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Molecular detection and genetic diversity of bovine papillomavirus in dairy cows in Xinjiang, China

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ABSTRACT

Background: Bovine papillomatosis is a type of proliferative tumor disease of skin and mucosae caused by bovine papillomavirus (BPV). As a transboundary and emerging disease in cattle, it poses a potential threat to the dairy industry.

Objectives: The aim of this study is to detect and clarify the genetic diversity of BPV circulating in dairy cows in Xinjiang, China.

Methods: 122 papilloma skin lesions from 8 intensive dairy farms located in different regions of Xinjiang, China were detected by polymerase chain reaction. The genetic evolution relationships of various types of BPVs were analyzed by examining this phylogenetic tree.



Results: Ten genotypes of BPV (BPV1, BPV2, BPV3, BPV6, BPV7, BPV8, BPV10, BPV11, BPV13, and BPV14) were detected and identified in dairy cows. These were the first reported detections of BPV13 and BPV14 in Xinjiang, Mixed infections were detected, and there were geographical differences in the distribution of the BPV genotypes. Notably, the BPV infection rate among young cattle (< 1-year-old) developed from the same supply of frozen sperm was higher than that of the other young cows naturally raised under the same environmental conditions.

Conclusions: Genotyping based on the L1 gene of BPV showed that BPVs circulating in Xinjiang China displayed substantial genetic diversity. This study provided valuable data at the molecular epidemiology level, which is conducive to developing deep insights into the genetic diversity and pathogenic characteristics of BPVs in dairy cows.

Keywords: bovine papillomavirus; molecular detection; genetic diversity; Xinjiang, China

INTRODUCTION

Bovine papillomatosis (BP) is a chronic proliferative skin disease caused by bovine papillomavirus (BPV) [1], which results in cutaneous neoplastic lesions and reductions in animal constitution within the cattle industry [2]. As one of the transboundary and emerging diseases in cattle, BP circulates in many countries [3-8]. However, to date, there is no effective vaccine to prevent the occurrence of BP [2].

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Meng Q, Ning C, Wang L; Data curation: Zhang X; Formal analysis: Lian K; Funding acquisition: Qiao J; Investigation: Li Y, Li Z, He Z; Methodology: Wang L, Ren Y, Li J; Project administration: Qiao J, Xiao C; Resources: Ren Y, Qiao J; Software: Xiao C; Supervision: Qiao J; Validation: Wang L; Visualization: Li Z; Writing - original draft: Meng Q, Ning C, Wang L; Writing - review & editing: Cai X, Qiao J.

As a non-enveloped double-stranded DNA virus, BPV is a member of the Papillomaviridae family that commonly infects epithelial cells of bovine skin, mucous membranes, and other tissues [2,9]. The genome DNA of BPV is about 7800 bp, and the virus capsid is mainly composed of L1 protein [10]. So far, at least 27 genotypes of BPVs (BPV1-27) have been identified and subsequently classified into the following genera: *Deltapapillomavirus* genus (BPV1, BPV2, BPV13, and BPV14), *Xipapillomavirus* genus (BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV12, BPV15, BPV17, and BPV20), *Epsilonpapillomavirus* genus (BPV5 and BPV8), *Dyokappapapillomavirus* genus (BPV16, BPV18, and BPV22), *Dyoxipapillomavirus* (BPV7) and unclassified genera (BPV19, BPV21, and BPV27) [8,11-17]. Furthermore, some unclassified papillomaviruses have been subsequently reported [13,15,18,19]. Recently, a novel type, designated as BPV28, from Japan was identified and characterized [17] and classified in the *Xipapillomavirus* genus.

