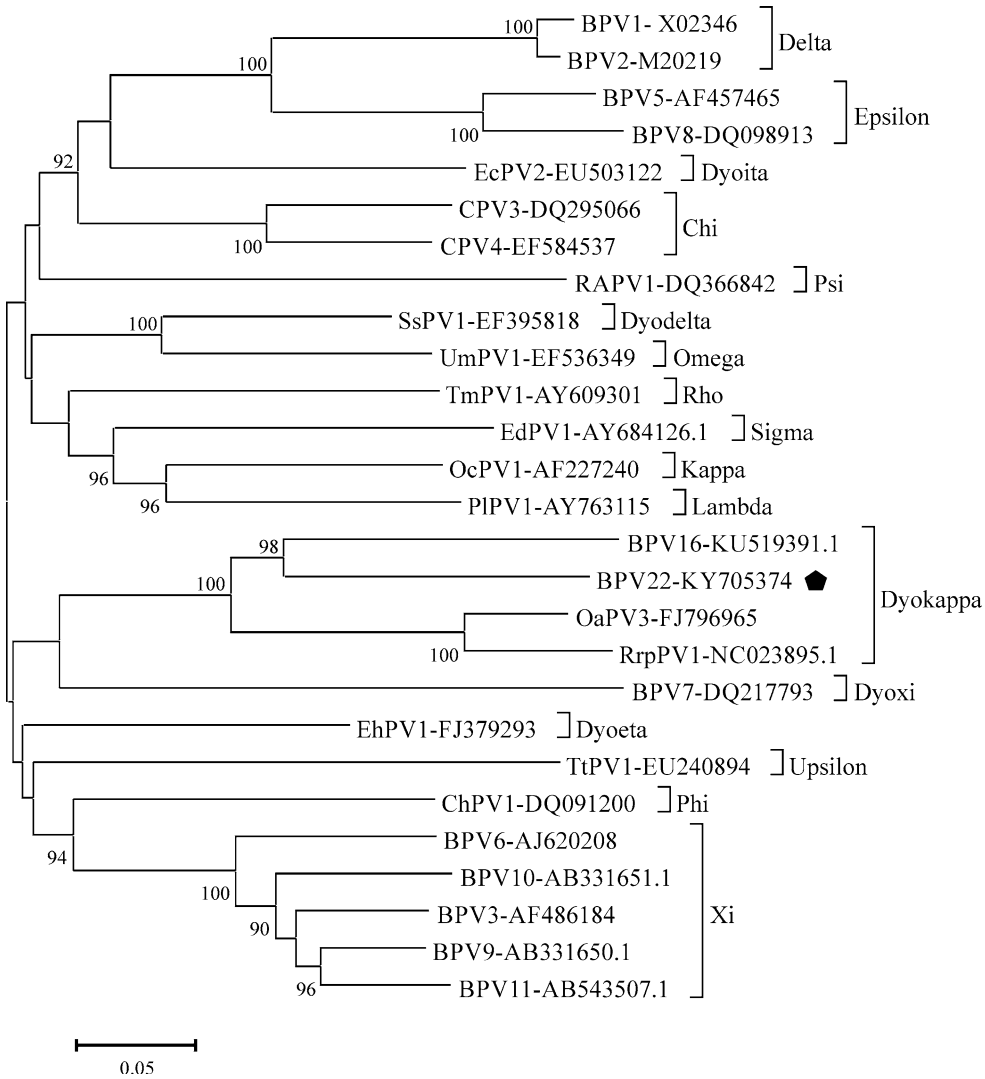

Fig. 1 Phylogenetic tree based on the papillomavirus L1 gene. The evolutionary history was inferred using the neighbor-joining method based on the nucleotide sequence. The optimal tree with the sum of branch lengths = 3.69 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (10,000 replicates) is shown next to each branch. The tree is drawn to scale. Evolutionary analysis was conducted in MEGA 6 [9]

first nucleotide of the NCR. The NCR contains a single TATA box (TATAA) at position 457, three canonical E2 binding sites (ACCGN4CGGT at positions 151, 187 and 260), and one non-canonical E2 binding site (ACCN6GGT; position 416). Both E6 and E7 contain one conserved zinc-binding domain (CXXC-X29-CXXC), and the latter also has a retinoblastoma (RB) protein binding site (LXCXE), which is associated with oncogenesis in human papillomavirus [10]. The E1 protein shows two cyclin interaction motifs (RXL), which are involved in efficient replication of papillomavirus [11]. Additionally, this protein also has an ATP-dependent helicase GX4GK(T/S) domain.

