pISSN 1598-298X / eISSN 2384-0749
J Vet Clin 33(6) : 346-350 (2016)
http://dx.doi.org/10.17555/jvc.2016.12.33.6.346

Journal of Veterinary Clinics
www.ksvc.or.kr

PCR-based Detection of Bovine Papillomavirus DNA from the Cutaneous Papillomas and Surrounding Environments in the Korean Native Cattle, Hanwoo

Sang-Hyun Han, Yong-Sang Park*, Jong-Pil Seo* and Tae-Young Kang*[1]

Educational Science Research Institute, Jeju National University, Jeju 63243, Korea
**Department of Veterinary Medicine, College of Veterinary Medicine, Jeju National University, Jeju 63243, Korea*

(Received: May 17, 2016 / Accepted: August 17, 2016)

Abstract : Two 1-year old calves of Korean Native cattle (Hanwoo) presented cutaneous papillomas on the face and neck. Type 2 bovine papillomavirus (BPV-2) was identified in the cutaneous papillomas based on BPV-specific PCR and subsequent DNA sequencing analysis results. Using DNA samples extracted from two affected calves and unaffected animals reared in the same stable, BPV-2 was not only detected in the cutaneous papillomas of affected animals based on BPV-specific PCR analysis, but also detected in normal skins, hairs, and their environments based on nested PCR analysis. BPV-2 was also detected in DNA samples isolated from animals and environments of that distinct stable with affected calves. However, no BPV-2 was detected in the drinking water of both stables (infected and unaffected). These findings concluded that BPV-2 was transmitted by direct or indirect contact, not by drinking water. This is the first report to show molecular evidence of BPV-2 infection. Rapid and precise molecular identification can be used to screen BPV-2 in cattle farms to understand the biological roles of BPV in animal diseases.

Key words **:** bovine papillomavirus, polymerase chain reaction, BPV-2.

Introduction

Papillomaviruses (PVs) are double-stranded DNA viruses belonging to family Papillomaviridae. They infect mammals, birds, and reptiles (1,4,5). These viruses have been associated with the development of lesions in the epithelium and various carcinogenic processes in humans and other animals including cattle, yaks, water buffaloes, and horses (4,7,9,26, 31). Up to date, thirteen types of bovine papilloma virus (BPV) have been classified into the following three genera based on their genetic and biological features: *Deltapapillomavirus* (BPV-1, -2, and -13), *Epsilonpapillomavirus* (BPV-5 and -8), and *Xipapillomavirus* (BPV-3, -4, -6, -9, -10, -11, and -12) with an unassigned group (BPV-7) (16,20,24). BPV infections are common in cattle. Approximately 50% of cattle are estimated to carry BPV-induced lesions or wart in the UK (8) while over 60% of Holstein cattle in Korea are estimated to have BPV-induced lesions (2,3).

Among those BPV infections, BPV-1 and -2 cause hyperproliferative lesions such as fibropapillomas (warts) of paragenital areas and the skin, benign fibroplasias, and urinary bladder cancer in cattle, causing significant economic losses (9,16,17,26). BPV-2 infection is also considered as an etiological agent of enzootic bovine haematuria (EBH) of urinary bladder neoplastic disease in the presence of ptaquiloside of bracken fern (*Pteridium aquilinum*), an environmental carcinogen (11,15,23,25).

[1]Corresponding author.
E-mail : tykang87@jejunu.ac.kr

Previous reports have described BPV infection in lesions of teat warts using immunohistochemistry and electron microscopy in Holstein and Hanwoo collected in South Korea without classifying the BPV types (2,3). This study used polymerase chain reaction (PCR)-based methods to detect and identify BPV infection in the cutaneous papillomas and their surrounding environments in Hanwoo.

