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Next-generation sequencing for the genetic characterization of Maedi/Visna virus isolated from the northwest of China

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ABSTRACT

Background: Maedi/Visna virus (MVV) is a contagious viral pathogen that causes considerable economic losses to the sheep industry worldwide.
Objectives: In China, MVV has been detected in several regions, but its molecular characteristics and genetic variations were not thoroughly investigated.
Methods: Therefore, in this study, we conducted next-generation sequencing on an MVV strain obtained from northwest China to reveal its genetic evolution via phylogenetic analysis.
Results: A MVV strain obtained from Inner Mongolia (NM) of China was identified. Sequence analysis indicated that its whole-genome length is 9193 bp. Homology comparison of nucleotides between the NM strain and reference strains showed that the sequence homology of gag and env were 77.1%–86.8% and 67.7%–75.5%, respectively. Phylogenetic analysis revealed that the NM strain was closely related to the reference strains isolated from America, which belong to the A2 type. Notably, there were 5 amino acid insertions in variable region 4 and a highly variable motif at the C-terminal of the surface glycoprotein (SU5).
Conclusions: The present study is the first to show the whole-genome sequence of an MVV obtained from China. The detailed analyses provide essential information for understanding the genetic characteristics of MVV, and the results enrich the MVV library.

Keywords: Maedi/Visna virus; next-generation sequencing; phylogenetic analysis; sheep lung

INTRODUCTION

Maedi/Visna virus (MVV) belongs to the Retroviridae family and Lentivirus genus and is a monocyte/macrophage-tropic non-oncogenic virus [1]. MVV infection is persistent and chronic and is characterized by slow progressive degenerative inflammation in multiple organs, including lungs, brain, udder, and joints in sheep and goats [2]. Labored breathing associated with emaciation caused by progressive pneumonitis is the predominant feature in clinically affected sheep [3]. MMV is epidemic globally, except for Australia and New Zealand, resulting in considerable economic losses [4].

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Zhang L, Liu S; Data curation: Zhao L; Formal analysis: Zhao L, Zhang L; Funding acquisition: Liu S; Investigation: Shi X; Methodology: Zhao L, Duan X; Project administration: Liu S; Resources: Liu S; Software: Zhao L, Li H; Supervision: Liu S; Writing - original draft: Zhang L, Zhao L; Writing - review & editing: Zhang L, Liu S.

The MVV is a single-stranded RNA molecule with positive-sense polarity and contains 3 primary genes (gag, pol, and env) and several regulatory genes (e.g., vif, vpr-like, and rev) [5]. The gag gene encodes the internal structural proteins, such as the capsid protein (CA), which stimulates the host to produce antibodies. The pol gene encodes enzymes involved in replication and DNA integration, such as reverse transcriptase [6]. These 2 genomic regions, the gag-pol (1.8 kb) and the pol (1.2 kb), were initially proposed by Shah et al. [7] to be used in the classification of MVV subtype strains. Subsequently, many other strains were added to the phylogenetic tree, but the additions were often based on the sequence of only a small part of one of the fragments initially proposed by Olech et al. [8]. The env gene encodes surface and transmembrane glycoproteins that insert into the envelope. The surface glycoprotein (SU) contains genetically variable domains, thereby determining the antigenic variability of the different isolates.

The use of next-generation sequencing (NGS), also called high throughput sequencing, has rapidly expanded over recent years [9-11]. Due to their high accuracy and high throughput capacity, Illumina, Inc. is the dominant player in the NGS arena. Illumina NGS supports various protocols (e.g., genomic sequencing, RNA sequencing) and provides varying levels of throughput, including the MiniSeq, MiSeq, and HiSeq models [12]. The presence of Maedi-Visna disease in China was first reported in 1966. Serological diagnosis revealed that MVV was present in several regions of China, except Inner Mongolia (NM), which is one of the major provinces of sheep production in China. However, the lack of a complete genome sequence of the Chinese MMV strains has hampered comprehension of its genetic characteristics. We have worked on ovine viral pneumonia for several years, and a case of Maedi-Visna disease was recently identified in the western portion of NM. To elucidate the molecular characteristics of our isolate, NGS was applied to obtain a complete genome sequence. Moreover, phylogenetic information on our isolate was analyzed, which may assist in elucidating the genetic evolution of MVV.

