



NOTE

Clinical Pathology

Identification of bovine papillomavirus type 1 and 2 from bovine anogenital fibropapillomas

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ABSTRACT. Papillomavirus (PV) is a well-known pathogen associated with epithelial and mucosal neoplastic diseases. In contrast to human PVs, characterization of animal PVs from the aspect of anogenital neoplasm is still on a learning curve. In the present study, two vulval and one anal warts, histologically diagnosed as fibropapillomas, excised from dairy cattle were analyzed. PCR and sequencing revealed that bovine papillomavirus type 1 (BPV-1) and BPV-2 were detected from anal and vulval fibropapillomas, respectively. Immunohistochemistry detected PV antigen in a few differentiated keratinocytes of one vulval case. Reverse-transcriptase PCR detected the early region, but not the late region of BPV mRNA in all three cases. The present study will provide new insight into the relationship between BPV and anogenital papilloma in cattle.

KEY WORDS: anogenital tumor, bovine papillomavirus, cattle, fibropapilloma

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Papillomavirus (PV) is a double-stranded DNA virus which is known to induce epithelial and mucosal diseases. Papillomaviral genotypes are classified based on the sequence similarity of L1 open reading frame (ORF), the major capsid protein, and PVs present high species- and tissue-specificity [7, 29]. In humans, some high-risk human PV (HPV) genotypes, such as HPV type 16 (HPV-16) and HPV-18, are known to be associated with cervical cancers [34]. HPV is not only associated with cervical cancers, but certain genotypes also cause neoplastic diseases of external genitals [11]. In nonhuman primates, some PV genotypes have been detected from exfoliated cervicovaginal cells of cynomolgus monkeys (*macaca fascicularis*) designated as *macaca fascicularis* papillomavirus (MfPV) [4]. In other animals, anogenital wart-associated papillomavirus genotypes have been detected from genital or penile papillomas/warts from dolphins such as *delphinus delphis* papillomavirus type 1 (DdPV-1), *phocoena spinipinnis* papillomavirus type 1 (PsPV-1), *phocoena phocoena* papillomavirus type 1, 2, and 4 (PphPV-1,2,4) [26]. In domestic animals, *equus caballus* papillomavirus type 2 and 7 (EcPV-2, 7) have been associated with penile squamous cell carcinomas and penile mass, respectively [13, 16]. However, contrary to humans, information on animal PV-associated genital neoplasia is limited. Hence, it is essential to strengthen the relevance between animal PV genotype and clinical phenotype by accumulating papillomaviral information from spontaneous cases.

Although anogenital fibropapillomatosis outbreaks in cattle were reported [19, 31], very few attempts have been made to detect PV from bovine anogenital lesion. Bovine (*Bos taurus*) papillomavirus (BPV) infection is thought to be one of the factors for development of bovine cutaneous anogenital neoplasms, but it is not clear whether certain types of BPVs are involved in the lesion, and how BPVs are transmitted. In this study, to further understand the pathogenesis of bovine anogenital warts, one anal and two vulval warts, collected in Japan were pathologically and virologically analyzed.

Two warts in vulvas (Sample ID: B160303 and B160620) and one in anus (B160805) were collected from dairy cattle owned

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by different farms of Chiba and Ibaraki prefecture in Japan. Case B160303 was one-year-old at the time of papilloma excision. Case B160620 and B160805 were two years old. Macroscopic view of each sample is shown in Fig. 1 (A–C). Each dairy cattle did not show any symptoms at the time of papilloma excision. No significant observations of anogenital papillomatosis were noted in each herd besides the present cases. All three cases had a history of artificial insemination (AI) and calving before excision of the lesion. Each of the excised papilloma sample was fixed in 10% buffered formalin and embedded in paraffin following hematoxylin and eosin (H.E.) staining for histological diagnosis. Microscopically, the lesion in all cases was composed of a bland population of spindle-shaped mesenchymal cells proliferating in streams and was covered by acanthotic epidermis. The thickened epidermis occasionally extended into the underlying dermis as broad rete pegs (Fig. 1D–I). Based on these findings, all of the present cases were classified as fibropapilloma. Immunohistochemical staining for detecting BPV antigen was conducted as described previously [14] using a cocktail of two mouse monoclonal antibodies (clone BPV-1/1H8 + CAMVIR; Abcam, Cambridge, MA, U.S.A.), which was produced against the major capsid protein of BPV type 1 (BPV-1) and HPV-16. Bovine fibropapilloma lesion from the skin, not related to this study was used as positive control. For negative control, section was incubated with Tris-buffered saline instead of the primary antibody. The results of immunohistochemistry (IHC) showed that no positive signal was observed for case B160303 and B160805. In case B160620, intranuclear localization of papillomaviral antigen was detected in a few keratinocytes at the stratum granulosum of the hyperplastic epidermis (Fig. 2). Furthermore, IHC against the cell-cycle protein, p16 (cyclin-dependent kinase inhibitor 2A: CDKN2A), was conducted [14], since the previous findings of *felis catus* papillomavirus (FcaPV) show that the expression of p16 is one of the indicative observations of FcaPV infection [14, 23, 30]. In this study, none of the cases showed positive immunoreactivity against p16 protein (data not shown).