Materials and Methods

Animals and DNA isolation

Two healthy Hanwoo calves (1-year old) presented cutaneous papillomas on their face and neck in March 2015. They were freely released in a same stable in a cattle farm in Jeju Island, South Korea. Tissue samples of fibropapillomas were excised from the lesions of two affected calves. Swab samples were prepared using 3×3 cm^2 cotton from the skin of face and hairs of ventral part of each individual and the environment (feed tub, drinking water, and stable wall) according to the procedure of Bogaert *et al*. (6). Drinking water was freshly collected from tap water and immediately frozen at −80ºC until analysis. Animal swabs and environmental swabs were divided into two groups (Group-1 and Group-2) depending on whether they had direct contact with the calves affected by bovine papilloma (BP). Group-1 (n = 60) was for samples that had direct contact with affected calves within the same. Group-2 (n = 48) was for samples that had no direct contact with the affected calves. They were collected in the same farm but different stables. Both groups included all cows and calves in each stable. Group-1 and Group-2 ani-

Table 1. Primers used in PCR and nested PCR analysis for detection of BPV-2

Target BPV	Primer name	Primer sequence	Reference
BPV-1, -2, -13	BPV1-2F	5'-TTTTAGAGATCGCCCAGACG-3'	This study
	BPV1-2R	5'-CTTGCCTTTGACTTGGTGAT-3'	
BPV-1	BPV1sF	5'-GCCCACGGAAGATCCTGAAG-3'	This study
	BPV1sR	5'-AGGTGTTCTGAGGTAGCAGTCTA-3'	
BPV-2	BPV2sF	5'-CGTAACCTGCCTCAAACTG-3'	This study
	BPV2sR	5'-ATGACTTGGGGAGCAAGGC-3'	
BPV-13	BPV13sF	5'-AGAAGCTTTCCTACAAGTG-3'	This study
	BPV13sR	5'-ATTTGTGGCTGATGCTCTTCTTG-3'	
BPV-1,-2, -13	BPVE5F	5'-GCTACGAGAACTGCACCACC-3'	Chambers *et al*. (2003)
	BPVE5R	5'-TGGACATGTGCCCGCTTGC-3'	
BPV-2 in nested PCR	BPVf	5'-CAAAGGCAAGACTTTCTGAAACAT-3'	Bogaert *et al*. (2005)
	BPVr	5'-AGACCTGTACAGGAGCACTCAA-3'	

mals were separated for three months before sample collection. Total DNA was extracted from tissues and environmental swabs using DNeasy Blood and Tissue Kit (Qiagen, USA).

PCR and nested PCR analysis

To detect the presence or absence of BPV, PCR was used. BPV type-specific primer sets were designed using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) after multiple alignments to select genus-specific primer sequences among genome sequences of *Deltapapillomaviruses* (BPV-1, BPV-2, and BPV-13) previously deposited at the National Center for Biotechnology Information (NCBI) database. In addition, primer pair (BPVE5F and BPVE5R) designed by Chambers *et al*. (12) were used for BPV-specific PCR to amplify E5 ORF fragment of *Deltapapillomavirus* specific region. BPVf and BPVr primers designed by Bogaert *et al*. (6) were also used for nested PCR analysis. Primers used in PCR and nested PCR are summarized in Table 1. PCR was performed in 20 μl reaction volume containing approximately 50 ng of DNA, 10 pmole of each primer, and 2 units of *Taq* DNA polymerase (GenetBio, South Korea). PCR were performed with the following conditions: initial heating at 95°C for 2 min, 35 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 60 s, followed by a final step of extension at 72°C for 10 min. For screening BPV-1/-2/-13 in environmental samples, BPVf and BPVr designed by Bogaert *et al*. (6) were used for nested PCR. The nested PCR was carried out with different primer set (BPVf and BPVr) and 65°C annealing temperature. PCR products were purified using Agarose Gel Extraction Kit (Roche, Germany) and directly sequenced with bidirectional inner primers using ET-dye Terminator DNA Sequencing Kit (Amersham Biotechnologies, USA).