MATERIALS AND METHODS

Ethics statement

This work does not contain any studies performed on living animals. Collection of lung tissues conformed to the experimental practices and standards approved by the animal welfare and research ethics committee of NM Agricultural University (approval ID: 2020007).

Sample collection and hematoxylin and eosin (H&E) staining

A 3-year-old Dorper ewe with serve dyspnea, cough, and wheezing was provided by a large-scale sheep farm in NM and was sacrificed for sample collection. Lung samples were kept in 10% formalin or frozen at −80°C. The lung samples were fixed in 10% formalin and processed by applying standard procedures for pathological examination. After processing, 3–5 mm-thick sections were stained with H&E for microscope examination.

Polymerase chain reaction (PCR) identification

MVV identification by PCR was performed as previously described [13]. Briefly, a pair of primers (F: 5′-TGACACAGCAAATGTAACCGCAAG-3′; R: 5′- CCACGTTGGGCGCCAGCTG CGAGA-3′) were used to amplify a 291 bp fragment of the long terminal repeat (LTR) region of pro-viral DNA. MVV was proliferated in choroid plexus cells and, after 3 passages, were used as positive controls. Healthy lung tissue and RNase-free and DNase-free water were used as negative and blank controls, respectively.

Virus enrichment and identification

PCR-positive lung tissue was ground and then made into a suspension by adding a 5-fold volume of PBS. Viruses were released by performing 3 freezing and thawing cycles. The supernatants were collected after centrifugation at 4,000 r/min for 30 min, repeated 3 times, and then centrifuged at 12,000 r/min for 1 h. The obtained supernatant was centrifuged at 35,000 r/min for 3 h, and the pellet was resuspended in TNE buffer. After centrifugation of the suspension at 5,000 r/min for 10 min, the supernatant was passed through a 25% sucrose cushion and centrifuged at 35,000 r/min for 4 h. Viruses were purified by a 20%–50% gradient of sucrose and ultracentrifugation. The final acquisition was negatively stained with 2% uranyl acetate and examined by electron microscopy.

Sequencing and genome assembly

Viral RNA was extracted using the TRIzol (TAKARA, China) reagent, then reverse-transcribed into complementary DNA (cDNA) with the Prime ScriptTM RT reagent kit and g DNA Eraser (TAKARA), according to the manufacturer's recommendations. A viral cDNA library was constructed and sequenced by Shanghai Biozeron Biological Technology Co. Ltd. Briefly, 1 μg of cDNA was used with Illumina's TruSeq™ Nano DNA Sample Prep Kit for library preparation. Libraries were sequenced on the Illumina HiSeq 4000 platform at 2 × 150 bp read length. Genome assembly was performed using ABySS [14] to achieve optimal results, and corrections for single-base polymorphism and the infilling of remaining gaps were conducted in SOAPdenovo [15].

Sequence alignment and polygenetic analysis

Sequence alignment was performed using DNASTAR Lasergene (version 7.1.0). To evaluate the relationship between the reference strains and the NM strain, a phylogenetic tree was constructed using the neighbor-joining method provided in molecular evolutionary genetics analysis software (version 7.0). Bootstrap values were estimated for 1,000 replicates.

RESULTS

Characterization of MVV in sheep lung tissue

Gross appearance showed that the volume of the affected sheep lung was increased by 2- to 3-fold. Nodular lesions were scattered or densely distributed in local areas, and the surrounding tissue was a dark red color and provided a hard tactile sensation. Fibrotic focuses were scattered in the pleural surface (**Fig. 1A**). Histopathologically, lymphocytic and lymphofollicular hyperplasias were scattered or locally distributed in the pulmonary interstitium. Lymphoid follicles occurred before the peripheral bronchioles, and the germinal centers of some lymphoid follicles were obvious. There were significant proliferations of lymphocytes, macrophages, and a small number of fibroblasts in the alveolar septum. Type II epithelial cells on the inner surface of the alveolar wall had proliferated to varying degrees. Additionally, some alveolar cavities were filled with red homogeneous serous fluid as well as some macrophages and lymphocytes (**Fig. 1B**). A PCR procedure was performed to amplify the LTR sequence of the MVV. Detection by gel electrophoresis confirmed that the lung tissue was MVV-positive (**Fig. 1C**). Furthermore, electron microscope-based examination of purified MVV showed that the viral particles were approximately 80 nm (**Fig. 1D**).

