



## NOTE

Virology

# Full genome analysis of bovine papillomavirus type 1 derived from a calf with severe cutaneous multiple papillomatosis

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**ABSTRACT.** Severe papillomatosis occasionally causes astasia leading to euthanizing cattle. There are currently a limited number of reports on virologic approach in severe bovine papillomatosis. Here we report a full genome characterization of bovine papillomavirus type 1 (BPV-1) from the case of severe papillomatosis. A calf developed numerous papillomas on the skin and some nodules in the upper gastrointestinal tract at seven months old. The skin lesion was diagnosed as the epithelial papilloma with BPV antigen expression, while the gastrointestinal lesions were diagnosed as the fibropapilloma without BPV antigen. Full genome analysis revealed that BPV-1s detected in all the lesions were exactly the same. Compared with the reference BPV-1 sequence, there was a single nucleotide insertion in the upstream regulatory region.

**KEY WORDS:** bovine papillomavirus type 1, calf, full genome sequencing, severe papillomatosis

*J. Vet. Med. Sci.*

80(11): 1691–1695, 2018

doi: 10.1292/jvms.18-0289

Received: 26 May 2018

Accepted: 1 September 2018

Published online in J-STAGE:  
12 September 2018

Bovine cutaneous papillomatosis caused by bovine papillomavirus (BPV) usually regress spontaneously [7], while the severe cases are considered to uncommon. In fact, the actual prevalence of severe case is unknown, because the investigation for severe bovine papillomatosis is limited. In such a case, papillomas spread over a wide area of skin, and often recurrent even after the surgical excision. The severe papillomatosis causing astasia may lead to euthanizing cattle. So far, there is no effective treatment for the severe papillomatosis. To our knowledge, there is the only one study describing about severe bovine papillomatosis case and its causative BPV [2], although there is limited information from the aspect of BPV. Here we report a severe case of bovine papillomatosis, and a full genome characterization of BPVs detected from different lesions of the animal.

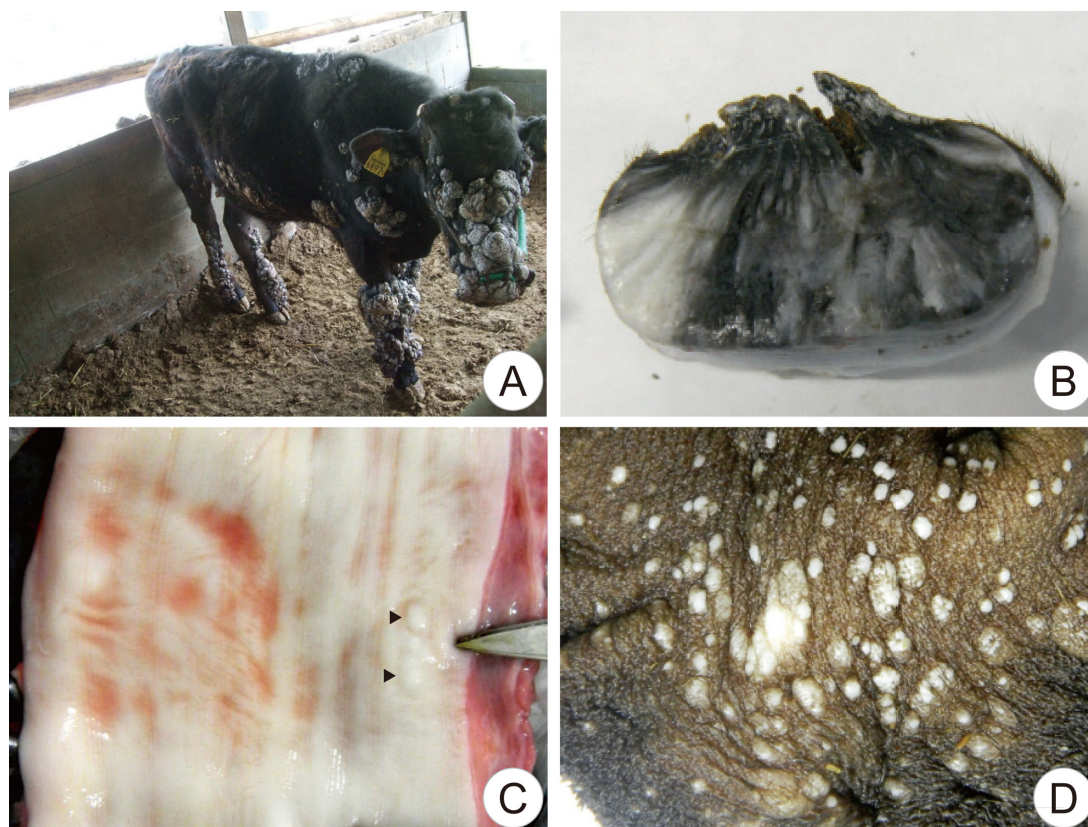
A Japanese Black calf (seven-month-old, castrated male) developed numerous warts on the skin (Fig. 1A). There were no other cattle showing the severe papillomatosis in the same farm. The warts were surgically excised from the animal but relapsed two months afterwards. The calf had recurrence of papillomas after several surgical treatments and was never cured. Due to the severe and aggressive development of the lesion, the calf was eventually euthanatized and examined postmortem at 13 months old. The cut surface of the warts on the skin showed cauliflower-like appearance (Fig. 1B). Some nodules were found in the esophagus and rumen (Fig. 1C and 1D, respectively). Other parts of the gastrointestinal tract were macroscopically normal. No abnormality was found in the urinary bladder and penis, where BPV occasionally produces lesions. Those nodular lesions were collected for