Xinjiang is one of five major cattle-farming areas in China. The dairy cattle inventory in Xinjiang province amounts to 2.8 million, BPV-like infections frequently occur in dairy cattle; however, to date, infection status and the genotypes of BPVs in dairy cows in Xinjiang, China remain unclear. Hence, this study aims to identify and characterize the genotypes of BPVs circulating in intensive dairy farms in Xinjiang, China. The results provide new insights into the molecular characteristics of BPVs, which will be conducive to studies into the pathogenicity of BPVs and the development of an effective vaccine to prevent this disease and its related cutaneous and mucosal tumors in dairy cows.

MATERIALS AND METHODS

Collection of clinical samples

During 2015–2019, a total of 122 clinical samples of Holstein cows with BP-like symptoms were collected from 8 intensive dairy farms located in 5 major cattle-farming areas of Xinjiang province, China (**Supplementary Fig. 1**), including the Changji (18 cases), Shihezi (22 cases), Tacheng (37 cases), Yili (29 cases), and Aksu (16 cases) areas. The skin samples were collected from several parts of dairy cow bodies, including face, neck, shoulder, back, and nipple. All efforts were made to minimize animal stress. Dairy information on age, sex, pathologically damaged sites, and papillomatosis size was also recorded (**Supplementary Table 1**). The obtained papilloma lesion samples were stored at -80°C freezer.

Design of primers

The degenerate primers FAP59-FAP64 (FAP59, 5'-TAACWGTGGICAYCCWTATT-3' and FAP64, 5'-CCWATATCWVHCATITCICCATC-3') were designed as described in the study by Forsslund et al. [20]. The primers were used for polymerase chain reaction (PCR) amplification of viral DNA from the skin samples.

Molecular detection

Briefly, 100 mg of each collected papilloma skin lesion sample were removed using a sterile knife and placed in a tissue grinder. After adding 1 mL of physiological saline, the sample was ground. The homogenate was centrifuged at 3,000 r/min for 1 min, and then 200 μL of supernatant was collected. Total DNA was isolated from the supernatant using a MiniBEST Viral DNA Extraction Kit (TaKaRa Bio, Japan) according to the manufacturer's instructions. The extracted DNA was used as a PCR template. The PCR was carried out using a Bio-Rad C1000 Touch 850W Thermal Cycler (Bio-Rad, USA). The PCR reaction mix included 21 μL of water, 1 μL (0.2 $\mu\text{mol/L}$) of each FAP59-FAP64 primer, 25 μL of $2\times$ Premix Ex Taq (TaKaRa

Bio), and 2 μ L of DNA template. The PCR sequence conditions were as follows: 95°C for 5 min followed by 30 cycles of 40 sec at 94°C, 40 sec at 64°C, and 1 min at 72°C, and a final 10 min at 72°C. The PCR products were separated on 2% agarose gel by electrophoresis, and the gels were stained with ethidium bromide (Takara Bio). Gels were then visualized under UV light and photographed. The DNA bands were recovered using an agarose gel DNA fragment recovery kit (Promega, USA) according to the manufacturer's instructions. The purified DNA fragment was then cloned into pMD19-T (Takara Bio) for sequencing.

Sequencing and comparison of L1 gene DNA sequences

Positive clones that had been screened and verified by PCR and restriction enzyme digestion as correct were randomly chosen to conduct DNA sequencing. When the DNA sequences of at least three clones were completely identical, they were recognized as the original sequence. Using both Clustal W (<https://www.genome.jp/tools-bin/clustalw>) and DNAMAN software (versions: 6.0; Lynnon Corporation, Canada), these DNA sequences were compared to L1 gene sequences of BPV1-28 that were downloaded from GenBank (<https://www.ncbi.nlm.nih.gov/>), and sequence identities were calculated. According to the nucleotide sequence identity of a relatively conserved region of the L1 gene, the different types of BPV share a genetic identity of more than 10%; differences between 2% and 10% identity define a subtype, while a variant is defined when the difference is $< 2\%$ [9,11,21]. Furthermore, relationship details for the detected BPV genotypes associated with age, sex, pathologically damaged sites, and papillomatosis size were also analyzed.

