INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
20–0953/2021/25–1–222–226
DOI: 10.17957/IJAB/15.1659
http://www.fspublishers.org

Short Communication

Detection and Genetic Characteristics of *Bovine leukaemia virus* in Holstein Cows in China

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Received 27 June 2020; Accepted 26 September 2020; Published 10 December 2020

Abstract

Bovine leukosis (BL) is a chronic tumor disease of dairy cow caused by *Bovine leukemia virus* (BLV), which seriously harms the healthy development of dairy farming. In order to understand the prevalence of BLV in Holstein cows in Xinjiang, China, serological survey was conducted on 462 clinical serum samples of Holstein cows collected from 5 large-scale dairy farms during the period of 2015–2018. Molecular detection of BLV *env* gene was performed on 109 lymph node samples, and then phylogenetic relationship was also explored. The results showed that the overall sero-positive rate of BLV antibody in Holstein cow samples was 8.44% (39/462), while PCR positive rate was 5.50% (6/109), indicating that BLV infection was emerging in Holstein cows from large-scale dairy farms in Xinjiang. Compared with other regional epidemic strains in China and other countries in the world, the nucleotide and amino acid sequences of *env* gene of 6 BLV Xinjiang strains shared 98.9–99.3 and 98.6–99.1% identities, respectively. Furthermore, a total of 55 mutation sites existed in the nucleotide sequence of *env* gene of 6 Xinjiang strains when compared with JPEH-2 strain from Japan, resulting in 18 mutations in amino acid residues. Phylogenetic analysis based on *env* gene showed that three genotypes, namely G1, G6, and G7, were substantively circulating in Holstein cows, showing significant genetic heterogeneity in epidemic strains of BLV in China.

Keywords: *Bovine leukemia*; *Bovine leukemia virus*; Molecular detection; Genetic heterogeneity; *env* gene

Introduction

Bovine leukosis (BL), also known as endemic bovine leukemia, is a chronic tumor disease of cattle (cows) caused by *Bovine leukemia virus* (BLV), which is characterized by malignant proliferation of lymphoid cells, progressive cachexia, and enlarged lymph nodes, reduced milk production and high mortality after onset (Norby *et al.* 2016; Yang *et al.* 2016a). Since BL was firstly reported in 1878, this disease was subsequently discovered in many countries, including the United States, Japan, Brazil, Argentina, Thailand and South Korea (Gutiérrez *et al.* 2011; Rola-Łuszczak *et al.* 2013; Lee *et al.* 2015, 2016). Nowadays, this infectious disease has widely spread to almost all cattle-raising countries in the world (Khudhair *et al*. 2016; Pandey *et al.* 2017), which has posed great threats to dairy industry. In 1974, BLV was first detected in China, and subsequently in more than 10 provinces across the country one after another. At present, BL has been classified as a second-class infectious disease in dairy cows in China.

BLV belongs to the single-stranded RNA virus of subfamily *oretroviridae* within family *Oncoviridae*, which is prone to rapid mutation (Camargos *et al.* 2014; Polat *et al*. 2015; Ochirkhuu *et al.* 2016). The genome of BLV is 8714 nucleotides in length and contains a long terminal sequence (LTR) at each end of its genome (Hirsch *et al.* 2015). The BLV genome from the 5' end to the 3' end is the structural group-specific antigen gene (*gag*), polymerase gene (*pol*), envelope gene (*env*) and the consensus sequence (*U3*) (Rovnak and Casey 1999), respectively. Among them, *env*

To cite this paper: Wang L, C Ning, C Ji, Y Guo, N Li, J Qiao, Q Meng, Y Ren, X Xia, X Zhang, Y Liu, K Cai, Z Zhang, J Zhang, Y Peng, X Cai (2021). Detection and genetic characteristics of *Bovine leukaemia virus* in holstein cows in China. *Intl J Agric Biol* 25:222–226

gene encodes the two glycoproteins, gp51 and gp30 (Marawan *et al.* 2017), respectively. As one of main structural proteins, Gp51 glycoprotein is located on the BLV capsule, which can induce the specific antibodies (Bruck *et al.* 1982, 1984) and is prone to variability under immune pressure. Initially, BLV epidemic strains in different regions could be divided into 7 genotypes (G1–G7) based on *env* gene (Yang *et al*. 2016b). Subsequently, the eighth genotype (G8) was identified and then two more new genotypes, G9 and G10, in Bolivia, Thailand and Myanmar, were found (Polat *et al*. 2016). To date, at least 10 BLV genotypes (G1–G10) have been identified (Polat *et al*. 2017).

