



## NOTE

Virology

# Detection and molecular characterization of equine infectious anemia virus in Mongolian horses

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**ABSTRACT.** The genetic characterization and actual prevalence of EIAV in Mongolian horse in the disease endemic region is currently unknown. Here, 11 of 776 horse serum samples from four Mongolian provinces tested positive on agar gel immunodiffusion test. Genomic DNA extracted from all seropositive samples was subjected to nested PCR assay. Among these, three samples tested positive with nested PCR assay and were identified by sequencing analysis based on long termination repeat and *tat* gene of the virus. Two of the three sequences were identical, with 94.0% identity with the third. These two independent Mongolian EIAV sequences were retained functional motifs, with no dramatic changes but some variability in the U5 region; they were clustered with genotypes from European countries but not with those from China, U.S.A., or Japan.

**KEY WORDS:** equine infectious anemia virus, horse, LTR-gene, molecular epidemiology, Mongolia

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Equine infectious anemia virus (EIAV) belongs to the genus of *Lentivirus* within the family of *Retroviridae* [12]. It is the etiological agent of equine infectious anemia (EIA) and causes acute and chronic infection in all members of the Equidae such as horses, ponies and donkeys [3]. EIAV infection is transmitted horizontally via vector-borne (bites of blood-feeding insects) and vehicle-borne (use of contaminated veterinary equipment) transmission of viruses. In particular, infected horses with high viral loads are a major source of infection within the herd [2]. In infected horses, the disease typically manifests in chronic form with mild clinical symptoms such as weight loss, recurrent fever, anemia, weakness; the disease may sometimes be inapparent and the host serves as a potential reservoir of infection [12]. The diagnosis and stamping out the infected horses is essential to the prevention and control of further transmission among the horse population owing to the lack of an effective vaccine [10].

Horses play a large role in the daily and national life of Mongolians, and the pathogens, such as EIAV, equine influenza virus, equid herpes virus 1, equine rhinitis A virus, equine arteritis virus, and equid herpes virus 3 were previously reported [14]. Surveillance for infectious diseases and molecular characterization of pathogens is essential to formulate strategies for prevention and control of infection. The current prevalence of EIAV infection in the country, and especially in Selenge Province, which has historically been a high-prevalence province is not clear. Besides, the molecular characterization of EIAV in Mongolian horse has not been performed to date. The aim of the present study was to survey EIAV infection among Mongolian horses from different provinces and to characterize its molecular biology based on the long termination repeat (LTR) and viral transcriptional trans-activator (*tat*) region of the virus.

During the summer of 2016, a total of 776 blood samples were collected from horses in the Selenge ( $n=688$ ), Bulgan ( $n=55$ ), Khuvsgul ( $n=21$ ), and Tuv ( $n=12$ ) Provinces in Mongolia. AGID assay was conducted with use of EIA antigen for Immunodiffusion test kit (Nisseiken Co., Ltd., Tokyo, Japan) and commercial agarose gel for EIA diagnosis (Nippon Bio-test laboratories Inc., Tokyo, Japan). Seropositive samples were further tested. Purified genomic DNA was extracted from EDTA-blood samples with use of a Genomic DNA Purification Kit (Promega, Madison, WI, U.S.A.), as recommended

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**Table 1.** EIAV detection in Mongolian horses

Province	No. of tested animals	No. of seropositive (%)	No. of PCR positive	No. of identified sequences	Accession number
Selenge	688	11 (1.6)	3 / 11	2	LC185347, LC190840
Bulgan	55	0	N.D.	–	–
Khuvsgul	21	0	0 / 21	–	–
Tuv	12	0	0 / 12	–	–
Total	766	11 (1.4)	3 / 44	2	

N.D.: not demonstrated.