Phylogenetic analysis based on the BPV L1 gene

The gene sequences of various genotypes of BPVs detected in this study, and those of BPVs 1–28 are presented in **Supplementary Table 1**. A phylogenetic tree was constructed by using the neighbor-joining method included in MEGA 5 software [22]. The bootstrap repeat was 1,000. The genetic evolution relationships of various BPV types were analyzed by examining this phylogenetic tree.

Statistical analysis of data

The data were analyzed using SAS software (version 9.1; SAS Institute, Inc., USA). The detection rates of the different BPV types were compared using the χ^2 test. A value of $p < 0.05$ was considered statistically significant, while a $p < 0.01$ was considered extremely significant.

Ethics statement

The experiments were carried out in accordance with the guidelines issued by the Ethical Committee of Shihezi University (No. SHZUA2015-120).

RESULTS

Papilloma in nipple samples had flat, round, and lobed shapes, while papilloma was nodular and cauliflower-like in head and neck samples (**Fig. 1**). Among the 5 major cattle-farming areas, the overall BPV detection rate was 100% in the samples collected from Changji (18 cases), Shihezi (22 cases), Tacheng (37 cases), Yili (29 cases), and Aksu (16 cases).

Among 122 samples from dairy cows with BP-like clinical symptoms, all were positive based on broad-spectrum PCR detection of papillomaviruses. Based on the sequencing results and



Fig. 1. Morphological characteristics of skin and nipple samples in dairy cows infected by bovine papillomavirus. Papilloma nodules (white arrow) were located in neck (A), lower jaw (B), face (C), and nipple (D).

according to the classification standard for papillomaviruses, 10 genotypes were identified (**Fig. 2**). The partial sequences of the L1 genes from ten BPV epidemic strains have been submitted to GenBank (accession numbers: BPV1-XJ05, MT974519; BPV2-XJ18, MT974518; BPV3-XJ21, MT974517; BPV6-XJ38, MT974516; BPV7-XJ42, MT974515; BPV8-XJ76, MT974514; BPV10-XJ91, MT974513; BPV11-XJ102, MT974512; BPV13-XJ29, MT974511; BPV14-XJ82, MT974510) (**Supplementary Table 2**).

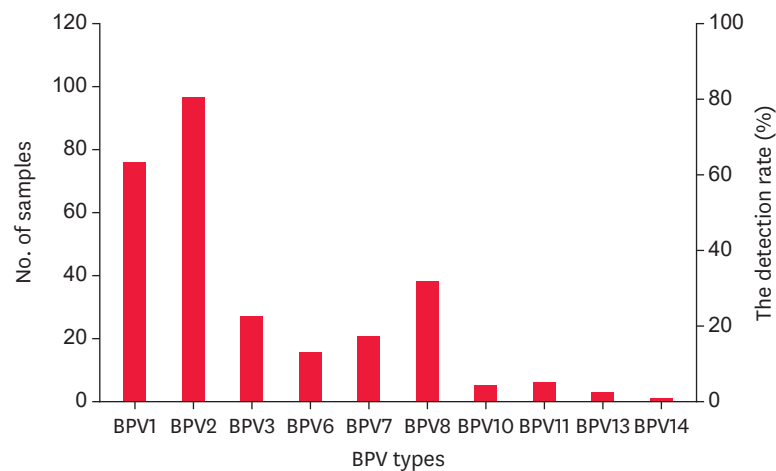


Fig. 2. Detection of BPV infection in young cattle (< 1-year-old) from a dairy farm located in Tacheng, Xinjiang province. BPV, bovine papillomavirus.