Xinjiang is one of China's most important dairy breeding bases, with a current dairy population of 3.6 million heads. In the recent years, Xinjiang has vigorously developed large-scale dairy farming through importing large number of Holstein cows and its' frozen semen from abroad and inland provinces of China. Unfortunately, with the large-scale introduction of dairy cows, transboundary infectious diseases have also been emerging, causing huge economic losses to the dairy farming industry. However, at the present, the infection status and molecular characteristics of BLV in Xinjiang are practically unclear. Thus, the main purposes of this study were to investigate the seroprevalence and genetic characteristics of BLV strains in large-scale dairy farms in Xinjiang China, for providing useful molecular epidemiological data for prevention and control of BLV infection in dairy cows.

Materials and Methods

Collection of samples

During the period of 2015–2018, a total of 462 clinical serum samples of Holstein dairy cows from 4 to 8 years old cows and 109 lymph node samples from dead Holstein cows were collected from 5 large-scale dairy farms in five different geographic farms of Xinjiang (Yili, Shihezi, Urumqi, Changji, and Aksu). The collected samples were placed in an ice box and transported at low temperature to Xinjiang Key Laboratory of Animal Disease Control and Prevention.

Serological testing

According to the instructions of the BLV ELISA antibody detection kit (IDEXX Leukosis Serum X2, Switzerland), a total of 462 Holstein dairy cow clinical serum samples were tested for BLV antibodies, and the test results of different dairy farms were statistically analyzed.

Design of primers

The genome sequences of different geographical strains of BLV in GenBank were compared, and the conserved sequence in the BLV LTR region was selected for the design of specific primers. The nested PCR primers FP1-RP1 (outer primers) and FP2-RP2 (inner primers) were designed to detect BLV proviral DNA (Table 1). The conserved sequence of *env* gene was selected to design the primer FP3-RP3 (Table 1). Theses primer sequences were sent to The Beijing Genomics Institute for synthesis (BGI, China).

Molecular detection

The lymph node samples of the dead cows were taken out and used for the molecular detection. Briefly, the sample was ground with a test tube mill and DNA was extracted with a DNA extraction kit (Qiagen, Germany). Using the extracted DNA as a template, the nested PCR amplification was performed with the primers OFP1-ORP1 (outer primer) and IFP2-IRP2 (inner primer), respectively. PCR reaction system was consisted of the following reagents: 2.0 μL of 10×buffer (containing $MgCl_2$), 0.6 μL of 2.5 mmol/L dNTP, 0.4 μL of 20 m*M* OFP1 and ORP1 primers, 1.0 μL of DNA template, 0.5 μL of TaqDNA (2.5 U/mL) polymerase (TaKaRa Bio, Japan), and the final volume was made up to 20 μL with H_2O. The reaction conditions were set as follows: pre-denaturation at 94℃ for 10 min, denaturation at 94℃ for 1.5 min, annealing at 50℃ for 1.5 min, extension at 72℃ for 1.5 min, a total of 30 cycles, followed by final extension at 72℃ for 5 min. After the first PCR amplification, 1.0 μL of the amplified product was taken, and IFP2-IRP2 was used to perform the second PCR amplification in the same reaction system. The PCR products were separated by electrophoresis on a 1.5% agarose gel, and then visualized under a UV lamp.

Cloning and sequencing of *env* gene of BLV epidemic strain

Briefly, PCR amplification of *env* gene fragment of provirus was performed on BLV nucleic acid positive samples with FP3-RP3 Primers. The amplified PCR product was purified and recovered using DNA recovery kit (TaKaRa, Japan). The recovered target fragment was cloned into the pMD18-T vector (TaKaRa, Japan), and the positive clones were selected by PCR method and sent to Shanghai Biotechnology Co., Ltd. for sequencing (Sangon, China). Three positive clones were selected from each sample, and each clone was sequenced three times.

Analysis of variation and genetic characteristics of *env* gene of BLV

The sequence completely consistent with the three sequencing results was taken as the target gene sequence. The *env* genes of different genotypes in different regions of BLV were downloaded from GenBank. DNAStar7.1 (DNASTAR Inc., USA) and Clustal X 2.1 software (http://www.clustal.org/) were applied to compare the

nucleotide sequence of *env* gene of BLV epidemic strain in Xinjiang with the reported BLV epidemic strains in different regions, and genetic variations of key sites such as A-G antigenic sites, $CD4^+$ T cell epitopes, $CD8^+$ T cell epitopes, and ND1-ND3 domain segments were analyzed. The polygenetic tree was constructed using Mega 6.0 software (https://www.megasoftware.net/), and the genetic evolution relationships among epidemic strains in different regions were explored.

