

## Co-infection of bovine papillomavirus type-1 and -10 in teat warts of dairy cattle

P. KUMAR, B.L. JANGIR, G. SAIKUMAR, R. SOMVANSHI

Indian Veterinary Research Institute, Izatnagar, Bareilly, Uttar Pradesh, India

**ABSTRACT:** The present study was carried out to investigate the involvement of different bovine papillomaviruses in the teat warts of cattle. A total of 11 teat wart samples showing rice grain-like and small, sessile elevated greyish or flesh-like growths were collected from dairy cattle. DNA was extracted from these teat wart samples and PCR and real time PCR techniques were applied using specific primers for BPV-1 and -10 to detect the presence of viral nucleic acid. PCR revealed the presence of viral DNA of BPV-1 and -10 in three and seven samples, respectively. Quantification using real time PCR revealed that the copy numbers of the viral DNA of BPV-1 and -10 DNA varied from  $1.12E + 04$  to  $2.99E + 04$  and  $3.56E + 02$  to  $5.23E + 06$ , respectively. From the present study it can be concluded that BPV-1 and -10 are involved in production of rice grain-like and sessile elevated growths on the teats of cattle.

**Keywords:** teat wart; bovine papillomavirus; cattle, PCR; real time PCR

Warts or papillomatosis is a common condition observed in cattle and caused by bovine papilloma virus (BPV). Warts have been observed mainly on the skin (Kumar et al. 2013a), teats (Maeda et al. 2007) and mucosal surfaces (Kumar et al. 2013b) of cattle. These BPV-induced lesions are usually benign in nature and regress spontaneously but can progress to cancer under the impact of environmental co-factors. To date, 13 different types of BPVs have been identified worldwide and grouped into three genera: Xi papillomaviruses (BPV-3, -4, -6, -9, -10, -11 and -12), Delta papillomaviruses (BPV-1, -2 and -13) and Epsilon papillomaviruses (BPV-5, -7 and -8) whose genomes seem to share similarities with both Xi and Delta papillomaviruses (De Villiers et al. 2004; Ogawa et al. 2007; Tomita et al. 2007; Hatama et al. 2008, 2011; Zhu et al. 2012; Lunardi et al. 2013).

Teat warts (TWs) are commonly observed in dairy cattle and appear as flat and round, rice grain and frond epithelial types (Jarrett et al. 1980). Warts of the teats and udders of cattle are reported to be caused by infection with BPV-1, -5 and -6 (Lindholm et al. 1984; Campo 2002; Maeda et al.

2007). Teat warts caused by BPV-6 have significant economic consequences, as papillomas of this type exhibit secondary and even tertiary spread around the primary tumours which ultimately disfigures teat architecture leading to difficulties in milking. In Indian studies cases of teat warts diagnosed as papilloma were reported but the involvement of different types of BPVs was not elucidated. Therefore, in the present study we aimed to identify the BPV types implicated in the teat warts of cattle.

### MATERIAL AND METHODS

**Teat wart tissue samples.** A total of 11 TW samples were collected in sterilised tubes from milking cows reared at an organised Dairy Station, IVRI and villages around Mukteswar, India. The TW samples were stored at  $-20^{\circ}\text{C}$  until used in molecular studies.

**DNA extraction.** DNA was extracted from TW samples using the Genomic DNA Mini Kit (Qiagen) according to the manufacturer's protocol and stored at  $-20^{\circ}\text{C}$  until used.

**PCR.** Oligonucleotide primers were commercially synthesised by Operon Biotechnologies, Genetix Biotech. Primers for BPV-1 (forward- 5'-gga gcg cct gct aac tat agg a-3'; reverse-5'-atc tgt tgt ttg ggt ggt gac-3') and BPV-10 (forward-5'-gag gtc cgc ggg atc gga ct-3'; reverse-5'-tga gca agg cgt gac gca gg-3') were designed to amplify DNA fragments of 301 and 192 bp, respectively. Amplified DNA fragments were electrophoresed in 1.2% agarose gel containing ethidium bromide (0.5 g/ml) and visualized using transillumination under UV light (Geldoc, USA).

**Sequencing.** The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen) and directly sequenced commercially at an ABI-PRISM dye terminator DNA sequencing facility at the Division of Biochemistry, Delhi University, South Campus, New Delhi. The generated sequences were analysed using the EDITSEQ and MegAlign modules of the DNASTAR software.