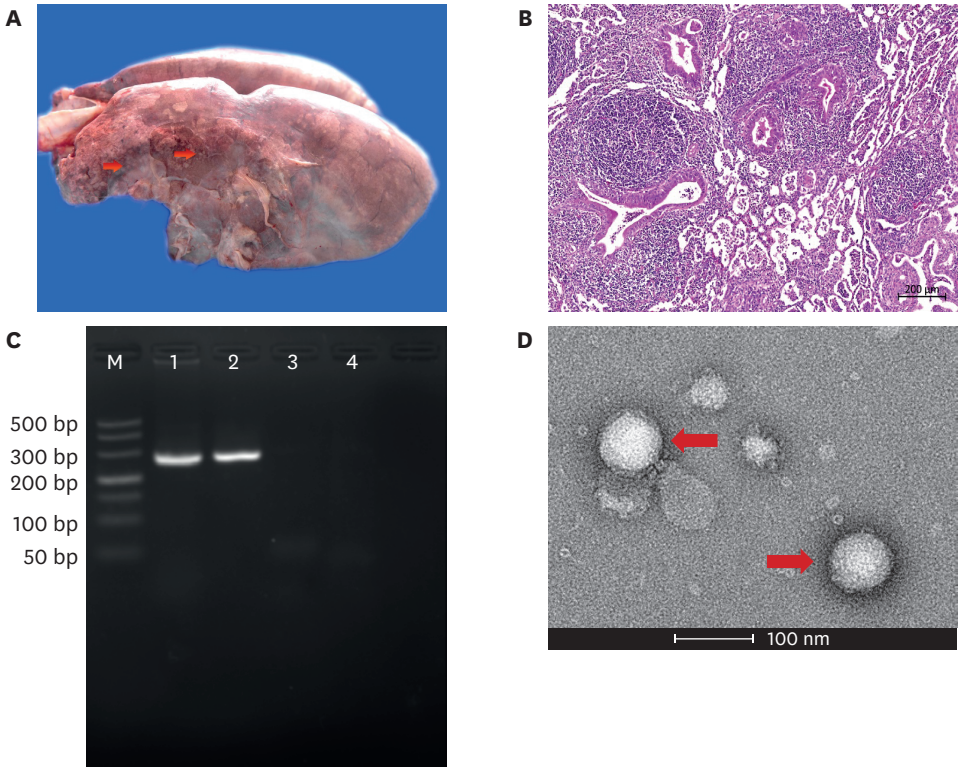

Fig. 1. Histological, molecular, and electron microscopy detection of MVV in sheep lung. (A) Gross appearance of sheep lung affected by MVV. The volume of the sheep lung was enlarged. Gray-white nodular lesions, ranging to as large as rice to soybean, were distributed diffusely or densely in local areas in the pleural surface with some transparency (red arrow). (B) Lymphocytic interstitial pneumonia. Lymphocytic or lymphofollicular hyperplasias were scattered or locally distributed in the pulmonary interstitium, hematoxylin and eosin staining (bar = 200 μm). (C) Polymerase chain reaction identification. Lanes 1–4 present the lung sample, positive control, negative control, and blank control, respectively. (D) Electron micrograph of MVV particles (red arrow), negatively stained with 2% uranyl acetate (bar = 100 nm).
M, marker; MVV, Maedi/Visna virus.

Complete genome sequence of the NM1111 strain

A total of 1.1 GB of valid data was obtained, and the data were uniformly distributed. The sequencing depth was up to 188,295 times. The values of Q20 and Q30 were 96.64% and 90.48%, respectively, and the G+C content was 40.43%. These results reveal that our data exhibited good sequencing quality. Finally, the isolate contained 9,193 nucleotides after splicing and optimization. Three primary encoding genes, the gag gene, position at 502 to 1,845, contained 1,344 nucleotides; the pol gene, position at 1716 to 5036, contained 3321 nucleotides; the env gene, position at 5,985 to 8,948, contained 2,964 nucleotides (**Table 1**, **Fig. 2**). The complete genome sequence of MVV was uploaded, named NM1111, to GenBank with the accession number MW248464 (**Supplementary Data 1**).