Data analysis

Similarity search was carried out to compare the newly determined BPV sequences with those previously reported in NCBI database using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The frequencies of positive BPV DNA samples between different groups were compared using Student's *t*-test SPSS ver. 19.0 (IBM Inc.). $p < 0.05$ was considered as statistically significant.

Results and Discussion

DNA samples (n = 11) of cutaneous papilloma from two calves showed 601-bp E5 ORF band of BPV using BPV-1/-2/-13 specific primers in primary PCR (Fig 1a). All sequences obtained from this study shared high identities (over 99%) with those of BPV-2. Therefore, we defined these sequences collected from cutaneous papillomas of the two calves as BPV-2. In this farm, DNA samples from clinically normal animals and their surrounding environments also yielded 247-bp BPV-2 based on nested PCR (Fig 1b) and subsequent DNA sequencing analysis. We concluded that BPV-2 was the causative agent for cutaneous papilloma development in this farm. Other BPVs including BPV-1 and BPV-13 belonging to the same *Deltapapillomavirus* were not detected.

For two different stables in the same farm, we tried to

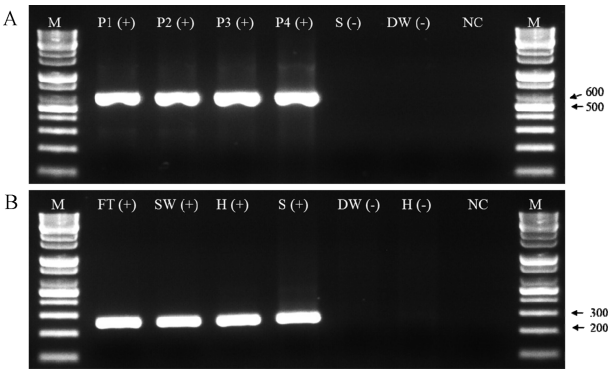

Fig 1. Detection of BPV DNA from papilloma and surrounding environments. a, PCR detection for BPV (601-bp PCR products) from DNA samples isolated from papillomas. b, nested PCR detection for BPV-2 (247-bp PCR products) from DNA samples prepared from normal skins and hairs as well as environmental swabs. P, papilloma; S, skin; H, hair; DW, drinking water; FT, feed tub; SW, stable wall; NC, negative control. (+) and (-) indicate the positive and negative for BPV. M is 1-kb plus DNA ladder.

Table 2. PCR and nested PCR detection of BPV-2 DNA in cattle and surrounding environments

DNA sample		Group-1 (n = 60)			Group-2 (n = 48)			*p*-value	Significance
		No. of test	No. of positive	Frequency	No. of test	No. of positive	Frequency		
Animal	Papilloma[1]	11	11	1.000	-	-	-	-	-
	Normal skin[2]	60	17	0.283	48	11	0.229	0.199	n.s.
	Hairs[3]	60	14	0.233	48	5	0.104	0.014	*
Surroundings	Feed tub	8	2	0.250	8	1	0.125	0.554	n.s.
	Drinking water	8	0	0.000	8	0	0.000	-	-
	Stable wall	8	1	0.125	8	0	0.000	0.351	n.s.

Note: different letters in the same row are significantly different at 5% significance thresholds. n.s. indicates not significant. [1], DNA samples of papilloma were isolated from two affected calves. [2] and [3], DNA samples of normal skin and hairs were prepared using the swabs from each individual in Group-1 and Group-2.