**Real time PCR.** A quantitative SYBR Green PCR assay was performed on a real time thermocycler (Cepheid) using commercial reagents procured from Qiagen. TW DNA samples were run together with a DNA standard and a standard curve was constructed in order to calculate copy numbers. The PCR protocol for BPV-1 and -10 was comprised of identical steps except for the annealing temperatures, which were 51 °C and 55 °C, respectively. The standard curve, amplification curve and dissociation curve were analysed after completion of the reaction.

## RESULTS

A total of 11 teat wart cases were observed in the dairy cows which appeared grossly as rice grain-like, small, sessile elevated greyish or flesh-like growths. These were usually multiple in numbers and had high vascularity. All cases of teat papillomatosis were without any secondary bacterial infection or injury. Out of 11 TW samples, seven were positive for BPV-10 and three for BPV-1. Among these samples, two were found to have mixed BPV-1 and -10 infection. Gel electrophoresis in 1.2% agarose gel showed the specific PCR product sizes of 301 and 192 bp for BPV-1 and -10, respectively (Figure 1). Detailed results are presented in Table 1.

The specific PCR products were extracted and purified from the agarose gel using a commercial available kit. Purified PCR products were directly sequenced at an ABI-PRISM dye terminator DNA sequencing facility using both forward and reverse primers. Generated sequences were submitted to NCBI and allotted accession numbers (FR874934, FR874937, FR874938, FR874939). The generated sequences in this study showed close homology with earlier published sequences.

Real time PCR of TW DNA samples revealed that copy numbers of BPV-10 DNA varied from  $3.56E + 02$  to  $5.23E + 06$ . The highest copy number was observed in TW-11 which was a small, sessile elevated growth. Three samples of TWs positive for BPV-1 showed DNA copy numbers of  $2.62E + 04$ ,  $2.99E + 04$

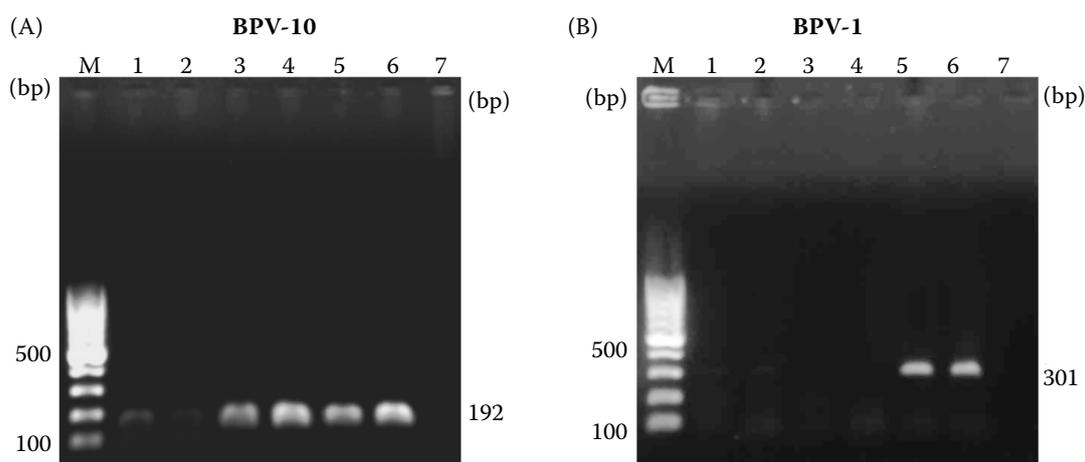


Figure 1. (A) Teat warts: PCR products in ethidium bromide-stained 1.2% agarose in TBE gel electrophoresis. M = 100 bp marker; L1 to L6 = BPV-10 – 192 bp, L7 = NTC. (B) Teat warts: PCR products in ethidium bromide-stained 1.2% agarose in TBE gel electrophoresis. M = 100 bp marker; L5 to L6 = BPV-1 – 301 bp, L7 = NTC

Table 1. Detection and quantification of BPV-1 and BPV-10 in teat warts of cattle using real time PCR