The detection rates of the different BPV types were significantly different. Among the 122 dairy cow samples, 98 (80.33%) were infected by BPV2; 77 (63.11%, 77/122) were infected by BPV1; 39 (31.97%, 39/122) were infected by BPV8; 27 (22.13%, 27/122) were infected by BPV3; 21 (17.21%, 21/122) were infected by BPV7; 16 (13.11%, 16/122) were infected by BPV6; 6 (4.92%, 6/122) were infected by BPV11; 5 (4.10%, 5/122) were infected by BPV10; 3 (2.46%, 3/122) were infected by BPV13; 1 (0.82%, 1/122) was infected by BPV14 (**Fig. 2, Supplementary Table 1**). These were the first reported detections of BPV13 and BPV14 in Xinjiang. The results indicate that BPV2 and BPV1 are the predominant genotypes circulating in dairy cows in Xinjiang province, China.

As shown in **Table 1**, there were some geographical differences in the distribution of BPV genotypes. Among them, BPV10, BPV11, and BPV14 were uniquely detected in Tacheng, Changji, and Shihezi, respectively, while BPV1, BPV2, and BPV3 were the most widely distributed in Xinjiang province (**Table 1**). Notably, molecular detection results confirmed that BPV13 and BPV14 were present in Xinjiang, China, and mixed infections of different BPV genotypes were common in dairy cows (**Supplementary Table 1**), accounting for 98.08% (116/122) of all samples. By contrast, the BPV detection rate in young cattle (< 1-year-old) developed from the same supply of frozen sperm (43.75%, 21/48) was significantly higher ($p < 0.05$) than that of young cattle naturally reproduced (14.29%, 6/42) at the same dairy farm located in Tacheng environment (**Fig. 3, Supplementary Table 3**).

When compared with those of the reference strains, the L1 genes of Xinjiang strains BPV1-XJ05, BPV2-XJ18, BPV3-XJ21, BPV6-XJ38, BPV7-XJ42, BPV8-XJ76, BPV10-XJ91, BPV11-XJ102, BPV13-XJ29, and BPV14-XJ82 shared 95.2–100% identities with reference strains (BPV1, NC_001522; BPV2, KC256805; BPV3, AJ620207; BPV6, AJ620208; BPV7, NC_007612; BPV8,

Table 1. Distribution of BPV types detected in 122 clinical skin samples from dairy cows

Feature	Number of papilloma lesions	BPV genotype
Area		
Yili	29 (23.77)	1, 2, 3, 7, and 8
Tacheng	37 (30.33)	1, 2, 6, 7, 8, 10, and 11
Changji	18 (14.75)	1, 2, 3, 7, 8, and 13
Shihezi	22 (18.03)	1, 2, 3, 6, 7, 8, and 14
Aksu	16 (13.11)	1, 2, 3, 6, 7, and 8
Age		
≥ 1-year-old	37 (30.33)	1, 2, 3, 6, 7, 8, 10, 11, 13, and 14
< 1-year-old	85 (69.67)	1, 2, 3, 6, 7, and 8
Sex		
Female	109 (89.34)	1, 2, 3, 6, 7, 8, 10, 11, 13, and 14
Male	13 (10.66)	1, 2, 3, 6, 7, and 8
Lesion location		
Neck	47 (38.53)	1, 2, 6, 7, 8, 10, 11, 13, and 14
Face	28 (22.95)	1, 2, 3, 7, 8, 10, 11, and 13
Shoulder	26 (21.31)	1, 2, 3, and 8
Back	14 (11.48)	1, 2, 3, 6, and 8
Nipple	5 (4.10)	3, 7, and 8
Other sites	2 (1.63)*	2, 3, and 7
Papilloma size		
Large (≥ 5 cm)	12 (9.84)	1, 2, 3, and 8
Medium (2–5 cm)	21 (17.21)	1, 2, 3, 6, 7, 8, 10, 11, 13, and 14
Small (< 2 cm)	89 (72.95)	1, 2, 3, 7, 8, 10, 11, 13, and 14

Values are presented as number (%).

BPV, bovine papillomavirus.

*The asterisk indicates a significant difference.