For papillomaviral genomic analysis, DNA was extracted from each sample with QIAamp DNA Mini Kit (Qiagen, Hilden,

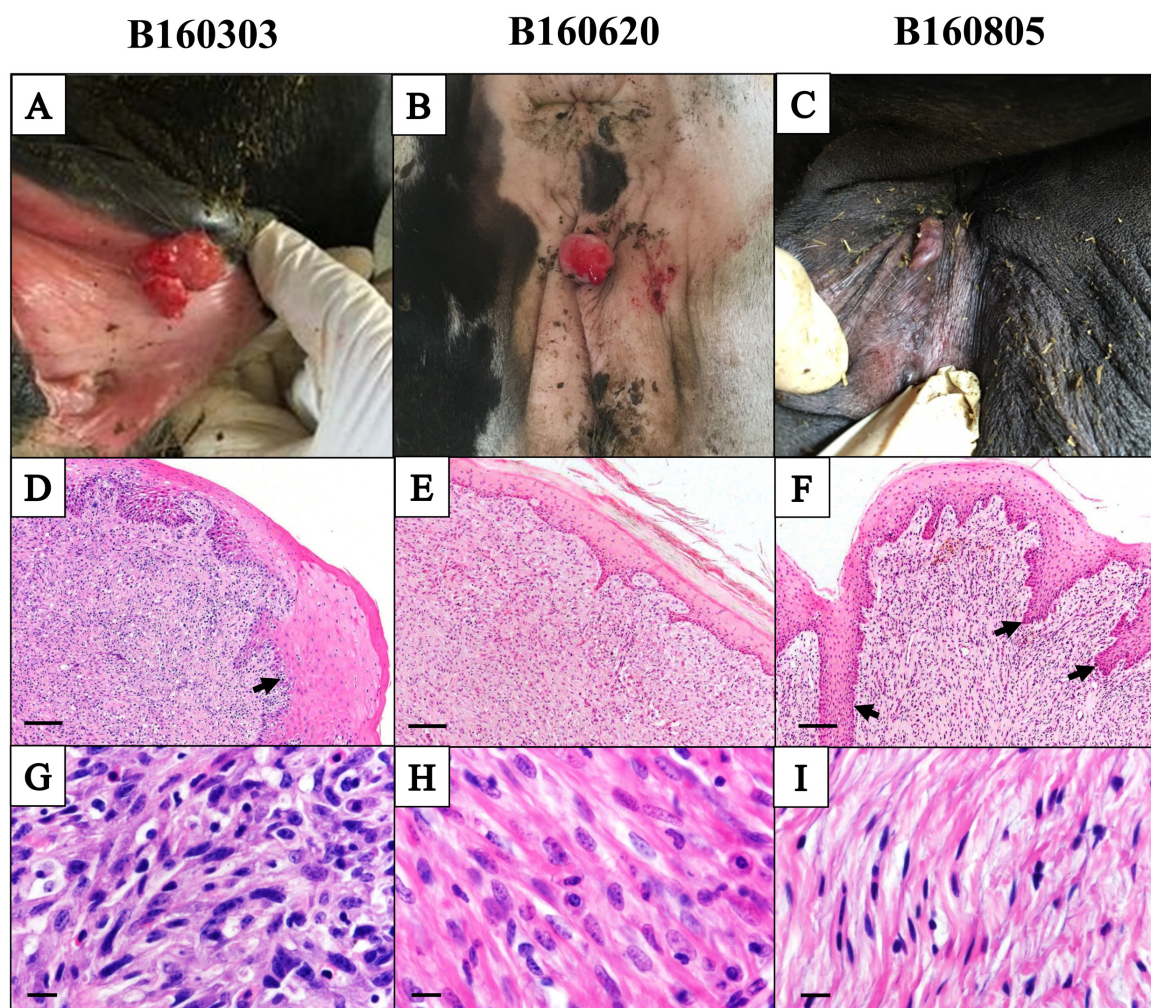


Fig. 1. Macroscopic and histopathological findings of one anal and two vulval fibropapilloma cases. Macroscopic and histopathological observation of each lesion is shown. (A–C) Exophytic nodular mass was observed on the vulva/anus. (D–I) The lesion in all cases consists of a bland population of spindle-shaped mesenchymal cells proliferating in streams and is covered by acanthotic epidermis, which occasionally forms broad rete pegs (arrows). (A, D, G) Case number B160303, vulval. (B, E, H) Case number B160620, vulval. (C, F, I) Case number B160805, anal. Bars, 100 μ m (D–F) and 10 μ m (G–I).

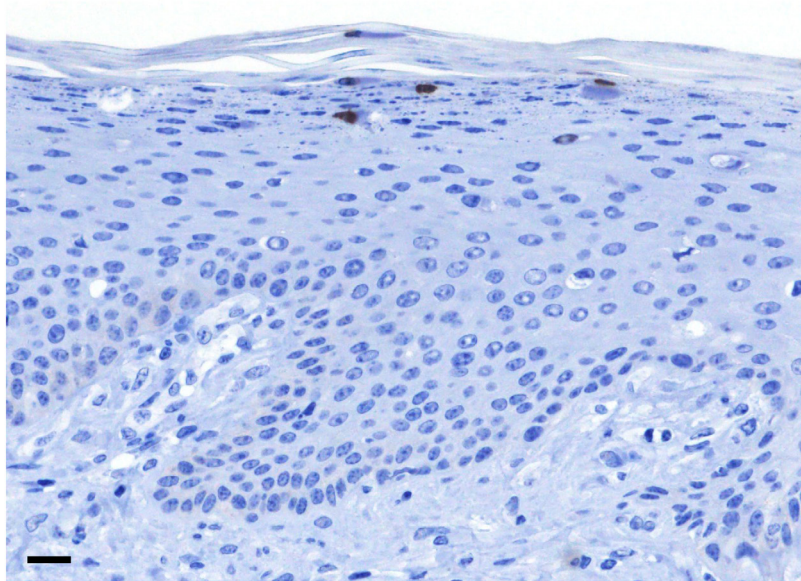


Fig. 2. Immunohistochemical detection of papillomaviral antigen in case B160620. Immunohistochemistry of the specimen of case B160620. Immunoreactivity for papillomaviral antigen is observed in the nucleus of few keratinocytes within the stratum granulosum of the hyperplastic epidermis. Bar, 20 μ m.

Germany) following the manufacturer's instruction. Detection of PV DNA was performed by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP). Two degenerate primer sets named subAup/subAdw and subBup/subBdw were used for detecting L1 genome of BPV [12]. For the positive control for subAup/subAdw and subBup/subBdw primers, BPV-1 positive DNA from bovine teat papilloma case, and a plasmid containing BPV-9 L1 DNA was used, respectively. Distilled water instead of template DNA was applied for the negative control. PCR and electrophoresis