| Number of sample | Species | Age (years) | Location of growth                    | PCR results |        |            |            |
|------------------|---------|-------------|---------------------------------------|-------------|--------|------------|------------|
|                  |         |             |                                       | BPV-1       | BPV-10 | BPV-1      | BPV-10     |
| TW-1             |         | 4.5         | rice grain-like growth on teat        | +ve         | +ve    | 2.62E + 04 | 5.91E + 05 |
| TW-2             |         | 6           | rice grain-like growth on teat        | +ve         | -ve    | 2.99E + 04 | -          |
| TW-3             |         | 5           | small elevated sessile growth on teat | -ve         | -ve    | -          | -          |
| TW-4             |         | 6           | rice grain-like growth on teat        | -ve         | -ve    | -          | -          |
| TW-5             |         | 6           | small elevated sessile growth on teat | -ve         | +ve    | -          | 3.56E + 02 |
| TW-6             | cattle  | 5.5         | rice grain-like growth on teat        | -ve         | +ve    | -          | 8.12E + 02 |
| TW-7             |         | 7           | rice grain-like growth on teat        | +ve         | +ve    | 1.12E + 04 | 1.91E + 06 |
| TW-8             |         | 5           | small elevated sessile growth on teat | -ve         | -ve    | -          | -          |
| TW-9             |         | 5.5         | rice grain-like growth on teat        | -ve         | +ve    | -          | 8.60E + 05 |
| TW-10            |         | 4           | rice grain-like growth on teat        | -ve         | +ve    | -          | 3.27E + 06 |
| TW-11            |         | 5.5         | small elevated sessile growth on teat | -ve         | +ve    | -          | 5.23E + 06 |

and  $1.12E + 04$ . Detailed results of the Ct values and quantity (copy numbers)/ $\mu\text{l}$  obtained in the SYBR Green qPCR of BPV-1 and -10 are shown in Table 1.

## DISCUSSION

A total of 11 teat wart samples were processed in this study. Most were rice grain-like and small, sessile elevated greyish or flesh-like growths, usually multiple in numbers. Earlier investigators reported teat warts with similar morphological features along with flower-like and frond type growths (Singh and Somvanshi 2010). Teat warts with elongated spine-like growths were also reported.

Out of 11 TW samples, seven were positive for BPV-10 and three for BPV-1. Two samples were found to have mixed infection of BPV-1 and -10. The detection of BPV-10 using PCR in the teat warts of Indian cattle is a novel finding as earlier studies detected only BPV-1 and -2 in the cutaneous warts of cattle (Pangty et al. 2010; Kumar et al. 2013a), buffalo (Singh and Somvanshi 2010; Kumar et al. 2013a) and yak (Bam et al. 2012). The association of BPV-10 with the teat warts of cattle was also observed in cattle papillomatosis studies of other countries (Hatama et al. 2008). BPV-1 was detected in the three cases of teat warts with rice grain-like morphological appearance, while BPV-10 was detected in both rice grain-like and small, elevated sessile growths.

Real time PCR allowed us to determine copy numbers of the BPV-10 DNA in these samples

which varied from  $3.56E + 02$  to  $5.23E + 06$ . The highest copy number was detected in TW-11 which was a small, sessile elevated growth. There was no correlation between type of growth and viral DNA copy number as both types of growth showed a range of copy numbers and both the highest and lowest copy numbers were observed in small, sessile elevated growths. The three TW samples positive for BPV-1 had copy numbers of  $2.62E + 04$ ,  $2.99E + 04$  and  $1.12E + 04$ .

In this study, we have found an association of teat warts with BPV-10 and -1. This is supported by earlier studies. However, there are also studies that have reported the presence of DNA of other BPV types such as BPV-1, -2, -3, -5 and -6 in the teat warts of cattle (Lindholm et al. 1984; Ogawa et al. 2004; Maeda et al. 2007). It is believed that BPVs exhibit site specificity since some are associated with cutaneous warts and some with mucosal warts; however, different studies have reported contrasting findings and various types of BPVs have been detected both in the cutaneous and mucosal warts of cattle (Jarret et al. 1984; Bloch et al. 1994, 1996; Carvalho et al. 2012). The present study indicates that BPV-10 and -1 are associated with teat warts and supports the idea that BPVs are not strictly site-specific.

## Acknowledgements

We are thankful to the DDG (Education), ICAR, New Delhi, for a financial grant through the ICAR-NAE Project. We also thank the Director of the IVRI,

Izatnagar, UP, India for providing all necessary facilities for carrying out this research work.