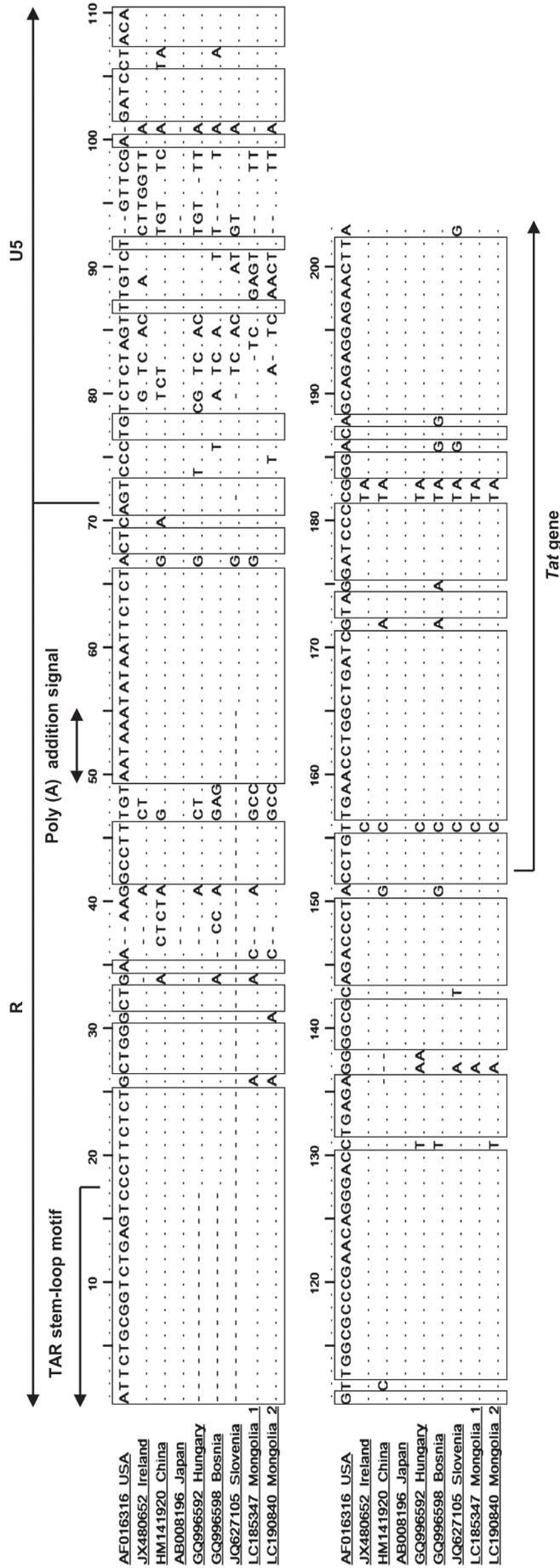
by the manufacturer. EIAV provirus was detected by nested-PCR targeting the LTR-*tat* primer pairs as EIAV ltr-1F 5'-GACAGTTGGGCACTCAGATT-3' and EIAV ltr-1R 5'-CAG GAA CAC CTC CAG AAG AC-3' for the initial PCR, and EIAV ltr-2F 5'-ATT CTG CGG TCT GAG TCC CT-3' and EIAV ltr-2R 5'-TAAGTTCTCTCTGCTGTCC-3' for the second PCR as previously described [6]. A total of 1  $\mu$ l (100 ng) of DNA sample was used as a template for the initial PCR, and 3  $\mu$ l of the first PCR products were reamplified. The amplified PCR products were confirmed using the MUPID-exU Electrophoresis System (Takara Bio Inc., Otsu, Japan) on 2.0% agarose gel. PCR products were extracted using the FastGene gel/PCR Extraction Kit (Nippon Genetics, Tokyo, Japan). The extracted PCR products were ligated into the pGEM-T Easy vector (Promega), and the plasmid was introduced into the *Escherichia coli* strain DH5 $\alpha$  (Takara Bio Inc.), plated on a Luria-Bertani (LB) agar (Invitrogen, Carlsbad, CA, U.S.A.), and cultured in an LB broth (Invitrogen). The plasmid DNAs from the positive clones were extracted from the LB culture using the FastGene Plasmid Mini Kit (Nippon Genetics). The sequencing amplifications of the plasmids were performed using the GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, U.S.A.). The nucleotide sequences of the amplified plasmids were determined using a CEQ8000 DNA analysis system (Beckman Coulter, Fullerton, CA, U.S.A.). All identified pathogens were analyzed with the BioEdit software [9] and basic local alignment search tool application (BLAST). Phylogenetic trees were constructed by MEGA 7 software [11] with the neighbor-joining method [17].