detect BPV DNA using PCR-based analyses. BPV was detected in DNA samples isolated from normal skins, hairs, and environmental swabs except drinking water (Table 2). The skin and hair DNA samples in Group-1 showed 28.3% and 23.3% detection rates of BPV-2, respectively. However, the skin and hair DNA samples in Group-2 showed 22.9% and 10.4% detection rates of BPV-2, respectively. The presence of BPV-2 DNA in hairs was significantly ($p < 0.05$) different between Group-1 and Group-2. For surrounding environmental DNA samples, BPV-2 DNA was detected in both groups, indicating that the surrounding environments might have been contaminated by BPV particles. However, DNA samples of drinking water did not show BPV DNA in either group. Because drinking water was collected freshly from tap water, we concluded that the virus was not introduced from the water source at that time. Group-1 showed higher ($p < 0.05$) levels of BPV-2 DNA than Group-2, suggesting that these virus particles could be easily released to the environment from papilloma affected animals. It is also possible that BPV-2 DNA found in Group-2 might have derived from Group-1 by human activities during management or through cross contamination during migratory time during outside grazing. In this farm, there are three different stables. However, cattle graze in the same grassland every summer, which can introduce direct contact to each other. In addition, BPV-2 has been detected from sarcoids, normal skin, and surrounding environments of horse farms (6,7). Two types of BPV-1 and BPV-2 have been detected in several groups of horses and surrounding environments. However, the locations of BPV found and the expression levels of BPVs were significantly different, corresponding to grouping conditions by contact opportunity with affected animals (6,7). They also found the presence of BPV-positive animals and environments that were strictly isolated without direct contacting pathogen-harboring animals. These results indicate that animals and their body parts might have more chances of viral transmission when they are near the BPV affected calves via direct or indirect contact than those far away from the affected calves. The occurrence of horizontal transmission of BPV-2 has been described in healthy cattle from an inoculating experiment with peripheral blood from EBH animals (30). On the other hand, the presence of viral DNA in a stable without papilloma affected animal indicates that viral latency is also possible (6,10,23). In animals and humans, it has been suggested that peripheral blood mononuclear cells may serve as the source of papillomavirus of the epithelial cells as well as simultaneous viral infections in cancer and healthy tissues of the same animals, thus suggesting a haematogenous virus spread (7,21,23,25).

The presence of BPV has been reported using immunohistochemical (IHC) and electron microscopic (EM) analyses in the teats of Holstein and Hanwoo in South Korea (3), and the detection rates of papilloma lesions by direct observation were 60.8% in Holstein and 7.4% in Hanwoo, respectively. Whereas the detection rates of BPV in the teats of Holstein cows was 41.9% in EM, 26.3% in IHC, but 71.4% in PCR assays, and twenty-two BPV sequences classified into three groups in nucleotide similarities corresponding to *Deltapapillomavirus* (BPV-1 and -2), *Epsilonpapillomavirus* (BPV-5), and *Xipapillomavirus* (BPV-3, -4 and -6) (2). However, we only identified BPV-2 in this study. Differences in BPV types detected between previous reports and the present study might be due to difference in survey sites. The previous reports used samples from Korean Peninsula. However, this study was carried out by only using samples from Jeju Island. Four types of BPVs (-1, -2, -3, and -9) were described from 71 lesions of cows and a donkey (16), and nine types of BPV except BPV-2, -5, -11, and -13 in cattle herds in Emilia Romagna region in Italy (27). Locations of animal farms, cattle breeds, and genetic influence are associated with the distribution pattern of BPV types (13,14,19,22,28,29). For example, Holstein cattle have higher prevalence (60.8%) of papillomas in teats than Hanwoo (7.4%) in South Korea (2,3). In addition, BPV-1 has been reported to be the main viral type causing papilloma-like lesions in Italy (16). However, BPV-6 and BPV-8 are the main ones in Japan (18) and Germany (28), respectively. These results suggest that BPV distribution is affected by type-specificity according to genetic background of the cattle population, such as disease susceptibility and environmental conditions. From the results of the present study we just identified BPV-2 in a single cattle farm in Jeju Island. For more clear understanding distribution of BPV types, it should be necessary to examine broad samples from cattle and horse farms in this island. Whether co-pastur-

ing with other species such as horses that are well known as hosts for BPV-1 and BPV-2 would affect BPV distribution merits further study.