revealed that single band was observed around the size of 440 base pairs (bp) with subAup/subAdw primer set, while no band was observed with subBup/subBdw primer set for all three samples (Fig. 3). To determine the genotype of PCR product amplified with subAup/subAdw primer set, amplicons were digested with *AfaI* (Takara, Kusatsu, Japan) following the manufacturer protocol. The success in *AfaI* digestion was confirmed using BPV-1 positive sample unrelated to the present case. After digesting each PCR product with *AfaI*, the band patterns were referred from Kawauchi *et al* and BPV genotype were predicted [12]. Sample B160303 and B160620 showed the same band pattern, observed around 440 bp, indicated as BPV-2 (Fig. 3). Sample B160805 showed two bands, around 330 bp and 110 bp, indicated the possibility of either BPV-1 or BPV-13 (Fig. 3). To confirm the BPV genotype of each sample, sequencing was conducted with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and 3130xl Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's instructions. Sequencing results were analyzed with MEGA7 software, and sequence identity with the reference alignment was confirmed with BLAST tool of the National Center for Biotechnology Information (NCBI). Sample B160805 was BPV-1, and B160303 and B160620 were BPV-2 by sequencing the PCR product amplified by subAup/subAdw primer pair. Furthermore, PCR and sequencing were conducted to characterize each of the whole L1 sequence identified in this study with primer pair: BPV1&2 E5 (forward: 5'-CACTACCTCCTGGAATGAACATTTCC-3') and BPV1&2 757 LCR (reverse: 5'-GATGGTGTGATTATTGTTAAC-3').