## REFERENCES

- Bam J, Kumar P, Leishangthem GD, Saikia A, Somvanshi R (2012): Spontaneous cutaneous papillomatosis in yaks and detection and quantification of bovine papillomavirus-1 and -2. *Transboundary and Emerging Diseases*, doi:10.1111/j.1865-1682.2012.01361.x
- Bloch N, Sutton RH, Spradbrow PB (1994): Bovine cutaneous papillomas associated with bovine papillomavirus type 5. *Archives of Virology* 138, 373–377.
- Bloch N, Breen M, Irvin ZV, Spradbrow BP (1996): Bovine papillomavirus type 4 DNA isolated from a skin lesion in a steer. *Veterinary Record* 138, 414–416.
- Campo MS (2002): Animal models of papillomavirus pathogenesis. *Virus Research* 89, 249–261.
- Carvalho CCR, Batista MVA, Silva MAR, Balbino VQ, Freitas AC (2012): Detection of bovine papillomavirus types, co-infection and a putative new BPV 11 subtype in cattle. *Transboundary and Emerging Diseases* 59, 441–447.
- De Villiers EM, Faquet C, Brooker TR, Bernard HU, Hausen H (2004): Classification of papillomaviruses. *Virology* 324, 17–27.
- Hatama S, Nobumoto K, Kanno T (2008): Genomic and phylogenetic analysis of two novel bovine papilloma virus BPV-9 and BPV-10. *Journal of General Virology* 89, 158–163.
- Hatama S, Ishihara R, Ueda Y, Kanno T, Uchida I (2011): Detection of a novel bovine papillomavirus type 11 (BPV-11) using xipapillomavirus consensus polymerase chain reaction primers. *Archives of Virology* 156, 1281–1285.
- Jarrett WFH, Mcneil PE, Laird HM, O'neil BW, Murphy J, Campo MS, Moar MH (1980): Papilloma viruses in benign and malignant tumors of cattle. In: *Viruses in naturally occurring cancers*. Cold Spring Harbor Conference on Cell Proliferation 7, 215–222.
- Jarret WF, Campo MS, O'neal B, Laird H, Coggins L (1984): A novel bovine papilloma virus (BPV-6) causing true epithelial papilloma of the mammary gland skin: A member of a proposed new BPV subgroup. *Virology* 136, 255–264.
- Kumar P, Nagarajan N, Kumar D, Bind RB, Somvanshi R (2013a): Detection and quantification of bovine papillomaviruses (BPVs) in cutaneous warts of cattle and buffaloes. *Advances in Animal and Veterinary Science* 1, 53–58.
- Kumar P, Nagarajan N, Saikumar G, Arya RS, Somvanshi R (2013b): Detection of bovine papillomaviruses (BPVs) in war-like lesions of upper gastrointestinal tract of cattle and buffaloes. *Transboundary and Emerging Diseases*, doi:10.1111/tbed.12127.
- Lindholm I, Murphy J, O'neil BW, Campo MS, Jarrett WFH (1984): Papillomas of the teat and udder of cattle and their causal virus. *Veterinary Record* 115, 574.
- Lunardi M, Alfieri AA, Otonel RA, de Alcantara BK, Rodrigues WB, de Miranda AB, Alfieri AF (2013): Genetic characterization of a novel bovine papillomavirus member of the Deltapapillomavirus genus. *Veterinary Microbiology* 162, 207–213.
- Maeda Y, Shibahara T, Wada Y, Kadota K, Kanno T, Uchida I, Hatama S (2007): An outbreak of teat papillomatosis in cattle caused by bovine papilloma virus (BPV) type 6 and unclassified BPVs. *Veterinary Microbiology* 121, 242–248.
- Ogawa T, Tomita Y, Okada M, Shinozaki K, Kubonoya H, Kaiho I, Shirasawa H (2004): Broad-spectrum detection of papillomaviruses in bovine teat papillomas and healthy teat skin. *Journal of General Virology* 85, 2191–2197.
- Ogawa T, Tomita Y, Okada M, Shirasawa H (2007): Complete genome and phylogenetic position of bovine papillomavirus type 7. *Journal of General Virology* 88, 1934–1938.
- Pangty K, Singh S, Saikumar G, Goswami R, Somvanshi R (2010): Detection of BPV-1 and -2 and quantification of BPV-1 by real-time PCR in cutaneous warts in cattle and buffaloes. *Transboundary and Emerging Diseases* 57, 185–196.
- Singh V, Somvanshi R (2010): BPV-2 associated papillomatosis in Indian Water Buffaloes (*Bubalus bubalis*). *Indian Journal of Animal Science* 80, 956–960.
- Tomita Y, Literak L, Ogawa T, Jin Z, Shrivastava H (2007): Complete genomes and phylogenetic positions of bovine papillomavirus type 8 and a variant type from a European bison. *Virus Genes* 35, 243–249.
- Zhu W, Dong J, Shimizu E, Hatama S, Kadota K, Goto Y, Haga T (2012): Characterization of novel bovine papillomavirus type 12 (BPV-12) causing epithelial papilloma. *Archives of Virology* 157, 85–91.

Received: 2013–09–11

Accepted after revision: 2013–12–01

Corresponding Author:

Pawan Kumar, Indian Veterinary Research Institute, Division of Pathology, Izatnagar, Bareilly, Uttar Pradesh, 243122 India  
Mobile +91 9023469447, E-mail: chauhan2k1@gmail.com