First report of border disease virus in *Melophagus ovinus* (sheep ked) collected in Xinjiang, China

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Abstract

*Melophagus ovinus* (sheep ked) is a blood-sucking ectoparasite that is parasitic primarily on sheep. It is widely distributed in different geographical regions worldwide. In China, it has been mainly found in Xinjiang, Gansu, and Tibet in recent years. In addition to causing direct damage to the animal hosts, *M. ovinus* also carries pathogens and serves as a vector for disease transmission. Border disease virus (BDV) is a positive-sense, single-stranded RNA pestivirus that mainly infects and causes border disease (BD) in sheep and goats worldwide. Since 2012, this disease has been reported in 4 provinces in China. In the present study, we investigated the presence of BDV in *M. ovinus* from Xinjiang and Gansu. Frozen *M. ovinus* collected during 2017 and 2018 from Xinjiang and Gansu and preserved in our laboratory were studied. First, total RNA of *M. ovinus* was extracted, followed by reverse transcription, PCR (RT-PCR) amplification of the 5' -UTR of BDV, and sequencing of the amplified products. Finally, the sequencing results were analyzed using DNAStar, MEGA 5.0 molecular biology software, and the BLAST online platform. The results from RT-PCR and sequencing analyses showed that among the samples included in the study, only the *M. ovinus* collected from Qinghe County in Alta, Xinjiang in 2018 tested positive for BDV. BLAST analysis showed that the viral strain with the most similar nucleotide identity to the sequence of the China/BDV/2018 fragment was the goat-derived BDV strain AH12-02 collected in Anhui, China, in 2012. A phylogenetic-tree analysis showed the strain to exhibit a BDV-3 genotype. This is the first report globally on BDV detected in *M. ovinus* and is also the first report of BDV discovered in Xinjiang, China. This study reconfirms the presence of BDV in China.

Background

*Melophagus ovinus* (sheep ked) is a member of Hippoboscidae (Diptera: Hippoboscoidea) and is a blood-sucking ectoparasite of livestock and wild animals. *Melophagus ovinus* has a small
head, strong mouthparts, no wings, dense bristles on the body surface, and 3 pairs of legs tipped with pointed claws [1–3]. The presence of *M. ovinus* has been reported in many countries in Africa, North America, Europe, Oceania, and Asia [2]. In China, *M. ovinus* has recently been reported mainly in Tibet [4], Xinjiang [2, 3, 5], and Gansu [6]. The direct and indirect damage caused by *M. ovinus* has led to huge economic losses in the sheep industry. In particular, *M. ovinus* can carry and transmit multiple pathogens and has thus become a worldwide concern [2, 3]. China has reported at least 13 pathogens detected in *M. ovinus* [2–6].

**Methods**

**Study areas and *M. ovinus* collection**

In June 2017, *M. ovinus* was collected from 5 sheep from a trading market in Yaha Town of Kuqa County in Aksu, Xinjiang (1029 m above sea level; 41˚44'0"N, E83˚14'0"E). Approximately 150 *M. ovinus* were collected from each sheep and were preserved at −70˚C in our laboratory. Fifteen *M. ovinus* were randomly sampled from each sheep for use in the present study.

In March 2018, 12 *M. ovinus* were collected from 2 sheep from a peasant household in the Yumai Township of Aketedu County in Kizilsu Kirghiz Autonomous Prefecture, Xinjiang (1325 m above sea level; 39˚13'0"N, 75˚97'0"E) and were preserved at −80˚C.

In March 2018, more than 400 *M. ovinus* were collected from 9 sheep from animal breeders in the Qinghe County in Alta, Xinjiang (1218 m above sea level; 46˚67'0"N, 90˚38'0"E) and were preserved at −70˚C. Forty *M. ovinus* were randomly selected for use in the present study (Up to 5 sheep keds per sheep).
In June 2018, 130 *M. ovinus* were collected from 11 sheep from animal breeders in the Zhangyi Town of Liangzhou District in Wuwei, Gansu (2125 m above sea level; 37°56′N, 102°74′E) and were preserved at −70°C. Thirteen *M. ovinus* were randomly selected for use in the present study (Up to 2 sheep keds per sheep).

In this study, 140 (75 + 12 + 40 + 13) *M. ovinus* (Fig 1a and 1b) were processed individually.

