NOTE *Virology*

Papillomavirus in yaks: the isolates of bovine papillomavirus type 1 have a high possibility of belonging to a novel type

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(Received 17 August 2015/Accepted 14 February 2016/Published online in J-STAGE 27 February 2016)

ABSTRACT. Although papillomaviruses (PVs) have been widely reported in vertebrates, there have been only a few PV reports in yaks (*Bos grunniens*). In 2012, Bam *et al.* reported bovine papillomavirus type 1 (BPV-1) and BPV-2 associated with cutaneous papillomatosis in yaks, which provided genomic and pathology information for yak PVs. However, nucleotide identity and phylogenic analyses revealed that there are two isolates with a high possibility of belonging to a novel type that is not BPV-1. The argument was thought to be caused by type-specific primers. Our analysis showed that BPV-1 type-specific primers can detect not only BPV-1 but also other PVs. It suggests that identification results using type-specific primers should be confirmed with more robust methods in molecular epidemiological studies.
KEY WORDS: novel type, papillomavirus, type-specific primer, yak

doi: 10.1292/jvms.15-0479; *J. Vet. Med. Sci.* 78(6): 1059–1061, 2016

Papillomaviruses (PVs) are non-enveloped, double-stranded DNA viruses and have been identified as infecting mammals and also amniotes, such as birds, snakes and turtles [3, 9, 10, 12, 14]. Since the first animal PV was identified in cottontail rabbits in the 1930s, more than 200 PV types have been identified [3, 9], and novel types were recently discovered one by another. PVs are thought to be highly species-specific to their host based on the epidemiological studies. However, *Deltapapillomavirus*, especially BPV type 1 (BPV-1) and type 2 (BPV-2), showed the cross-species infection. BPV-1 and BPV-2 not only caused skin warts in cattle, but also can infect equine and lead to the development of benign tumors known as sarcoid [6, 16]. Meanwhile, BPV-1 can infect rabbits, hamsters and mice experimentally [4, 18]. Although PVs have been widely reported in vertebrates, there are only a few PV reports in yak (*Bos grunniens*). In 2012, Bam *et al.* reported BPV-1 and BPV-2 associated with cutaneous papillomatosis in yaks in the North-East Region of India [1]. Subsequently, our group identified the first novel PV type in yaks, *Bos grunniens* papillomavirus type 1 (BgPV-1) [21]. Both studies provided genomic and pathology information for yak PVs.

However, the analysis of the viral genomic sequences reported in the Indian article showed that a putative novel PV type was possibly misclassified as BPV-1. In that study, 301 bp and 165 bp fragments of the L1 region were amplified and sequenced for the BPV-1 and BPV-2 isolates, respectively. Sequences of 2 BPV-1 isolates [PKC-24 (HE603637) and PKC-25 (HE603638)] and 2 BPV-2 isolates [PKC-22 (HE603639) and PKC-23 (HE603640)] were deposited in GenBank [1]. Homology analysis was performed with MegAlign program in DNAstar software package (DNAStar, Madison, WI, U.S.A.), and the result showed that PKC-24 and PKC-25 possessed only 80.4% and 79.7% nucleotide identity with the BPV-1 isolate (X02346), while PKC-22 and PKC-23 possessed 95.7% and 97.6% identity with the BPV-2 isolate (M20219) [11]. According to the criteria of the Papillomavirus Study Group for the International Committee on Taxonomy of Viruses (ICTV), a new PV strain can be designated as a novel type, if the complete genome has been cloned and it shows less than 90% nucleotide identity in complete gene coding for the major capsid protein (L1 gene) with respect to the closest known PV type [3, 9]. The BLAST results for PKC-24 and PKC-25 showed that BPV-1 is the closest similar type, with an approximately 80% nucleotide sequence identity. Although only a partial sequence of L1 was determined for the two isolates, it is suggested that PKC-24 and PKC-25 likely belong to a novel type, not BPV-1.