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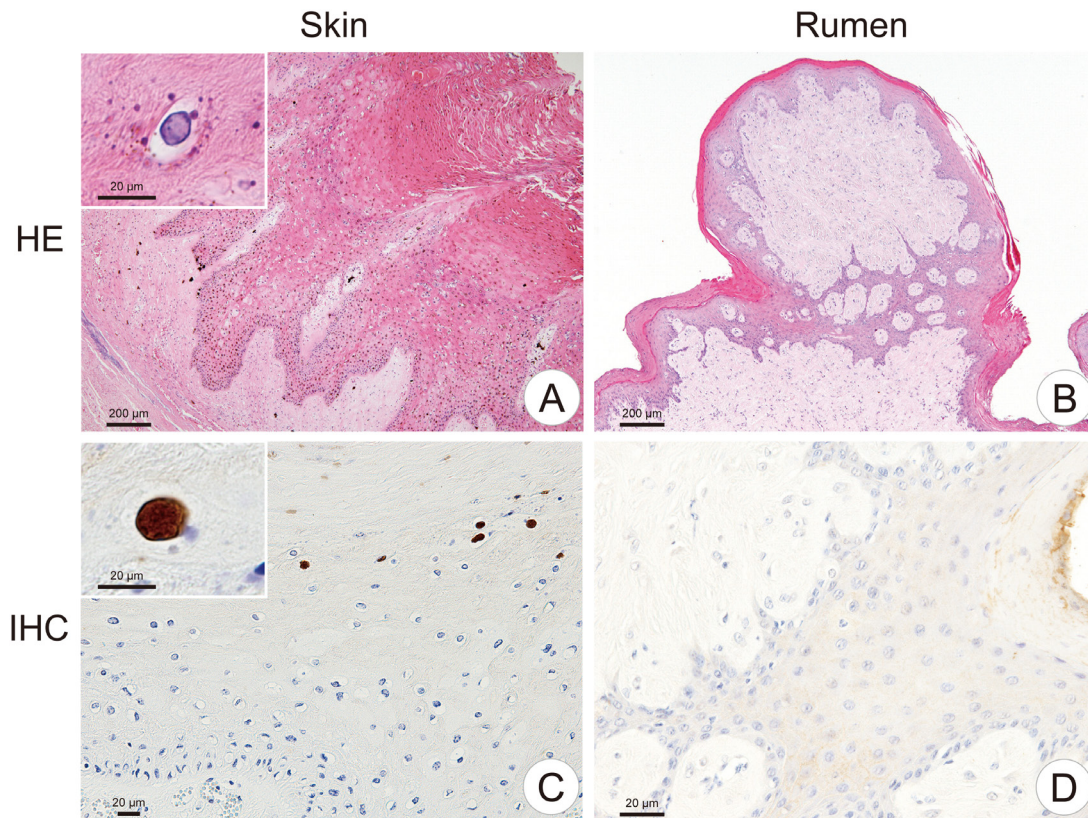
**Fig. 1.** Gross lesions in the calf with severe cutaneous multiple papillomatosis. The numerous warts around the face and limbs (A). Cut section of a wart collected from the skin (B). Nodules in mucosal surface of the esophagus (C, arrowhead) and rumen (D).

histopathological and molecular virological analysis. The omasum was also collected for evaluating the BPV infection.