In this study, we applied PCR-based methods to detect BPV from bovine cutaneous papillomas, normal body parts, and surrounding environments. We clearly detected BPV-2 infection in papilloma lesions. The presence of viral DNAs was also found in normal body parts and surrounding environments. Such rapid and precise molecular detection might result in proper management plan and disease control on farms. Further research is needed to reveal the genetic characterization of BPVs from different regions of South Korea, including the surrounding islands and their associations with viral pathogenesis in livestock animals.

References

1. Antonsson A, Hansson BG. Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. J Virol 2002; 76: 12537-12542.
2. Bae YC. Pathological and molecular biological studies of bovine papilloma. PhD Dissertation, University of Chonnam National University. 2005.
3. Bae YC, Yoon SS, Park JW, Lee CS, Jean YH and Kang MI. Bovine papillomavirus detection from bovine teats using immunohistochemistry and electronmicroscopy. Korean J Vet Res 2005; 45: 233-238.
4. Bernard HU, Burk RD, Chen Z, van Doorslaer K, zur Hausen H, de Villiers EM. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology 2010; 401: 70-79.
5. Bernard HU. The clinical importance of the nomenclature, evolution and taxonomy of human papillomaviruses. J Clin Virol 2005; 32: S1-S6.
6. Bogaert L, Martens A, De Baere C, Gasthuys F. Detection of bovine papillomavirus DNA on the normal skin and in the habitual surroundings of horses with and without equine sarcoids. Res Vet Sci 2005; 79: 253-258.
7. Bogaert L, Martens A, Van Poucke M, Ducatelle R, De Cock H, Dewulf J, De Baere C, Peelman L, Gasthuys F. High prevalence of bovine papillomaviral DNA in the normal skin of equine sarcoid-affected and healthy horses. Vet Microbiol 2008; 129: 58-68.
8. Campo MS. Infection by bovine papillomavirus and prospects for vaccination. Trends Microbiol 1995; 3: 92-97.
9. Campo MS. Bovine papillomavirus: old system, new lessons? in M.S. Campo (ed.). In: Papillomavirus Research: From Natural History to Vaccine and Beyond. Wymondham: Caister Academic Press. 2006:373-383.
10. Carr EA, Théon AP, Madewell BR, Griffey SM, Hitchcock ME. Bovine papillomavirus DNA in neoplastic and nonneoplastic tissues obtained from horses with and without sarcoids in the western United States. Am J Vet Res 2001; 62: 741-744.
11. Carvalho C, Freitas AC, Brunner O, Góes LGB, Yaguiu AC, Beçak W, Stocco dos Santos RC. Bovine papillomavírus type 2 in reproductive tract and gametes of slaughtered bovine female. Braz J Microbiol 2003; 34: 72-73.
12. Chambers G, Ellsmore VA, O'Brien PM, Reid SW, Love S, Campo MS, Nasir L. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. Virus Res 2003; 96: 141-145.
13. da Silva FR, Daudt C, Streck AF, Weber MN, Filho RV, Driemeier D, Canal CW. Genetic characterization of Amazonian bovine papillomavirus reveals the existence of four new putative types. Virus Genes 2015; 51: 77-84.
14. Escudero C, Vázquez R, Doménech A, Gómez-Lucía E, Benítez L. First report of a variant bovine papillomavirus type 2 (BPV-2) in cattle in the Iberian Peninsula. Vet Ital 2014; 50: 219-226.
15. Freitas AC, Carvalho C, Brunner O, Birgel Jr. EH, Libera AMD, Benesi FJ, Beçak W, Stocco dos Santos RC. Viral DNA sequences in peripheral blood and vertical transmission of the vírus: a discussion about BPV-1. Braz J Microbiol 2003; 34: 76-78.