Furthermore, a phylogenic analysis was performed based on the 301 bp sequences of PKC-24 and PKC-25 and the comparable L1 ORF of published BPV and BgPV types. The analysis revealed that PKC-24 and PKC-25 formed a new cluster at a significant distance from the clusters of BPV-1, BPV-2, BgPV-1 and other types (Fig. 1). Meanwhile, in order to avoid disturbance of recombination to phylogenic analysis, the possibility of recombination along with potential breakpoints was investigated using the DnaSP algorithm [15], and the minimum number of recombination events Rm was established according to Hudson [13]. As a result, there was no evidence of recombination (Rm=0) with BgPV-1 and BPV-1 to -14. Taken together, these results strongly support the conclusion that PKC-24 and PKC-25 belong to a novel

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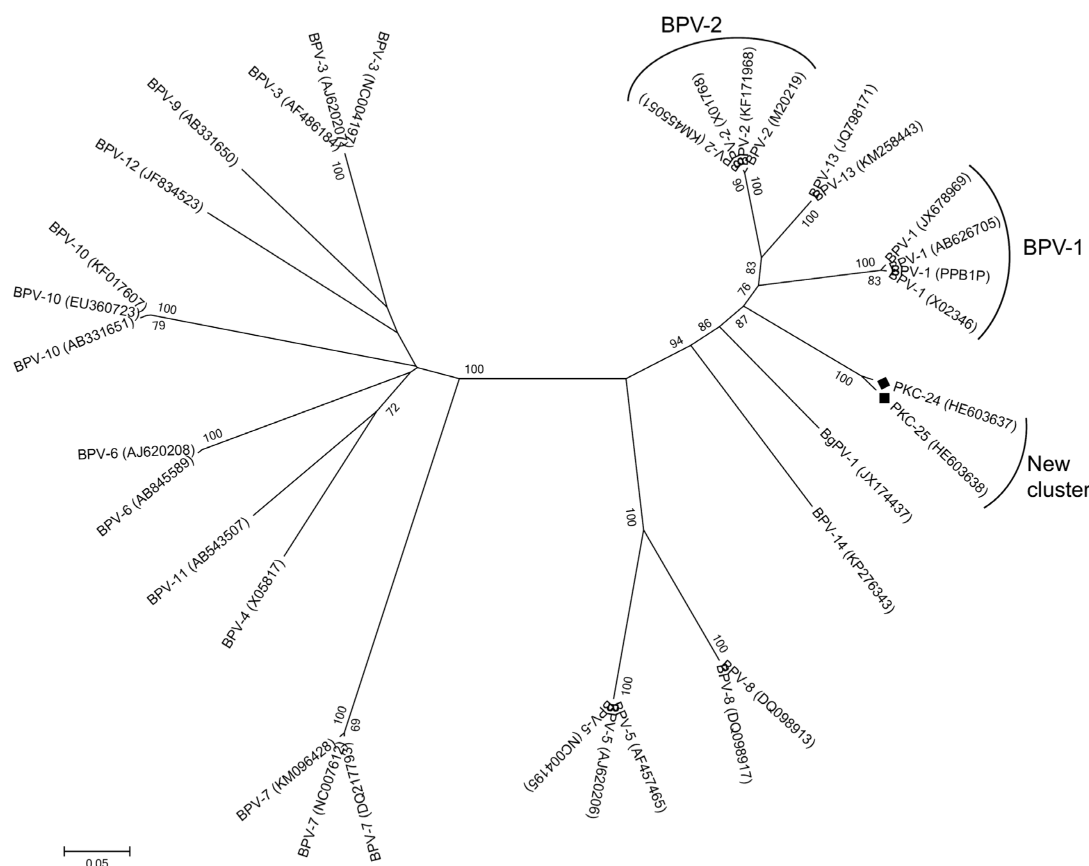

Fig. 1. Phylogenetic tree of PKC-24, PKC-25, BgPV-1 and reported BPVs. The analysis was performed based on a 301 bp nucleotide sequence of PKC-24 and PKC-25 and comparable L1 ORFs of BgPV-1 and BPVs. The tree was constructed with the neighbor-joining method in conjunction with the maximum-composite likelihood algorithm to compute evolutionary distance using MEGA version 6 [19]. The numbers at the nodes show the percentage occurrence in 1,000 bootstrap replicates. Bar, 0.1 nucleotide substitutions per site.