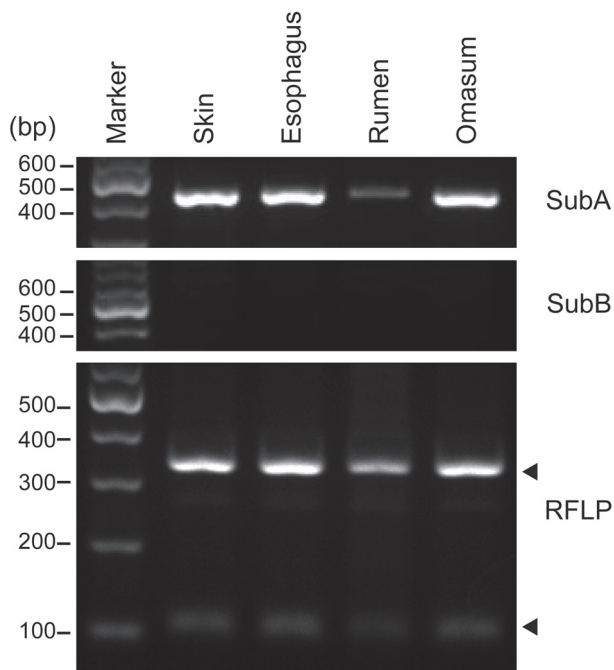
Histopathological and immunohistochemical analyzes of these specimens were performed. In addition to the hematoxylin and eosin staining, immunohistochemical staining for detecting BPV antigen was conducted as described previously [9]. The warts in the skin consisted of squamous epithelial cells with irregular papillary proliferation and orthokeratotic hyperkeratosis (Fig. 2A). Eosinophilic intranuclear inclusion bodies were observed in the squamous epithelial cells (Fig. 2A, inset). In the rumen, fibroblasts proliferated in lamina propria and formed a fibropapilloma (Fig. 2B). The inclusion bodies in the specimen of skin were strongly positive for BPV antigen by immunohistochemistry (Fig. 2C). In contrast, the BPV antigen was not detected in the rumen (Fig. 2D), where no inclusion body was found. Similar histopathological findings were observed in the esophagus as well as the rumen (data not shown). Depending on the anatomical site, both morphological and biological features of PV-infected lesions may differ [5]. In this study, inclusion bodies corresponded to the site of viral antigens detected by immunohistochemistry. This may be due to that BPV can replicate in differentiated epithelial cells where the inclusion bodies formed, although BPV in fibroblast cells is thought to remain premature [1].

Detection and typing of papillomavirus were performed by polymerase chain reaction and restriction fragment length polymorphism (RFLP). Two primer sets, subA and subB, were used for the detection of BPV [3]. The subA amplifies the genus, deltapapillomavirus (BPV-1, 2, 13), epsilonpapillomavirus (BPV-5, 8) and dyoxipapillomavirus (BPV-7), and the subB amplifies xipapillomavirus (BPV-3, 4, 6, 9, 10, 11, 12). With the subA, single bands were observed at approximately 440 base pairs (bp) in all specimens including skin, esophagus, rumen and omasum (Fig. 3), while no bands with the subB. The subA amplicons were digested into approximately 330 and 110 bp fragments by the restriction enzyme, *Afa* I (Takara Bio Inc., Kusatsu, Japan), indicating the pattern of either BPV type 1 or 13 (Fig. 3) [3].

To further analyze the genome of BPVs derived from four different tissues (skin, esophagus, rumen and omasum), the full genome sequencing of each BPV was performed as previously reported [9]. The three primer sets were designed to amplify full genome: BPV1\_whole\_1 (forward: 5'-CCGTGCCATTTCGGCCGTCCT-3' and reverse: 5'-CCACCGGTACCGTGCCTGC-3'), BPV1\_whole\_2 (forward: 5'-GCACCATGGCCGGTGCTGGA-3' and reverse: 5'-ACTGCCACACCAGCCGCTC-3') and BPV1\_whole\_3 (forward: 5'-TGCAGAAACGGAGCGCCTGCT-3' and reverse: 5'-ACACCTGCCCCGGGTTTCCTCA-3'). These amplicons were purified and then sequenced with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) and 3130 × 1 Genetic Analyzer (Thermo Fisher Scientific), according to the manufacturer's instructions. The results showed that the BPVs from four different tissues were completely the same (sequence deposited under DDBJ accession number LC333380). The blast analysis showed that 99.9% nucleotide identity compared with the reference sequence of BPV-1



**Fig. 2.** Histology (A and B) and immunohistochemistry (C and D) of the specimens of the skin and rumen. (A) Papillary proliferation of squamous epithelial cells. The inset shows nuclear inclusion body. (B) The nodules in the rumen mucosa are composed of squamous epithelial cells and fibrous tissue. (C) Nuclear inclusions in the squamous cells of the skin are positive for BPV antigen. (D) Squamous epithelial cells and fibroblasts in the rumen are negative for BPV antigen. A and B, Hematoxylin and eosin staining (HE); C and D, Immunohistochemistry for BPV (IHC).