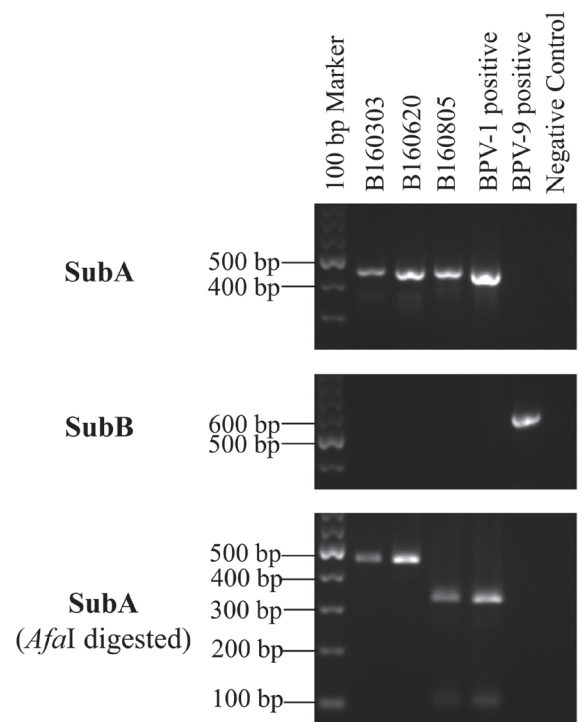


Fig. 3. Detection of BPV-1 and BPV-2 by PCR. Agarose gel electrophoresis of PCR product amplified with subAup/subAdw and subBup/suBAdw primer pairs are shown (upper, middle). The observed band patterns of subAup/subAdw-amplified products digested with *AfaI* is shown (bottom). A single band around 440 bp was observed in sample B160303 and B160620 indicated as BPV-2 (bottom). Sample B160805 showed two bands, around 330 and 110 bp, indicated the possibility of either BPV-1 or BPV-13 (bottom). Sequencing results of sample B160303 and B160620 were BPV-2, and B160805 was BPV-1. Abbreviations: bp, base pairs.