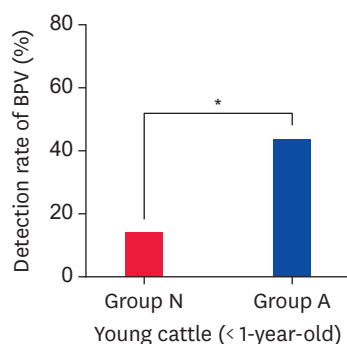


Fig. 3. Detection rates of BPVs in young cattle (<1-year-old) reproduced via artificial fertilization and natural reproduction in a dairy farm. Group N: Natural reproduction; Group A: Artificial fertilization. BPV, bovine papillomavirus. *The asterisk indicates a significant difference.

DQ098913; BPV10, AB331651; BPV11, AB543507; BPV13, JQ798171; BPV14, KR868228, respectively), indicating the presence of obvious genetic diversity.

The phylogenetic tree based on L1 gene of BPVs revealed that BPV1-XJ05, BPV2-XJ18, BPV13-XJ29, and BPV14-XJ82 were members of the *Deltapapillomavirus* genus, while BPV3-XJ21, BPV6-XJ38, BPV10-XJ91, and BPV11-XJ102 were classified as members of the *Xipapillomavirus* genus. BPV8-XJ76 was a representative of the *Epsilonpapillomavirus* genus, whereas BPV7-XJ42 was a *Dyoxipapillomavirus* genus member (**Fig. 4**). Based on genetic distances, BPV13-XJ29 was shown to be closely related to the BPVBR-UEL4 strain, while BPV14-XJ82 had a close relationship with the BPV/UFPE04BR strain.

DISCUSSION

The L1 protein is not only a major component of the BPV capsid but also a major protective antigen for inducing the release of neutralizing antibodies, and it is prone to mutation under immune system pressure [22-24]. At present, 28 types of BPVs have been identified based on the nucleotide sequence differences of the L1 gene [1,17,25]. However, the predominant types of BPVs vary among the different geographic regions of the world [12,26]. Ogawa et al. [21] investigated 122 samples of bovine tumor skin and normal healthy skin and detected 11 BPV types (BAPV1-10 and BAPV11my 41). Lunardi et al. [12] identified BPVs 6-10 and 2 unreported putative papillomavirus types (BPV/BR-UEL6 and BPV/BR-UEL7 40) in teat lesions from cattle farms in Brazil. In this study, we detected 10 genotypes of BPVs, including BPV1, BPV2, BPV3, BPV6, BPV7, BPV10, BPV11, BPV13, and BPV14 in dairy cattle from intensive dairy farms in Xinjiang, China. The distribution of the BPV genotypes varied within the geographical regions of Xinjiang.

The relationship between the genetic diversity of BPVs and their pathogenicity remains unclear [27]. Therefore, the pathogenesis and immunity of different genotypes of BPVs should be further explored in the future.

It is reported that BPV can infect cattle through various routes [2,28], among which direct contact is commonly considered responsible for transmission [1]. It was reported that viral DNA could be detected in milk, blood, urine, semen, and spermatozoa of BPV-infected