**Ethics approval and consent to participate**

Ethical treatment of animals was practiced in this study. Permission was obtained from the farm owners before collection of the specimens.

**Isolation of RNA, cDNA synthesis, PCR of the 5′-UTR, sequencing of PCR products and sequence analysis**

The preserved and frozen *M. ovinus* were retrieved and placed in an autoclaved, chilled mortar. Liquid nitrogen was added and the samples were rapidly ground into powder. Next, total RNA from *M. ovinus* was extracted using the TaKaRa RNAiso Plus Kit (TaKaRa, Beijing, China, Code No. 9108) according to the manufacturer’s protocol. The precipitates were dissolved in 20 μL of RNase-free water in the final step. Next, cDNA was synthesized using the extracted RNA and according to the manufacturer’s protocol of the TaKaRa PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Beijing, China, Code No. 6210A). Subsequently, the 5′-UTR of BDV was amplified according to the manufacturer’s protocol of Premix Taq™ II (TaKaRa Taq™ Version 2.0) (TaKaRa, Beijing, China, Code No. R004A) and using the KOD-Plus amplification enzyme (Toyobo Co. Ltd, Osaka, Japan). The amplified product was approximately 225 bp.

Each 50 μL PCR reaction mixture contained 25 μL of the 2× PCR solution for Premix Taq™, 1 μL each of the forward and reverse primers (PBD1: 5′−TCGTGGTGAGATCCCTGAG−3′;
**Results**

The results from total RNA extraction of *M. ovinus*, cDNA synthesis, PCR amplification of the 5′-UTR of BDV, sequencing, and sequence analyses showed that only 7 *M. ovinus* from the Qinghe County in Xinjiang collected in 2018 were positive for BDV-specific PCR amplification. The sequencing results showed that the sequence of the 5′-UTR gene was identical in 7 samples, and the sequence was named China/BDV/2018.

In the GenBank database, the nucleotide identity between the China/BDV/2018 sequence and the goat-derived BDV strain AH12-02 isolated in Anhui, China in 2012, the pig-derived BDV strain Gifhorn isolated in Germany in 2000, the sheep-derived BDV strain 297 isolated in Slovakia in 2007, the goat-derived BDV strain AH12-01 isolated in Anhui, China in 2012, and the goat-derived BDV strain JS12/04 isolated in Jiangsu, China in 2012, were 94%, 93%, 93%, 93%, and 92%, respectively.

The 38 nucleotide sequences (Table 1) analyzed in this study included sequences from BDV-1 to BDV-8, BDV Turkey, BDV Tunisian, CSFV, BVDV-1, BVDV-2, and an outgroup. Based on the 191 positions in the 5′-UTR, MEGA 5.0 was used to perform the analysis on the evolutionary history of the strains. Viral strains from different countries, origins, and time periods could be clustered into 1 branch. China/BDV/2018 was classified as the BDV-3 genotype (Fig 2). However, the 10 BDV-3 genotype sequences were clearly divided into 2 smaller branches. Therefore, the subdivision of the BDV-3 genotype into BDV-3a and BDV-3b genotypes is recommended.

Based on the analyses of the 5′-UTR of pestivirus, the nucleotide identity between the sequences of China/BDV/2018 and BVDV was 69.4% to 75.7%, between that of China/BDV/2018 and CSFV was 83.6% to 84.5%, between that of China/BDV/2018 and BDV-3 was 90.5% to 94.1%, and between that of China/BDV/2018 and other BDV subtypes was 78.8% to 89.6%. Analyses of the conserved and variable regions of the pestiviruses (VR II and VR III) showed that in the first conserved region, only BDV-2, BDV-7, and BDV Turkey had 1–4 base changes among that of all BDV strains, while in the second conserved region, only BDV-6 had a single base change among that of all BDV strains. The changes of BDV in VR II were prominent and the changes in VR III were minimal. Similar changes in the variable regions were observed in the same subtype of viral strains, including BDV-3b and BDV-3a. The viral strains of the BDV-3b subtype might be further classified into smaller divisions or exhibited greater variation (Fig 3).
Border disease virus (BDV) causes prenatal and postnatal infections in animals, resulting in reproductive disorders, birth of unviable lambs, and persistent infections [14]. In addition, mortalities can range from 40% to 85% in certain populations during epidemics [8, 20–22] and outbreaks in some countries [31], leading to huge economic losses. Furthermore, pestiviruses possess a high degree of genetic variability and extensive interspecies transmissions can occur between domestic and wild animals [32, 33]. Moreover, genetic changes in viruses can lead to changes in virulence [34]. A comprehensive analysis of reports from China since 2012, and