Statistical analysis of data

S.P.S.S. 18 software (Version 18.0, IBM, U.S.A.) was used for conducting statistical analysis. Chi-square test was used to compare the sero-positive rates of different farms. The difference with $P < 0.05$ was considered statistically significant, while $P < 0.01$ was considered extremely significant.

Results

The seroprevalence rates of Holstein cows in various large-scale dairy farms in Xinjiang ranged from 5.15 to 16.16%, respectively, with the overall seropositivity being 8.44% (39/462) (Table 2). Among 109 lymph node disease materials tested, 6 positive samples were detected, and the PCR positive rate was 5.50% (6/109) (Fig. 1, Supplementary Fig. 1), indicating that BLV infection was prevalent in dairy farms in Xinjiang.

Env gene fragments were amplified from 6 PCR-positive samples (Fig. 2) and these sequences were submitted to Genbank (GenBank accession numbers: BLV-XJ-4, MN765152; BLV-XJ-26, MN765153; BLV-XJ-65, MN765154; BLV-XJ-87, MN765155; BLV-XJ-91, MN765156; BLV-XJ-102, MN765157) (Supplementary Table 1). The nucleotide sequences of *env* gene of 6 BLV Xinjiang epidemic strains shared 99.1–99.8% identities, while they shared 96.2–99.6% identities when compared with other BLV strains in the world.

Among the BLV epidemic strains in different regions, *env* gene has a total of 73 nucleotide mutation sites, and the encoded *gp51* protein amino acid sequence has 21 mutation sites. Compared with JPEH-2 strain from Japan, a total of 55 mutation sites were identified in the nucleotide sequence of *env* gene of 6 Xinjiang strains, which caused 18 mutations in the amino acid residues of gp51 protein (Supplementary Fig. 2). Most importantly, the ND2 domain of BLV-XJ-65 and BLV-XJ-91 strain were substantively altered; the $CD8^+$ epitope of BLV-XJ-26, BLV-XJ-65, BLV-XJ-87, BLV-XJ-91, BLV-XJ-102 strains were also genetically mutated when compared with JPEH-2 strain from Japan.

Phylogenetic analysis based on *env* gene showed that the different geographical strains of BLV could be divided into 10 genotypes (G1–G10), and different genotypes include epidemic strains in different regions (Fig. 3).

Table 1: Primers used in this study

Primer's name	Nucleotide sequence (5' to 3')	Position in reference sequence	Size of amplified product (bp)
OFP1	CCTAGGAAACCAACAATGGATG	116-137	640
ORP1	CGTGTTGACCCAGAAGATTTGG	734-755	
IFP2	TCACCTTTCTGTGCCAAGTCTC	204-235	321
IRP2	CTTATGTAAAGAAAAGGTGATC	503-524	
FR3	ATGCCTAAAGAACGACGGTCCCGAA	1-25	897
RR3	GACCCGGGTAGGAGGGGCGGAGGA	873-897	

Table 2: Serological detection results of BLV infection by indirect ELISA in five different geographic farms in China

Farm	Number of samples	Number of positive samples	Positive rate (%) of BLV
Farm 1	91	7	7.69 (7/91) [a]
Farm 2	85	6	7.06 (6/85) [a]
Farm 3	97	5	5.15 (5/97) [a]
Farm 4	99	16	16.16 (16/99) [b]
Farm 5	90	5	5.56 (5/90) [a]
Total	462	39	8.44 (39/462)

Note: Different superscript letters in one column means significant difference ($P < 0.05$)

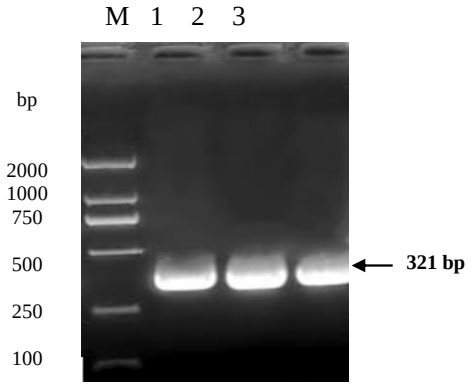

Fig. 1: Molecular detection of provirus DNA of BLV in lymph node from Holstein cows in Xinjiang China by nested PCR
M:DNA marker standard DL-2000(2000,1000,750,500,250,100 bp);1-3:positive samples