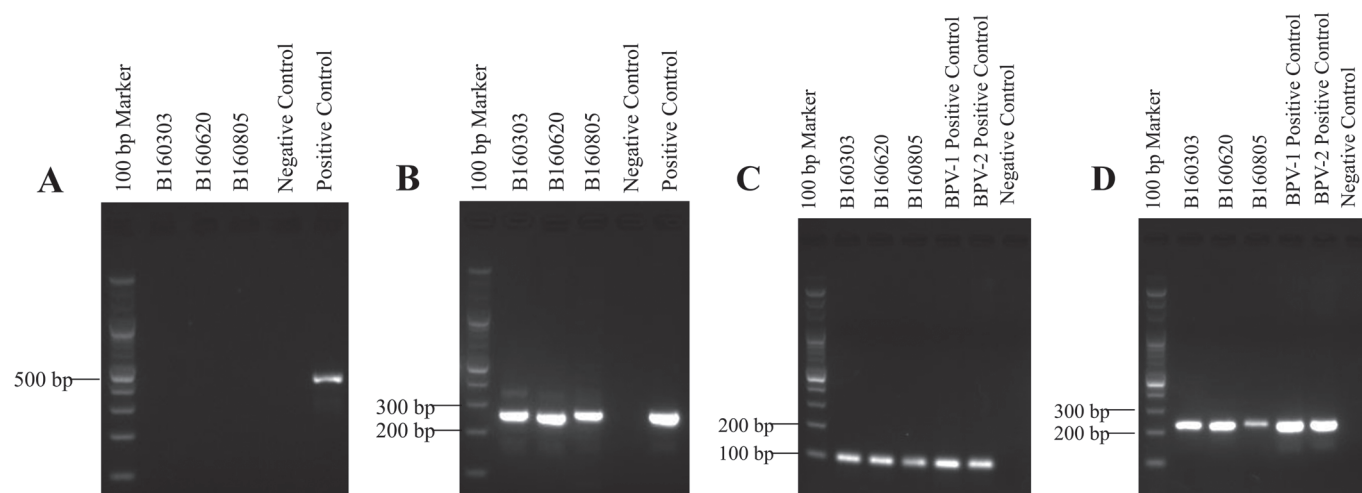


Fig. 4. RT-PCR result of L1 and early region (E2, E5, E6) mRNA from the anogenital papilloma samples. Agarose gel electrophoresis of RT-PCR products are shown. RT-PCR result of (A) mRNA L1, (B) mRNA E2, (C) mRNA E5, and (D) mRNA E6. mRNAs of three early regions (E2, E5, E6) were detected in all three samples, while L1 was not.

L1 sequence of sample B160805 showed 100% identity with four reference BPV-1s (GenBank accession number: KY886226.1, J02045.1, X02346.1, and U13843.1). L1 sequence of sample B160303 and B160620 showed 100% identity with one reference BPV-2 (GenBank accession number: MF045490.1 and X01768.1, respectively). Sequence of sample B160303 and B160620 were identical except for the 1456th nucleotide of L1 (“A” for B160303 and “T” for B160620). Due to the difference of 1456th nucleotide, substitution of the 486th amino acid in L1 was also observed (“T” for B160303 and “S” for B160620). L1 sequence of B160303, B160620, and B160805 were deposited in GenBank (Accession number: LC426021, LC426022, and LC426023, respectively).

As BPVs can be detected from healthy skin [25], and to test the hypothesis of active contribution of BPV, we sought to detect mRNA of BPV-1 and BPV-2 by performing reverse-transcriptase (RT)-PCR to confirm the transcription of BPV genome. By detecting BPV mRNA, we could confirm whether the viruses are biologically active and to exclude the possibility of incidental detection [1]. Total RNA was purified from each sample (B160303, B160620, and B160805) by using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacture’s instruction. Genomic DNA was eliminated with recombinant DNase I (Takara), and the success in eliminating genomic DNA was confirmed by PCR using subAup/subAdw primer pairs (data not shown). Subsequently, reverse transcriptase was conducted by PrimeScript RT Reagent Kit (Takara). RT-PCR was performed with four pairs of primers for amplifying mRNA of three early genes (E2, E5, E6) and one late gene (L1). The sequence of each primer pair are as follows. E2 (BPV1rnaE2-F/R) [33]. E5 (BPV1,2 E5-F/R: 5'-TTGTTCTTGGGACTAGTTGCTG -3'/ 5'-CTGTACAGGAGCACTCAAATGA-3'), E6 (BPV1,2 E6-F/R: 5'-GTGCAGRGAGCCTCTYACAGAA -3'/ 5'-AGTTTCCCCACAGTAGCA-3'), and L1 (subAup/subAdw). The product size for each primer pair for early region are as follows (BPV1rnaE2-F/R: 265 bp, BPV1,2 E5-F/R: 100 bp, BPV1,2 E6-F/R: 234 bp). Distilled water was applied for negative control, and genomic DNA extracted from bovine fibropapilloma lesion from the neck (positive for BPV-1), and the eyelid (positive for BPV-2) which were not related to this study, were used for positive controls. RT-PCR confirmed the detection of early regions (E2, E5, E6), while L1 mRNA was not detected in all three cases (Fig. 4). Sequencing results of the RT-PCR products from early regions (E2, E5, E6) confirmed that each case had the same BPV genotype detected with subAup/subAdw primer pair (B160303, BPV-2; B160620, BPV-2; B160805; BPV-1) (data not shown).