### Table 1. List of pestivirus strains used in this study.

| GenBank Accession No. | Strain       | Year | Country | Host                      |
|-----------------------|--------------|------|---------|---------------------------|
| AB122085              | Casimir      | 2003 | Germany | Wisent and reindeer       |
| AF037405              | X818         | 1987 | Australia | Sheep lamb               |
| AF1144618             | reindeer-1 V60-Krefeld | 1996 | Germany | Rangifer tarandus         |
| AF220247              | CP7-5A       | 1999 | Germany | Bos                       |
| AJ829444              | 712/02       | 2004 | Italy   | Capra hircus              |
| AM418427              | BDV/Aydin/04-TR | 2006 | Turkey  | Sheep                     |
| AM418428              | BDV/Burdur/05-TR | 2006 | Turkey  | Goat                      |
| AY453630              | BM01         | 2003 | Tunisia | Sheep                     |
| AY781152              | /            | 2004 | America | Pronghorn antelope        |
| DQ361072              | LE31C2       | 2001 | Spain   | Sheep                     |
| EF693988              | 89-F-5415    | 1989 | France  | Sheep                     |
| EF693989              | 90-F-6227    | 1990 | France  | Sheep                     |
| EF693991              | 90-F-6338    | 1990 | France  | Sheep                     |
| EF693993              | 91-F-7014    | 1991 | France  | Sheep                     |
| EF694003              | 06-F-0299/477 | 2006 | France  | Sheep                     |
| EU637006              | chemnititz   | 1999 | Germany | Sheep                     |
| FJ040215              | Th/04_KhonKaen | 2004 | Thailand | Bovine                   |
| FM163379              | LA/82/04     | 2010 | Italy   | Ovies aries               |
| GQ902940              | Gilhorn      | 2000 | Germany | Pig                       |
| GU270877              | H2121 (Chamois-1) | 2002 | Andorra | Chamois                  |
| HQ231763              | Italy-1/10-1 | 2010 | Italy   | Cattle                   |
| HQ380231              | CSFV-GZ-2009 | 2009 | China   | Pig                       |
| J04358                | Alfort/Tuebingen | 1989 | Germany | Unknown                  |
| JQ946320              | AH12-01      | 2012 | China   | Goat                      |
| JX437132              | AH12-02      | 2012 | China   | Goat                      |
| JX437133              | JSL512-01    | 2012 | China   | Sheep                     |
| JX683184              | JS12/04      | 2012 | China   | Goat                      |
| KF918753              | Aveyron      | 1984 | France  | Sheep                     |
| KT072634              | Italy-103761 | 2014 | Italy   | Capra hircus              |
| KT327869              | JSYZ15       | 2015 | China   | Sheep                     |
| KT327870              | AHHX15       | 2015 | China   | Sheep                     |
| L49347                | P97          | 1993 | Taiwan  | Pig                       |
| M96751                | SD-1         | 1992 | America | Heifer                   |
| NC_003678             | giraffe-1 H138 | 1967 | Kenya   | Giraffa camelopardalis   |
| NC_024018             | /            | 2004 | America | Pronghorn antelope       |
| U18059                | 890          | 1994 | America | Heifer                   |
| U65022                | Moredun cp   | 1976 | Scotland—Lothian | Sheep |

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**Discussion**

Border disease virus (BDV) causes prenatal and postnatal infections in animals, resulting in reproductive disorders, birth of unviable lambs, and persistent infections [14]. In addition, mortalities can range from 40% to 85% in certain populations during epidemics [8, 20–22] and outbreaks in some countries [31], leading to huge economic losses. Furthermore, pestiviruses possess a high degree of genetic variability and extensive interspecies transmissions can occur between domestic and wild animals [32, 33]. Moreover, genetic changes in viruses can lead to changes in virulence [34]. A comprehensive analysis of reports from China since 2012, and
Fig 2. Phylogenetic tree of pestivirus based on 5'-UTR region. The evolutionary history was inferred by using the NJ method based on the Maximum Composite Likelihood method [27, 28]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [29]. The analysis involved 38 nucleotide sequences. There were a total of 191 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [30]. Sequences of this work were marked with black circular (●).