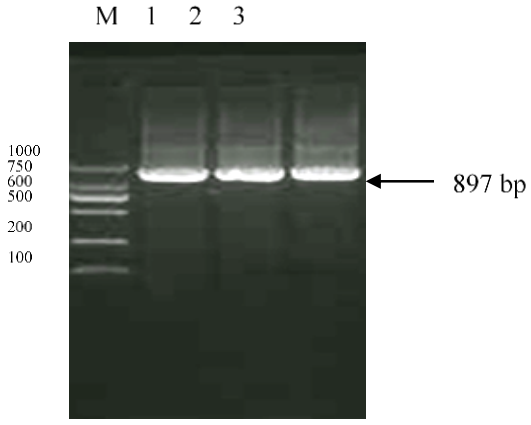

Fig. 2: Amplification of *env* gene of different strains of BLV from positive samples
M: DNA marker standard DL-1000 (1000, 750, 600, 500, 200, 100 bp);
1-3: Amplification of *env* gene from positive samples

Among the 6 BLV epidemic strains in Xinjiang, BLV-XJ-4, BLV-XJ-26, BLV-XJ-91 and BLV-XJ-102 strain belong to

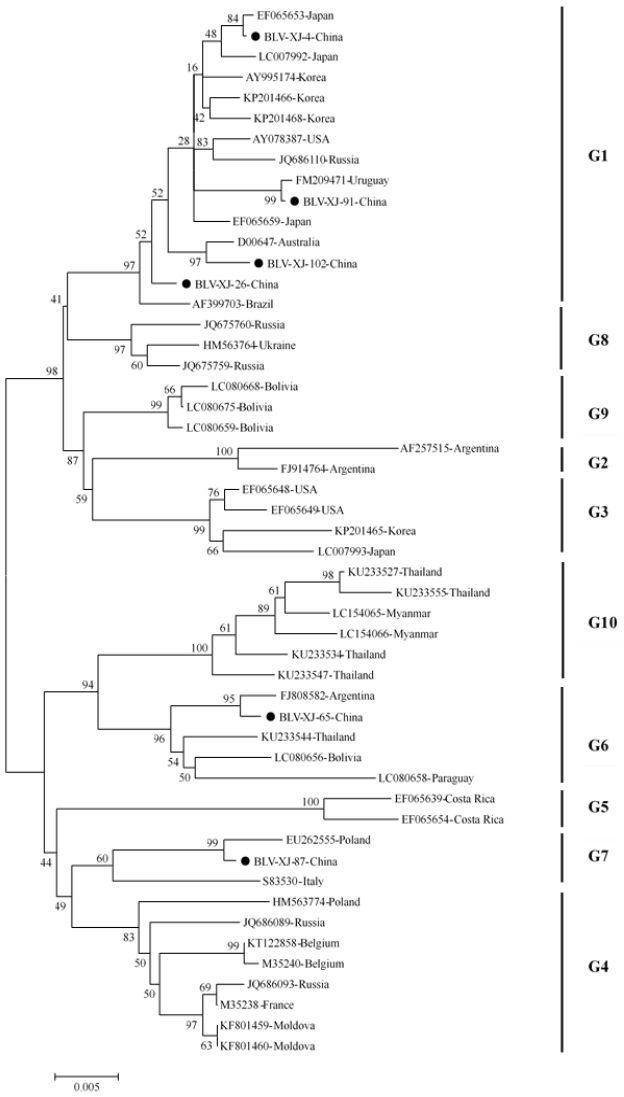

Fig. 3: Phylogenetic analysis of different geographical strains of BLV based on the nucleotide sequences of *env* genes

Phylogenetic tree based on the nucleotide sequences of *env* genes was constructed by the neighbor-joining methods using 1000 bootstrap replicate values. These *env* genes were obtained in this study and available in GenBank (Supplemental Table 1). Different genotypes were indicated by vertical lines. The black dot represents the different strains of BLV identified in this study. The GenBank accession numbers of BLV *env* genes of different geographical strains in Xinjiang China were as follows: BLV-XJ-4, MN765152; BLV-XJ-26, MN765153; BLV-XJ-65, MN765154; BLV-XJ-87,MN765155; BLV-XJ-91, MN765156; BLV-XJ-102, MN765157

G1, while BLV-XJ-65 strain belongs to G6, and BLV-XJ-87 strain belongs to G7, which indicated that significant genetic heterogeneity had occurred in epidemic strains of BLV in China.