Out of the 776 samples, 11 samples were serologically positive for EIAV and overall prevalence of the infection was 1.4% among the horse samples which was relatively low to the previous report as 24.5% in 2007 [14]. Interestingly, these seropositive horses were derived from 5 different herds only in Selenge Province and none of seropositive sample was detected from other areas but number of the tested horse from other provinces (Bulgan, Huvsgul and Tuv) were insufficient to conclude the three provinces were free from EIAV infection. In addition, the nested PCR assay was performed by using purified genomic DNA from all seropositive horses, and only 3 of them from two different herds were positive but other 8 samples were negative for EIAV. To note, two seropositive horse were foals and it was possible that they may had maternal antibodies for the infection (Table 1). The failure of the nested PCR assay to detect virus in remaining samples may be associated with genomic mutation of Mongolian EIAV or oligonucleotide primer because limitations of PCR-based detection for EIAV is the genetic variability between the strains endemic in different geographical areas, especially for unknown EIAV genotypes [7]. A variety of PCR assays have been performed using primers designed from various EIAV strains, largely of American, Chinese, and European origin [1, 13, 15]. However, some of these failed to detect EIAV due to genetic variability.

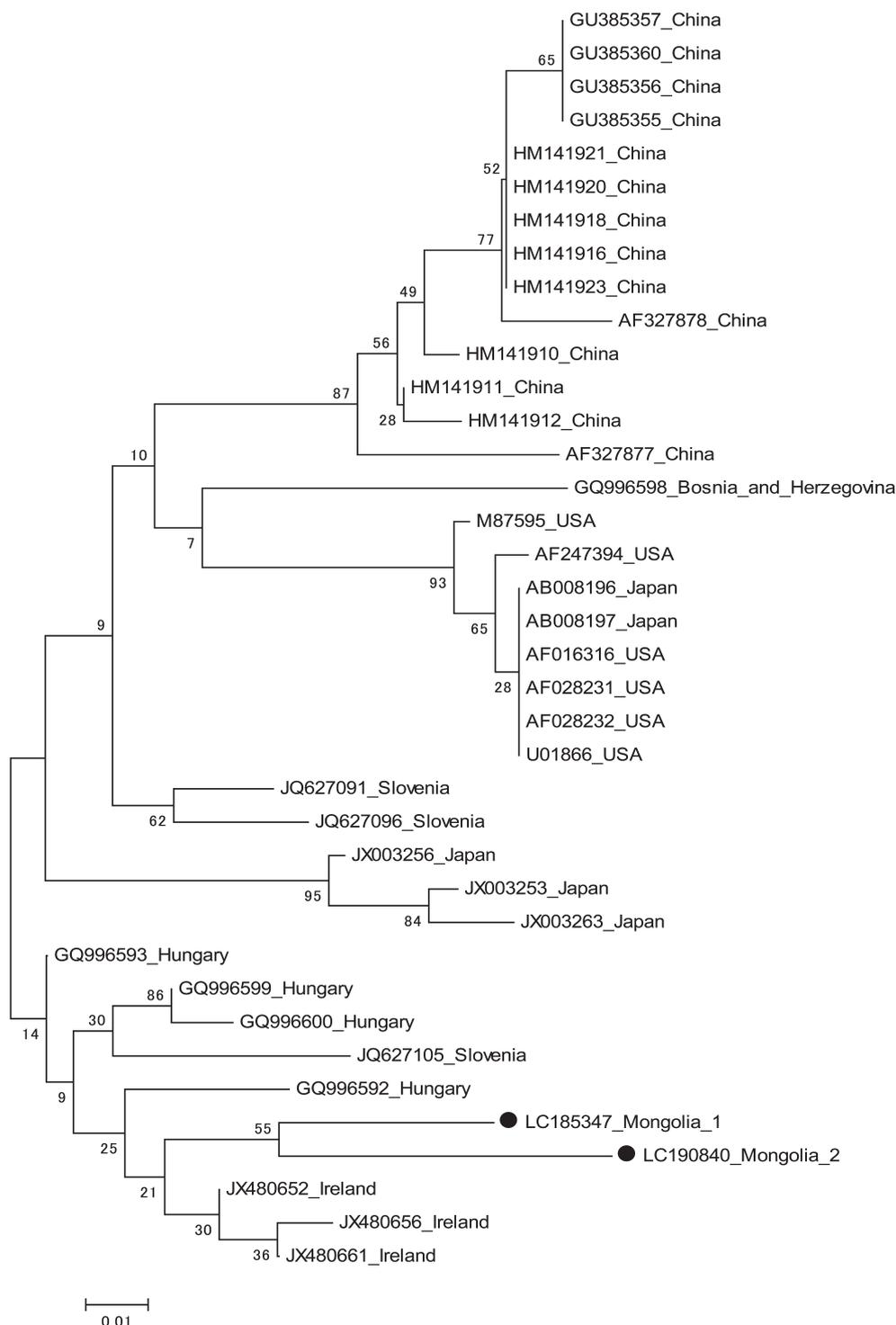
According to the previous report EIAV was quite prevalent as 24.5% with 49/200 samples from Selenge Province in 2007 but the result of this study indicate that prevalence of EIAV has been decreased among the horse population in Selenge Province. The reason for the dramatic decrease of infection rate may be associated with “Animal-Health disease control program” implemented by Mongolian government from 2000 through 2010. This control program was composed of 2 steps. The first step was annual active seroprevalence survey that covering 85–96% of horse population in 5 provinces including Selenge. The second step was a compensation for the culling EIAV-infected horses [18].