BPV transcription is regulated by E2 [21], and cellular transformation is known to be generated by oncogenes, E5 and E6 [24]. In general, viral replication of BPV occurs only in differentiating cells [4] and may differ between anatomical site, tissue, and/or cells [27]. The detection of mRNA of three early regions (E2, E5, E6), while no L1 mRNA, suggests that the active virus was present, however, none or very few complete (encapsulated) viruses were being produced. The negative result for BPV IHC of case B160303 and B160805 can be explained together with the results of RT-PCR. In this study, none of the cases were positive for L1 mRNA, so theoretically, L1 protein should not be expressed within the lesions. Since the primary antibody used for IHC was produced against BPV-1 L1 antigen, the negative results for BPV IHC were acceptable. For case B160620, BPV L1 antigen was observed within the terminally differentiating keratinocytes in the stratum granulosum (Fig. 2). The positive signal was limited to very few keratinocytes, so we consider that BPV L1 mRNA was below the detection limit of RT-PCR.

The combined studies of p16 IHC and PV detection have been widely conducted in cats [14, 23, 30] and humans [5, 15]. To our knowledge, the relationship between p16 expression and BPV infection has not been evaluated in cattle. The p16 protein is also used as a marker to evaluate the malignancy of cervical neoplasms in humans [15]. The negative results for p16 could be explained that all cases are histologically benign. One study showed the expression of p16 by IHC in bovine ocular squamous cell carcinoma cases, however, no PV was detected by PCR [9]. The relationship between PV infection and p16 expression in cattle needs to be further examined.

PVs have been known to show high tissue-specific characters by each genotype [7]. In dairy cattle, BPV-1 and BPV-2 have been suggested to be common in cutaneous papillomas [6]. BPV-1 and BPV-2 have also been detected from urinary bladder cancer of bracken fern-fed cattle [3, 32]. A recent study showed the detection of BPV-2 DNA in anal warts of heifers in New Zealand [22], suggesting the association of BPV and anal warts. Munday *et al* reported the detection of BPV-2 in anal fibropapilloma [22], while the present study confirmed the detection of BPV-1 from one case (B160805), and BPV-2 from two vulval fibropapilloma cases (B160303 and B160620). It has been shown that BPV-1 and BPV-2 are associated with anogenital fibropapillomas [18], however, the information on PV genomic characteristic detected from bovine anogenital fibropapilloma is very limited. This study provides the genomic characteristics of BPV-1 and BPV-2 L1, following the criteria established by the International Committee on the Taxonomy of Viruses (ICTV) [29]. Since additional BPV types were reported and the major method of PV detection changed after the first observation of genital BPV [8], the present study could update the knowledge on relationship between BPV-1 and BPV-2 with anogenital fibropapillomas in cattle.

In humans, anogenital wart-associated HPV genotypes have been identified. HPV-6 and HPV-11 are highly associated with genital warts [10], and nearly 40 HPV genotypes have also been associated with anogenital warts [34]. As HPV-6 and HPV-11 are known to cause approximately 90% of genital warts [20], HPV prophylactic vaccine, known as Gardasil has been developed to prevent HPV-6 and HPV-11 infections [2]. Although HPV-associated genital warts are generally benign in contrast with cervical cancers caused by HPV-16 and HPV-18, HPV-associated genital warts have potential to show sexual transmission [17]. The present study confirmed the detection of BPV-1, and BPV-2 from bovine anogenital warts, suggesting that these two BPV genotypes may have potential to develop proliferative lesions (warts) of external genitals in cattle. In domestic cattle, rectal palpation, ultrasonography, the use of controlled internal drug release device and AI are routinely conducted for managing breeding. A previous study reported an outbreak of fibropapillomatosis following rectal examination in a herd of beef heifers [28]. Although PV detection was not conducted in that report, BPV transmission by breeding routine could become one of the risk factors for an outbreak of anogenital papillomatosis. Furthermore, additional sample numbers are needed to determine the association of BPV-1 and BPV-2 with bovine anogenital warts epidemiologically.

In the present study, BPV-1 and BPV-2 were detected from anal and vulval fibropapilloma cases, respectively. This report will update the knowledge on the relationship between anogenital fibropapilloma and BPV genotype. Further investigations are needed to define the role of BPV infection and the development of anogenital fibropapillomas in cattle.

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