Phylogenetic analysis

A neighbor-joining tree was constructed using the whole-genome sequence of NM1111 and the available reference strains. The phylogenetic analysis showed that the NM1111 strain was clustered in a branch of MMV type A2 (**Fig. 3**). Meanwhile, homology comparisons showed that the gag and pol of NM1111 had a close relationship with American strain MH916859 (**Table 2**). In contrast, the env had a lower sequence homology with the reference strains, suggesting the development of antigenic variability with evolution. Amino acid homology analysis indicated that the gag protein is highly conserved, especially in 2 dominant epitopes

Table 1. Genome information of the NM1111 isolate

Name	Position	Fragment length (bp)	Amino acid length
5′LTR	1–157	157	N/A
Gag	502–1,845	1,344	447
Pol	1,716–5,036	3,321	1,106
Vif	4,993–5,682	690	229
Vpr-like	5,685–5,972	288	95
Rev1	5,985–6,111	127	42
Rev2	8,568–8,875	308	102
Env	5,985–8,948	2,964	987
TATA box	9,091–9,017	7	N/A
PolyA	9,173–9,178	6	N/A
3′LTR	8,864–9,193	300	N/A

LTR, long terminal repeat; N/A, not applicable.

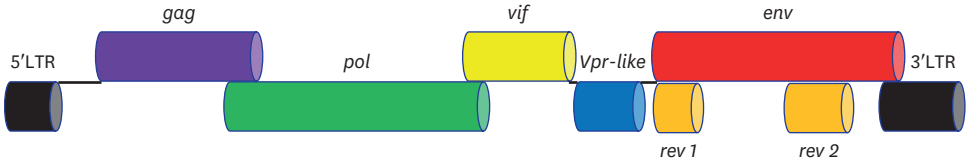

Fig. 2. Schematic representation of gene distribution in the Maedi/Visna virus genome.
LTR, long terminal repeat.

(epitope2 and epitope3). However, within the highly homologous epitope2, the Sichuan strain (KR011757) mutated from alanine (A) to valine (V) at position 221. Notably, the major homology region (MHR) region of NM1111 mutated from aspartic acid (D) to glutamic acid (E) at position 296 (**Table 3**). The SU region had a high degree of sequence variation, especially in variable region 4 (V4) with 5 amino acid insertions (position at 554-555, 564-566) (**Table 4**). Immunodominant epitope SU5 had a conserved motif (VRAYTYGV) located at the N-terminal part and a highly variable motif at the C-terminal (**Table 4**).

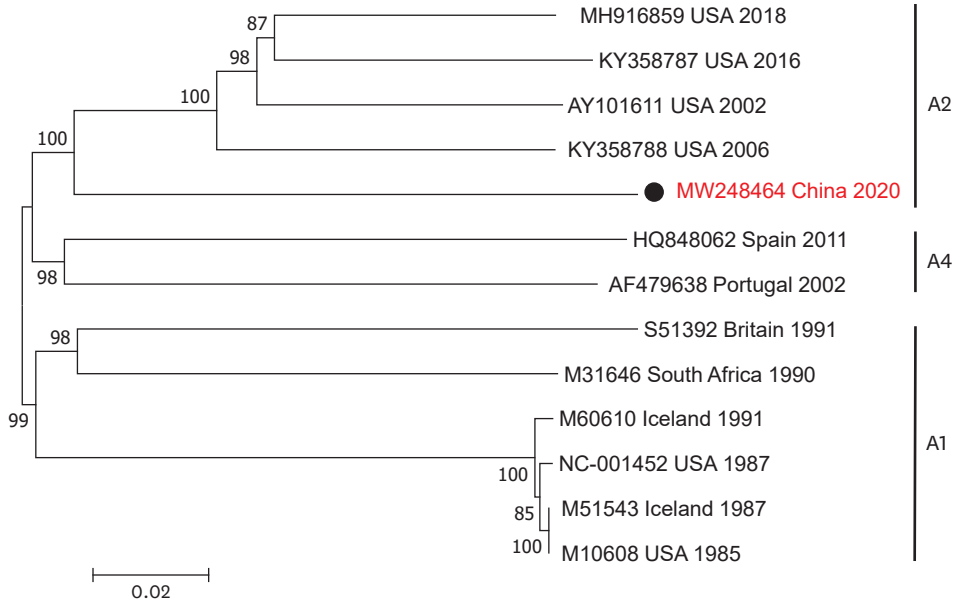

Fig. 3. Phylogenetic tree based on the alignment of whole-genome sequences from the NM and reference strains. The tree was generated using the neighbor-joining method coupled with the p-distance model and bootstrap analysis of 1,000 replicates. The virus isolated from NM, China, is indicated in red. The genotypes of each Maedi/Visna virus strain are marked on the right. The scale bar represents the number of substitutions per nucleotide.
NM, Inner Mongolia.