PV type.

The cause of the misclassification of the novel type is potentially the type-specific primers, which are often used to evaluate individual types in molecular epidemiological studies in the bovine PVs. Although there are high diversities among genomes of different PV types, there are some conserved regions in L1 ORFs in one genus. For example, in the genus *Deltapapillomavirus*, L1 ORFs showed several highly conserved regions in BPV-1, BPV-2, BPV-13 and BgPV-1. Therefore, nucleotide sequence alignment should be thoroughly analyzed, and type-specific primers should be designed out of the conserved regions. In 2003, a pair of type-specific primers for BPV-1 was first reported [8], and it was widely used in molecular epidemiological studies [1, 2, 5, 7, 20]. Because BPV-1 belongs to the genus *Deltapapillomavirus*, we analyzed the priming efficiency of the type-specific primer set to detect other PV types in the same genus by using Oligo Primer Analysis Software version 6.31 (Molecular Biology Insights, Inc., Colorado Springs, CO, U.S.A.). The concept of priming efficiency was introduced to provide an accurate way to predict false priming. Primers work well, if their priming efficiency is above the threshold [17]. Our result suggested that this BPV-1 type-specific primer set not only can amplify the fragment of BPV-1, but it can also amplify the corresponding fragments from BPV-13 (JQ798171) (Fig. 2). Meanwhile, the results of a study of Bam *et al*. (2012) showed that the BPV-1 type-specific primer set can amplify the corresponding fragments from unknown PVs. Taken together, the evidences suggest that the widely used type-specific primers for BPV-1 are not type-specific.

In conclusion, the PKC-24 and PKC-25 strains in the study by Bam *et al*. are likely a novel type, not BPV-1. In addition, although the type-specific primer set for BPV-1 has been widely used in molecular epidemiological studies, it is not type-specific. This study suggests that the identification results with type-specific primers should be confirmed with more robust methods in molecular epidemiological studies of PVs.

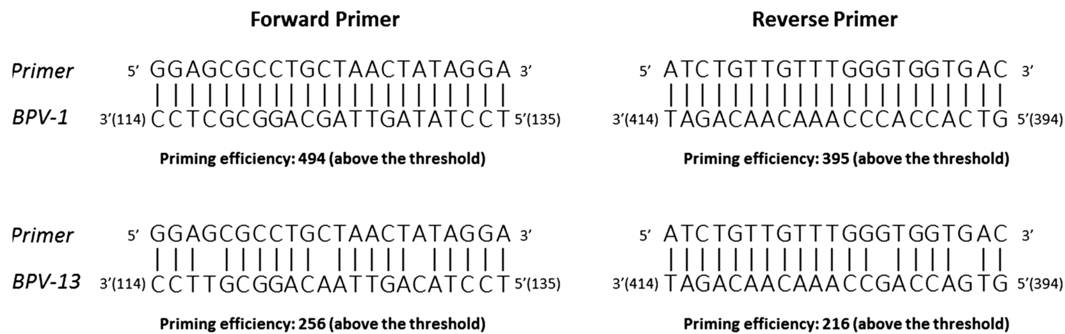

Fig. 2. A priming efficiency analysis of the BPV-1 type-specific primer set for detecting BPV-1 and BPV-13. The analysis was performed using Oligo Primer Analysis Software version 6.31 (Molecular Biology Insights, Inc.). The BPV strains used for the analysis were BPV-1 (X02346) and BPV-13 (JQ798171). The primer binding sites are indicated with nucleotide locations in the L1 ORF of BPVs.

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