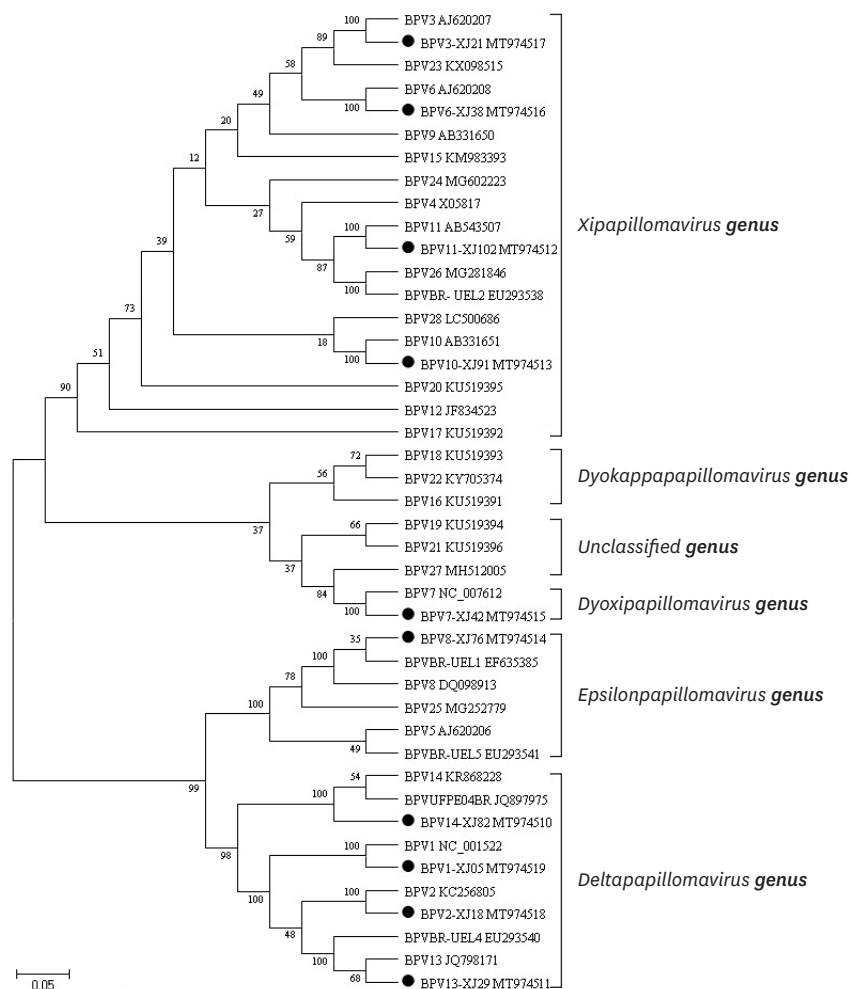


Fig. 4. Phylogenetic analysis of the different BPV genera based on partial sequences of the BPV L1 gene. The phylogenetic tree was constructed using the neighbor-joining method within MEGA 6.0 software. The numbers at the internal nodes represent the bootstrap support values determined in 1,000 replications. The GenBank accession numbers of the reference BPVs are shown in the tree. The black dot represents the epidemic strains of BPVs identified in this study. BPV, bovine papillomavirus.

animals, implying that lymphocytes, seminal fluid, and spermatozoa may have potential roles in BPV transmission [29]. In our study, it is worth noting that the BPV infection rate among young cattle (< 1-year-old) developed from the same supply of frozen sperms was significantly higher than that of other young cattle naturally raised in the same environment. This high infection rate may be closely related to the extensive use of frozen sperm to reproduce Holstein cows in Xinjiang, China, implying that artificial insemination with frozen semen may be an important route of BPV transmission in dairy cows.

To date, at least 27 BPV genotypes have been identified and characterized [13,18,19]; however, numerous putative and new genotypes of BPVs have already been detected in dairy cows [16]. Yamashita-Kawanishi et al. [17] identified and characterized a novel BPV (named BPV28) in a facial cutaneous papilloma lesion on Holstein dairy cattle in Japan. Herein, we report the first occurrence of BPV13 and BPV14 in Xinjiang, China. Mixed infections with different BPV genotypes were common in the same papillomatosis sample, indicating the widespread presence of genetic diversity. This study provides valuable epidemiological data for BPV in

Xinjiang, China, and elucidates the genetic diversity and pathogenic characteristics of BPVs in dairy cattle.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

General information on BPV types detected in 122 clinical skin samples from dairy cows

[Click here to view](#)

Supplementary Table 2

GenBank accession information for nucleotide sequences of the BPV L1 gene

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Supplementary Table 3

Detection of BPV infection in young cattle (< 1-year-old) from a dairy farm located in Tacheng

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Supplementary Fig. 1

Geographic distribution of 122 clinical skin samples infected by bovine papillomaviruses of dairy cows in Xinjiang province, China.

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