Discussion

In the recent years, BLV infection has been widespread in many countries around the world, which has brought greater harm to dairy farming (Gutiérrez *et al.* 2011; Merlini *et al.* 2016; Norby *et al.* 2016; Ruiz *et al.* 2018). However, infection status and molecular characteristics of BLV in Holstein dairy cows in Xinjiang China still remain unclear. In 2014, BLV infection in Yaks (*Bos mutus*) in China was firstly reported (Ma *et al.* 2016). Recently, an epidemiological survey on yaks on the Qinghai-Tibet Plateau in China showed that the seropositivity rates were in the range between 14.94 and 18.93% (Wang *et al.* 2018). In this study, the overall seropositivity rate was 15.6–27.9% in 5 large-scale dairy farms, which suggested that BLV infection in Xinjiang is relatively common.

According to the identities of *env* gene sequences, BLV epidemic strains in different geographical regions of the world can be divided into at least ten genotypes, G1 to G10 (Polat *et al.* 2016, 2017). Confirmed that there were at least two genotypes, G6 and G10, in the yak epidemic strain in Tibet, China (Wang *et al.* 2018). In this study, three genotypes, G1, G6 and G7 were identified for the first time in Xinjiang, of which G1 is the dominant genotype in Holstein cows.

Gp51 glycoprotein protein encoded by *env* gene is located on the capsule of BLV, which is one of the main antigenic proteins and extremely susceptible to mutation (Bruck *et al.* 1982, 1984; Balić *et al.* 2012; Camargos *et al.* 2014; Pluta *et al.* 2017). It has been found that BLV gp51 glycoprotein contains three neutralization domains of ND1, ND2, and ND3 and five T cell epitopes ($CD4^+$ T cell epitope, $CD8^+$ T cell epitope, gp51N5, gp51N11, and gp51N12) (Bruck *et al.* 1982). However, the alterations of the ND2 domains in BLV-XJ-65 and BLV-XJ-91 strain and the $CD8^+$ epitopes of BLV-XJ-26, BLV-XJ-65, BLV-XJ-87, BLV-XJ-91, BLV-XJ-102 strain maybe affect the interaction between gp51 protein with the receptor on host cells, which will likely alter the pathogenicity and antigenicity of virus. Therefore, the impacts of genetic variations in key sites on infection and immune escape of BLV need to be further investigated (Lee *et al.* 2015; Brogniez *et al.* 2016).

It is currently believed that BLV can be transmitted horizontally and vertically (Gutiérrez *et al.* 2011). Transmission routes of BLV include contact transmission, secretory transmission (oral and nasal secretions, milk, urine, feces and semen), blood-borne transmission (virus-contaminated devices, injections and blood collection), artificial fertilization and embryo transfer (Mekata *et al.* 2015). Notably, one of the five geographic dairy farms investigated in this study owned a significantly higher infection rate than those of the other dairy farms, which may be related to the repeated use of syringes and needles during blood collection or tuberculin intradermal tests (epidemiological survey). Therefore, blood-borne transmission should be received more attention for the dairy farm breeders and veterinarians. In addition, regular serological and molecular detection to monitor the infected cattle is of high importance for the prevention and control of BLV infection in dairy farms.

Conclusion

This study for the first time confirmed that three genotypes of BLV, namely G1, G6, and G7, were substantively circulating in Holstein cows, showing significant genetic heterogeneity in BLV Xinjiang strains, which provided useful epidemiological data for the prevention and control of BLV infection in dairy cows.

Acknowledgments

The authors thank the field staff for providing the samples for this study. This work was supported by grant from the national key research and development program (No. 2016YFD0500900), International Science & Technology Cooperation Program of XPCC (No. 2019BC004), and International Science & Technology Cooperation Program of China (No. 2014DFR31310).

Authors' contributions

Lixia Wang, Jun Qiao, Qingling Meng and YanRen planned and designed the whole study. Chengcheng Ning and chunhui Ji carried out the whole work. Xingxing Zhang, Yucheng Liu, Kuojun Cai, Zaichao Zhang, Jinsheng Zhang and Yelong Peng collected sample. Lixia Wang, Jun Qiao and Qingling Meng wrote the manuscript. Yun Guo, Na Li, Xianzhu Xia and Xuepeng Cai helped during manuscript writing and revision. All authors read and approved the final manuscript.

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