Despite the fact that BPV22 was detected in a swab from vaginal mucosa of a heifer presenting chronic vulvovaginitis, it is important to note that no typical lesions of papillomavirus (proliferative lesions or tumors) were observed in the positive animals. A similar scenario has been reported in humans, where a new gammapapillomavirus was identified in women with vaginitis lacking the classic proliferative lesions caused by PVs [3]. Several PVs have also been detected in normal skin or mucous membranes, indicating that these viruses are ubiquitous and may or may not be associated with clinical disease [1–4]. Three PV proteins that have been implicated in malignant transformation and tumorigenesis are E5, E6 and E7 [13–15]. Notably, while BPV22 lacks the E5 oncoprotein, the virus encodes both E6 and E7. Interestingly, the closely related RrPV1, OaPV3 and BPV16, which also lack the E5 coding sequence, have been associated with neoplasia in target host species [5–7].

Whether BPV22 detection in cows with vulvovaginitis was incidental or the virus was a co-factor in the chronic clinical condition observed in the animals remains unknown. It is important to note that approximately 13,000 reads of the sequencing data mapped to the genome of the anaerobic bacterium *Porphyromonas levii*, which has been associated with vulvovaginitis in cows [12]. These observations, combined with the fact that BPV22 was detected

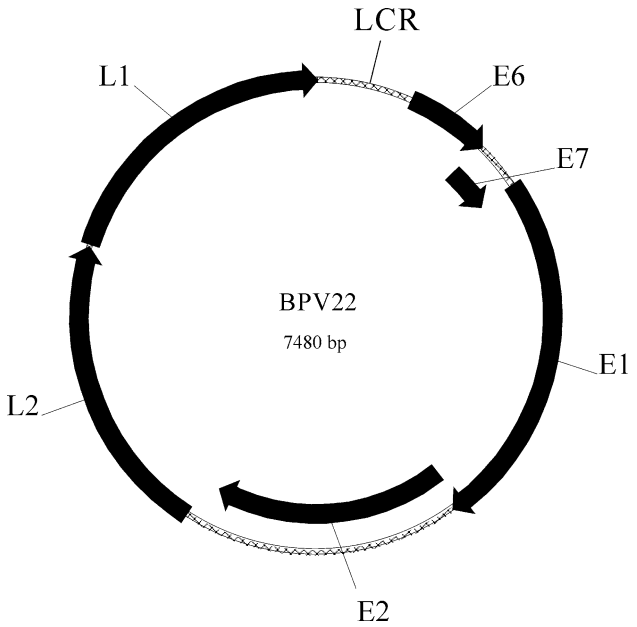

Fig. 2 Schematic representation of the BPV22 genome (GenBank accession no. KY705374). The long control region (LCR), with 484 bp, is followed by the early genes (E6-E7-E1-E2) comprising 3861 bp and the late genes (L2-L1) comprising 3049 bp. The positions of the ORFs are as follows: E6, nt 485-919; E7, nt 894-1169; E1, nt 1156-3003; E2, nt 2945-4345; L2, nt 4422-5966; and L1, nt 5975-7480

only in two out eight animals with vulvovaginitis (PCR run on vaginal swabs) suggest that the virus may not play a primary role in this particular clinical presentation. Collectively, these results suggest that BPV22 belongs to a new type within the genus *Dyokappapapillomavirus*, representing the second bovine papillomavirus within the genus.

Compliance with ethical standards

This work was funded by the U.S. Department of Homeland Security under the Center of Excellence of Emerging and Zoonotic Animal Diseases grant award number 2010-ST061-AG0001. The views and conclusions contained in this paper are those of the authors and should not be interpreted as necessarily representing the official policies, either explicit or implicit, of the U.S. Department of Homeland Security. This article does not contain any studies with human participants or animals performed by any of the authors. Field diagnostic samples were collected following standard collection techniques. All authors declare that they have no conflict of interest.

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