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Table 2. Homology comparison of nucleotides and amino acids (%) between NM1111 and reference strains

GenBank No.	Gag	Pol	Vif	Vpr-like	Env
MH916859	86.8/92.2	83.1/86.3	75.8/75.1	82.1/81.9	68.7/61.3
KY358787	85.2/90.8	82.0/85.7	77.1/75.1	80.0/78.7	67.8/60.2
AY101611	86.7/92.1	82.4/86.0	77.1/76.4	80.4/81.9	N/A
KY358788	85.9/92.4	83.5/88.2	77.2/76.0	78.9/79.8	67.7/60.3
HQ848062	82.8/91.0	80.9/84.8	73.6/70.3	76.8/77.7	68.5/63.2
AF479638	83.8/89.7	81.0/84.0	77.5/79.9	80.0/78.7	70.1/64.1
S51392	83.4/74.8	79.7/83.0	76.4/74.2	75.1/73.4	68.3/60.8
M31646	84.3/89.9	81.3/84.3	77.0/68.4	76.1/79.8	70.4/63.0
M60610	84.5/91.9	80.0/83.3	76.8/77.7	77.9/77.7	69.0/63.0
NC-001452	84.8/92.1	80.0/83.3	76.8/77.7	78.6/78.7	69.2/63.3
M51543	84.8/92.1	79.9/83.3	76.8/77.7	78.6/77.7	69.2/63.3
M10608	84.8/92.1	79.9/83.3	76.8/77.7	79.5/-	69.2/63.3
KR011757	85.9/91.7	N/A	N/A	N/A	N/A

N/A, not applicable.

Table 3. Representative amino acid variations in gag-encoded linear epitopes

Amino acid	Epitope2			MHR						Epitope3		
	K	A	N	P	V	T	D	I	A	V	T	E
MH916859	●	●	●	●	●	N	●	●	●	●	●	●
KY358787	●	●	●	●	●	S	●	●	●	●	S	●
KY358788	●	●	●	●	●	S	●	●	●	●	S	●
MW248464	●	●	●	●	●	●	E	●	●	●	●	●
HQ848062	●	●	●	●	●	N	●	●	●	●	●	●
AF479638	●	●	●	●	●	●	●	V	●	●	●	●
S51392	●	●	●	●	●	●	●	●	●	●	●	●
M31646	●	●	●	●	I	S	●	●	●	●	S	●
M60610	●	●	●	●	●	●	●	●	●	●	●	●
NC-001452	●	●	●	●	●	●	●	●	●	●	●	●
M51543	●	●	●	●	●	●	●	●	●	●	●	●
M10608	●	●	●	●	●	●	●	●	●	●	●	●
KR011757	●	V	●	●	●	●	●	●	●	●	●	●
Position	216	221	233	283	286	291	296	298	304	343	347	361

MHR, major homology region.

Table 4. Representative amino acid variations in the env-encoded V4 and SU5 regions

GenBank No.	V4												SU5												
	N	Y	R	N	T	S	N	L	R	K	A	N	V	R	A	Y	T	Y	G	V	R	R	N	L	Q
MH916859	E	L	G	K	R	N	E	I	K	Q	●	T	●	●	●	●	●	●	●	●	S	E	L	Q	L
KY358787	H	L	G	E	Q	G	K	E	K	L	P	●	●	●	●	●	●	●	●	●	T	E	L	S	L
KY358788	E	F	G	T	Q	M	G	E	L	Q	●	●	●	●	●	●	●	●	●	●	S	E	L	Q	L
MW248464	T	M	Q	T	●	T	S	I	K	Q	E	D	●	●	●	●	●	●	●	●	A	L	S	●	R
HQ848062	Q	●	E	N	I	Y	E	I	K	T	●	-	●	●	●	●	●	●	●	●	K	●	S	●	●
AF479638	R	●	-	R	●	N	●	P	I	Q	●	T	●	●	●	●	●	●	●	●	P	Q	S	●	●
M31646	G	●	E	K	●	N	●	I	Y	●	●	-	●	●	●	●	●	●	●	●	T	E	I	S	R
M60610	●	●	K	●	●	K	●	●	●	●	●	S	●	●	●	●	●	●	●	●	●	●	●	●	●
NC-001452	●	●	●	●	●	●	●	●	●	●	S	-	●	●	●	●	●	●	●	●	●	●	●	●	●
M51543	●	●	●	●	●	●	●	●	●	●	S	-	●	●	●	●	●	●	●	●	●	●	●	●	●
M10608	●	●	●	●	●	●	●	●	●	●	S	-	●	●	●	●	●	●	●	●	●	●	●	●	●
Position	545	547	553	557	567	569	576	578	585	590	595	605	653	654	655	656	657	658	659	660	678	679	680	681	682