**Fig. 3.** Detection and typing of papillomavirus in the four tissues. Two primer sets, subA and subB, were used for detecting BPV genomic DNA. Additionally, type of BPV was speculated by RFLP using amplicons of subA. The digested fragments are shown by arrowhead. bp: base pairs.

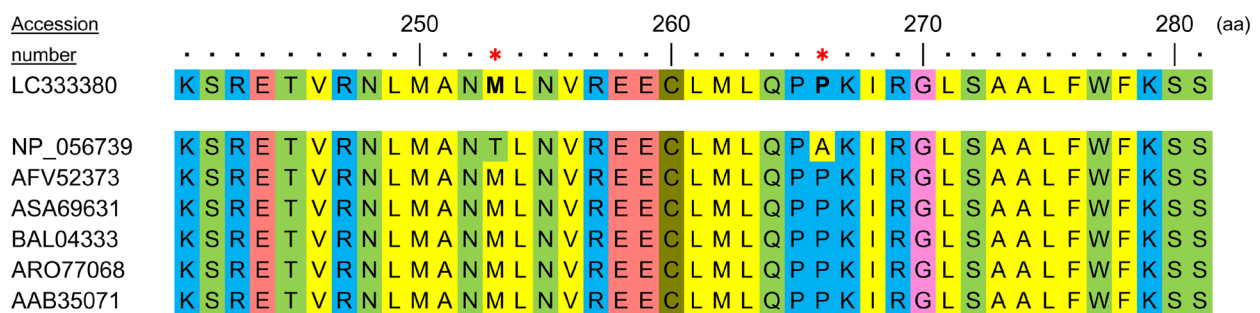
in the database of the PapillomaVirus Episteme (PaVE, <https://pave.niaid.nih.gov/index.html>). Eight nucleotide changes were identified, and two of these resulted in amino acid substitutions (Table 1). Search in the database of the National Center for Biotechnology Information (NCBI, <https://www.ncbi.nlm.nih.gov/nucleotide/>) showed that these substitutions in E1 protein were the same as the all registered sequences except for NP\_056739, which is the original source of the reference in PaVE (Fig. 4). Since the designated sequence of the reference BPV-1 (NP\_056739) was deposited based on the first BPV-1 report in 1982 as the draft sequence, the sequence may not strongly be reliable [8]. Therefore, the revision of the sequence of the reference may need to be considered. The insertion at 7,148 nucleotides (nt) located in the upstream regulatory region (URR) has not been found so far in the sequences of BPV-1 databases (Table 1, Fig. 5).

Host factors may be associated with the development of papillomatosis. The difference of susceptibility to BPV infection in individual cattle was reported previously [6], and immune status may affect the susceptibility to develop papillomatosis [2, 4]. In our case, the calf had not shown increased susceptibility to infections over the lifetime. The calf also had no history of long-term excess administration of immunosuppressive agents. Therefore, there is no evidence to suggest that the calf was congenital or acquired immunodeficient. Further studies on the host immune status are required for understanding the pathology