In addition, the nucleotide sequences of these three nested PCR positive samples were subjected to sequencing analysis targeting 5'-LTR-*tat* region of EIAV to determine their genetic characteristics. Two novel EIAV partial sequences of the virus were identified. The first EIAV sequence (LC185347, Mongolia 1) was derived from a herd with only one seropositive sample whereas the second EIAV sequence (LC190840, Mongolia 2) was derived from two horses of another herd which were 100% identical to each other. These EIAV-sequences from Mongolia showed 94% identity to each other and 84–90% identity with the isolates of EIAV from several countries. The primer pair used in this study covers the R/U5 region of 5'-LTR and *tat* genes of the virus [5, 7]. Most nucleotide variations were occurred in the U5 region compared with the retained partial TAR stem-loop motif and poly (A) tail regions of LTR gene. In addition, *tat* gene of EIAV had few single nucleotide substitutions and alignment of nucleotide sequences for each gene was shown with the boxes Fig. 1. The phylogenetic analysis revealed that Mongolian EIAV sequences were similar with the sequences from European countries such as Hungary, Slovenia, and Ireland while quite divergent from Asian isolates including China and Japan, as well as U.S.A. (Fig. 2).

According to a previous report, EIAV has been isolated from worldwide including China [19], Russia [8], Japan [7], U.S.A. [4] and Ireland [16]. Horse population in Mongolia was contracted by stamping out of seropositive animals under the state surveillance control program during EIAV outbreaks at the border region with Russia in mid 1990s. However, sporadic cases of EIAV infection among horses were frequently detected in the country but not systematically documented. The present study is the first genomic analysis of EIAV in Mongolia. We suspect that EIAV may have been reintroduced to Mongolia from Europe through



**Fig. 1.** Comparison of LTR nucleotide sequences between Mongolian EIAV and other isolates. Structural and putative transcriptional control elements were predicted based on previous reports [5]. Dashes (-) indicate deletions, and dots (·) indicate conserved residues. Identical residues are boxed.



**Fig. 2.** Phylogenetic trees of this study were constructed by MEGA 7 program with the neighbor-joining method by using the sequences based on the LTR and *tat* gene of EIAV identified in this study and database sequences in GenBank.

horse movement because the new cases have been found only in the Mongolia-Russia border regions; moreover, this hypothesis is also supported by phylogenetic analysis wherein the Mongolian EIAV sequences were found to be closely related to the European isolates. Phylogenetic analysis of the 1,018 bp proviral *gag* genes of from the three Russian EIAV strains (Zapozhye-1967, Novgorod-2011 or Omsk-2012) showed up to 98% identity with those from European strains [8]; unfortunately, LTR gene sequences of the EIAV isolates are not available. Further work is required to confirm the relationship of circulating EIAV in horse among Mongolia and Russia. This study is the first attempt to clarify the present situation of EIAV infection in Mongolian horses from four different provinces. Although number of tested horses was still limited, the infection rate of EIAV is relatively low and

its incidence is restricted to the northern borders of the country. The prevalence of infection in other areas of Mongolia needs to be investigated. Further, characterization of the genetic diversity of the pathogen will help to formulate control strategies for the disease in the country.

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