16. Grindatto A, Ferraro G, Varello K, Crescio MI, Miceli I, Bozzetta E, Goria M, Nappi R. Molecular and histological characterization of bovine papillomavirus in North West Italy. Vet Microbiol 2015; 180: 113-117.
17. Hatama S, Nobumoto K, Kanno T. Genomic and phylogenetic analysis of two novel bovine papillomavirus, BPV9 and BPV10. J Gen Virol 2008; 89: 158-163.
18. Kawauchi K, Takahashi C, Ishihara R, Hatama S. Development of a novel PCR-RFLP assay for improved detection and typing of bovine papillomaviruses. J Virol Methods 2015; 218: 23-26.
19. Lee T, Cho S, Seo KS, Chang J, Kim H and Yoon D. Genetic variants and signatures of selective sweep of Hanwoo population (Korean native cattle). BMB Rep 2013; 46: 346-351.
20. Lunardi M, Alfieri AA, Otonel RA, de Alcântara BK, Rodrigues WB, de Miranda AB, Alfieri AF. Genetic characterization of a novel bovine papillomavirus member of the Deltapapillomavirus genus. Vet Microbiol 2013; 162: 207-213.
21. Melo TC, Araldi RP, Pessoa NS, de-Sá-Júnior PL, Carvalho RF, Beçak W, Stocco RC. Bos taurus papillomavirus activity in peripheral blood mononuclear cells: demonstrating a productive infection. Genet Mol Res 2015; 14: 16712-16727.
22. Pang F, Shi Q, Du L, Zhao T, Cheng Y, Jiao H, Zhao J, Wang M, Rong H, Zhou H, Wang F. Complete genome sequence of bovine papillomavirus genotype 13 from local yellow cattle in hainan province, china. Genome Announc 2014; 2: e01087-14.
23. Pathania S, Dhama K, Saikumar G, Shahi S, Somvanshi R. Detection and quantification of bovine papilloma virus type 2 (BPV-2) by real-time PCR in urine and urinary bladder lesions in enzootic bovine haematuria (EBH)-affected cows. Transbound Emerg Dis 2012; 59: 79-84.
24. Rector A, Van Ranst M. Animal papillomaviruses. Virology 2013; 445: 213-223.
25. Roperto S, Brun R, Paolini F, Urraro C, Russo V, Borzacchiello G, Pagnini U, Raso C, Rizzo C, Roperto F, Venuti A. Detection of bovine papillomavirus type 2 in the peripheral blood of cattle with urinary bladder tumours: possible biological role. J Gen Virol 2008; 89: 3027-3033.
26. Roperto S, Russo V, Ozkul A, Corteggio A, Sepici-Dincel A, Catoi C, Esposito I, Riccardi MG, Urraro C, Lucà R, Ceccarelli DM, Longo M, Roperto F. Productive infection of bovine papillomavirus type 2 in the urothelial cells of naturally occurring urinary bladder tumors in cattle and water buffaloes. Plos One 2013; 8: e62227.
27. Savini F, Mancini S, Gallina L, Donati G, Casà G, Peli A, Scagliarini A. Bovine papillomatosis: First detection of bovine papillomavirus types 6, 7, 8, 10 and 12 in Italian cattle herds. Vet J 2016; 210: 82-84.
28. Schmitt M, Fiedler V, Müller M. Prevalence of BPV genotypes in a German cowshed determined by a novel multiplex

BPV genotyping assay. J Virol Methods 2010; 170: 67-72.
29. Staiger EA, Tseng CT, Miller D, Cassano JM, Nasir L, Garrick D, Brooks SA, Antczak DF. Host genetic influence on papillomavirus-induced tumors in the horse. Int J Cancer 2016; in press.
30. Stocco dos Santos RC, Lindsey CJ, Ferraz OP, Pinto JR, Mirandola RS, Benesi FJ, Birgel EH, Pereira CA, Beçak W. Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. J Gen Virol 1998; 79: 2127-2135.
31. Yuan Z, Gallagher A, Gault EA, Campo MS, Nasir L. Bovine papillomavirus infection in equine sarcoids and in bovine bladder cancers. Vet J 2007; 174: 599-604.