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evidence from etiological, molecular biology, and serological studies has confirmed that the sheep and/or goats in the Chinese provinces of Anhui, Jiangsu, Gansu, and Tibet have been infected with BDV [13, 23–25]. In summary, monitoring and research on BDV in China is necessary.

In recent years, *Melophagus ovinus* has been frequently reported in Tibet [4], Xinjiang [2, 3, 5], and Gansu [6] in China. At least 13 pathogens have been reported in *M. ovinus* found in China [2–6]. In this study, *M. ovinus* was collected from the body surface of sheep from Xinjiang and Gansu. The results showed that BDV was present in the *M. ovinus* isolated from sheep from Northern Xinjiang, which allowed us to make the connection that the sheep in Northern Xinjiang were infected with BDV. This confirms for the first time that *M. ovinus* is a carrier of BDV. This is also the first report confirming the presence of BDV in Xinjiang, China, which increases the number of BDV-positive provinces in China to 5.

Currently, the results of pestivirus genotyping using the 5′-UTR, NPro, or E2 sequences were identical and a consistent phylogeny [10, 14] was obtained. Among the 3 sequences, the 5′-UTR was the most conserved nucleotide sequence in pestiviruses. In addition, the NPro or E2 genes lacked consensus sequences for primer design and sufficient reference fragments for analyses [14]. Therefore, the 5′-UTR is more frequently used in genotyping studies. This study also utilized the 5′-UTR to classify 38 nucleotide sequences, including the target sequence of this study. The results showed that China/BDV/2018 belonged to the BDV-3 genotype, which was consistent with the BDV classification in previous reports from China. BDV-3 is widely distributed worldwide, including in goats and sheep in Austria [35], Germany [33], India [36], Slovakia [37], Italy [38], Switzerland [39], China [12, 23, 24], and France [14], as well as cattle in Austria [35]. However, the BDV-3 genotype sequence analyzed in this study is clearly divided into 2 smaller branches. In addition, viral strains of the same subtype displayed similar changes in the variable regions, and these changes also suggest that BDV-3 can be further divided into 2 groups. This study suggests dividing the BDV-3 genotype into BDV-3a and BDV-3b genotypes, which will also reflect the greater diversity of BDV compared with other pestivirus species reported in the literature [13].
In this study, the nucleotide identity between the sequences of China/BDV/2018 and different subtypes of BDV ranged from 78.8% to 94.1%, while the nucleotide identity between the sequences of China/BDV/2018 and CSFV was 83.6% to 84.5%. This indicates that the nucleotide identity between the same type of pestiviruses may be much lower than that between pestiviruses and other types of viruses. In other words, the classification of pestiviruses based on nucleotide identity is unreliable, and the establishment of a phylogenetic tree is required. Furthermore, data from the GenBank database show that the sequences with similar identity with China/BDV/2018 are the goat-derived BDV strain AH12-02 isolated in Anhui, China in 2012, the pig-derived BDV strain Gifhorn isolated in Germany in 2000, and the sheep-derived BDV strain 297 isolated in Slovakia in 2007. These are BDV isolates from different regions, time periods, and origins. This information limits our ability to deduce the source and origin of China/BDV/2018. The emergence of BDV in Xinjiang may be related to animal trading, as there were no base changes in the 2 conserved regions in China/BDV/2018. The changes in the conserved region in all of the BDV strains listed in this study are relatively small, and this region may be used as a target site for primer design for BDV studies. Molecular epidemiological research and additional genetic studies on BDV should be extensively investigated in China and Xinjiang to provide definitive evidence for the classification, determination of origin, and control of BDV. Nevertheless, future analyses on additional BDV-3 isolates collected from different geographical regions in the world will help to provide a clearer picture in this regard.

Conclusions
To our knowledge, this is the first report worldwide on the detection of border disease virus (BDV) in Melophagus ovinus. It is also the first report to confirm Xinjiang as the 5th BDV-positive province in China.

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