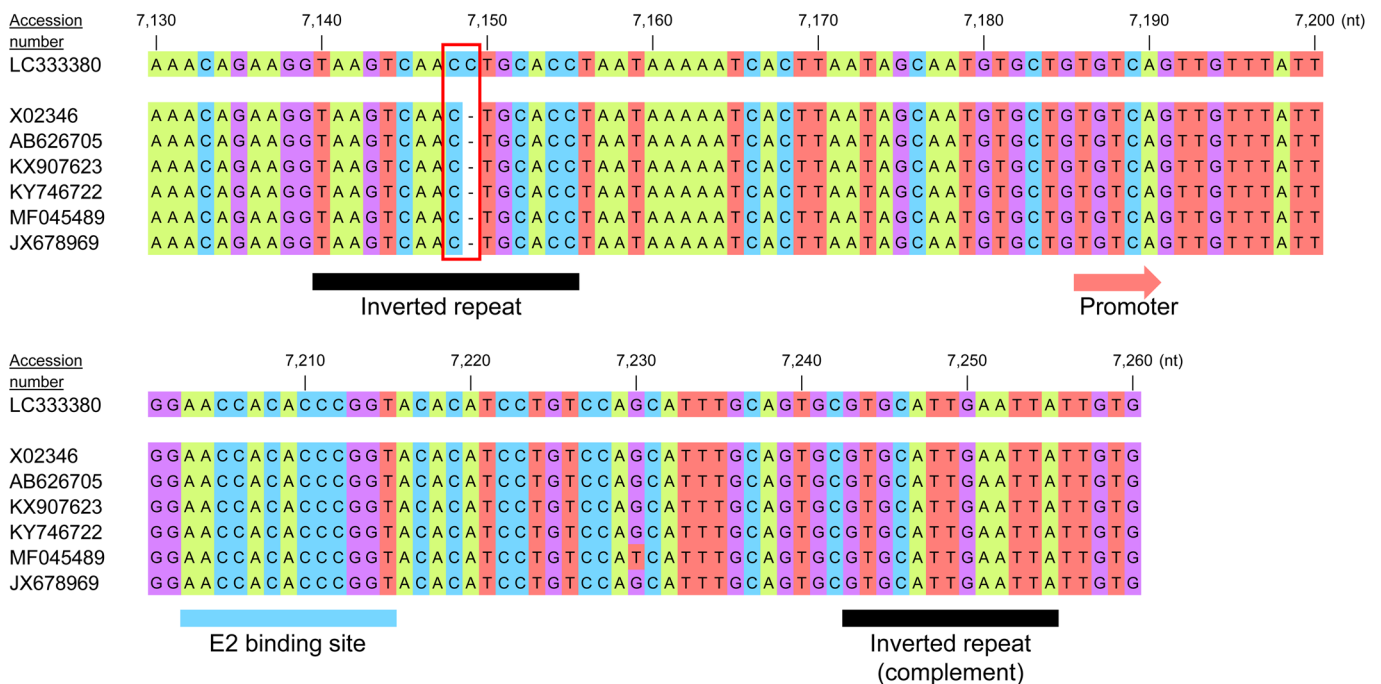
**Table 1.** Genetic changes of BPV-1 detected in this study by comparing with the reference

Position (nt <sup>a</sup> )	Reference genome <sup>b</sup>	BPV-1 genome in this study	Region	Amino acid substitution
138	T	C	E6 ORF <sup>c</sup>	Synonymous
144	G	T	E6 ORF	Synonymous
652	C	G	E7 ORF	Synonymous
1,606	C*	T	E1 ORF	T253M
1,644	G*	C	E1 ORF	A266P
4,016	C	T	NCR <sup>d</sup>	N/A <sup>f</sup>
5,341	T	A	L2 E1 ORF	Synonymous
7,148	C	CC	URR <sup>e</sup>	N/A

a) nt, Nucleotides. b) PaVE ID: BPV1REF. c) ORF, Open reading frame. d) NCR, Non-coding region. e) URR, Upstream regulatory region. f) N/A, Not available. \*The base was observed only in the reference genome.



**Fig. 4.** Multiple protein sequence alignment on the amino acid substitutions in E1 protein. Asterisk showed the position of substitution in LC333380 compared with the reference sequence in PaVE. aa: amino acid.



**Fig. 5.** Multiple DNA sequence alignment and annotations around the insertion at 7,148 nt. The cytosine at 7,148 nt was only duplicate in the sequence of this study (red frame). The insertion exists on inverted repeat (7,140–7,155 nt) which binds to complementary sequence (7,243–7,255 nt). Promoter exists in downstream from 7,186 nt. E2 protein binds sequences from 7,203 to 7,215 nt. nt: nucleotides.

of severe papillomatosis.

To our knowledge, the full genome analysis of BPV derived from severe bovine papillomatosis has not been reported previously. Therefore, we sought to characterize the full genome sequence of the BPV detected from this severe papillomatosis case by comparing those from benign cases in the databases. Although no significant mutation was found in the coding regions in this study, we found a single nucleotide insertion in URR (Fig. 5). Because the reference BPV sequences in databases are all from benign lesions, it is interesting that if the insertion in URR may have a potential to affect the pathogenicity of BPV-1 [3, 5, 7]. Further studies are needed to determine the significance of this insertion in URR.

In conclusion, the calf showed severe papillomatosis having recurrence and aggressive development of disease resulting in euthanasia. This study provides complete genomic information of BPV derived from the severe papillomatosis. Although histopathological findings were different between the skin and upper gastrointestinal tract lesions, BPV-1s detected in these lesions showed exactly the same genome sequence. The BPV-1s showed a single nucleotide insertion after position 7,148 nt in URR. This report would contribute to further understanding the pathology of severe bovine papillomatosis.

**ACKNOWLEDGMENTS.** This research was partly supported by a grant from the Project of NARO Bio-oriented Technology Research Advancement Institution (the special scheme project on vitalizing management entities of agriculture, forestry, and fisheries). No conflicts of interest have been declared.

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