DISCUSSION

NGS is a recent, powerful technique developed and applied for virus sequencing. In this study, the first complete genome sequence of MVV from China was obtained by NGS. Sequencing depth, high purity of the assembled data, the distribution of the GC content, and the sequencing coverage verified the reliability of the obtained results. Despite NGS being the

most prevalent technology for sequencing at present, future complete genome sequencing of MVV isolates with recently developed third-generation sequencing would also be desirable.

Gag is relatively conserved in MVV and encodes a major protective antigen. CA, encoded by gag, contains 3 linear epitopes (epitope 2, MHR, and epitope 3) and can cause a strong antibody reaction during infection, thus making it of great value for serodiagnosis. These epitopes are also important for maintaining cross-reactivity in gag antigen-based serological tests [16]. The amino acid sequence analysis showed that the linear epitopes were highly conserved, but there was a mutation from aspartic acid (D) to glutamic acid (E) at position 296 in the MHR region, an observation similar to that for a type A5 strain from Poland [17]. MVV has been serologically detected in 12 regions of China (Yunnan, Guizhou, Gansu, Ningxia, Shandong, Sichuan, Hunan, Guangdong, Chongqing, Guangxi, Jilin, and Anhui), and the infection rate in those regions ranged from 4.60% to 50.00% [18]. However, when Sun et al. [19] carried out a serological investigation on MVV in China, the detection rate in NM was zero. In addition to the limited sample collection area, it was inferred that a mutation in MHR region might also have contributed. Tanaka et al. [20] reported that, in addition to the epitope2 and epitope3, MHR is usually conserved in many retroviruses. Mutations in this region may destroy capsid protein assembly and reduce human immunodeficiency virus infectivity [20]. In this study, the MHR region of the strains isolated thirty years ago, except the south Africa strain, were shown to be conserved, but other strains exhibited varying degrees of mutation. Whether the mutations in this region could reduce the virulence of MVV needs to be further elucidated.

Env is the most mutated gene in MVV, which can often cause antigen drift. The V4 region of the SU domain can affect cell tropism and the synthesis of neutralizing antibodies [21]. Hence, mutations in this region have an important role in interactions between the virus and the host. Based on the analysis of env protein, the mutation rate of the V4 region was high in our isolate, which is consistent with the results in the study of Hötzel and Cheevers [22]. In addition, there were 5 amino acid insertions, suggesting a high probability of antigen drift. Skraban et al. [23] reported that the SU5 epitope is responsible for a specific immune response, and it could be used to realize a specific diagnosis for a virus strain. In this study, we observed a conserved motif at the N-terminal and a highly variable motif at the C-terminal of the SU5 region, observations that are consistent with the A5 strain of MVV isolated from Poland [17]. These observations emphasize the effectiveness of identifying different MVV subtypes within local genotypes [24].

Polygenetic analysis indicated that the NM1111 isolate was closely related to American stains. Meanwhile, based on the gag sequence construction of a neighbor-joining tree, the Sichuan strain (KR011757) was separated along the same branch from an American stain (AY101611) (**Supplementary Fig. 1**). The results suggest that the main epidemic MMV strain in China was probably MMV type A2. Characterizing more MVV strains isolated from China will help elucidate the genetic evolution of MMV.

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SUPPLEMENTARY MATERIALS

Supplementary Data 1

The whole-genome sequence of NM1111

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Supplementary Fig. 1

The phylogenetic tree based on the alignment of gag sequences from NM strain and reference strains. The tree was generated using the neighbor-joining method coupled with the p-distance model and a bootstrap analysis of 1,000 replicates. The virus isolated from NM of China indicated in red color, and the genotypes of each Maedi/Visna virus strain marked on the right. The scale bar represents the number of substitutions per nucleotide.

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