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Detection and molecular characterization of rabies virus in Mongolia during 2008-2010

Bumduuren Tuvshintulga[1], Enkhbaatar Batmagnai[1], Enkhbold Bazarragchaa[2], Purevtseren Dulam[2], Sengee Sugar[2] and Badgar Battsetseg[1]

1. Laboratory of Molecular Genetics, Institute of Veterinary Medicine, Mongolian, University of Life Science, Zaisan, 17024 Ulaanbaatar, Mongolia; 2. State Central Veterinary Laboratory, Zaisan, 17024 Ulaanbaatar, Mongolia.
Corresponding author: Badgar Battsetseg, e-mail: Bata07@gmail.com, BT: tuvshintulga@gmail.com, EBat: magnai1987@gmail.com, EB: bazarragchaa@scvl.gov.mn, PD: dulampurevtseren@yahoo.com, SS: ssugar352000@yahoo.com

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Abstract

Aim: We aimed to investigate the prevalence and molecular characterization of rabies virus (RABV) from wild and domestic animals in Mongolia during 2008-2010.

Materials and Methods: Brain tissue samples were collected from 24 rabid animals in Zavkhan, Omnogovi, Tov, Dundgovi, Govi-Altai, Selenge, Ovorkhangai, and Khentii provinces in Mongolia. Herein, samples were included from 13 domestic animals (dogs, cattle, camels, sheep, and goat) and 11 wild animals (wolves and foxes) in this study. Direct fluorescent antibody (DFA) test and reverse transcriptase polymerase chain reaction (RT-PCR) were performed for detection of RABV, and positive samples were further processed for molecular characterization of the virus using nucleoprotein gene. Subsequently, the molecular characterization was determined based on the nucleoprotein gene.

Results: Out of 24 samples, 22 samples were detected positive for RABV by DFA test, and its nucleoprotein gene was amplified in all of the 24 samples by RT-PCR. These Mongolian RABVs were classified within steppe-type virus clade by phylogenetic analysis of nucleoprotein gene sequences. This steppe-type virus clade was clearly divided by two Sub-clades (A and B). The most of Mongolian RABVs belongs to the Sub-clade A in the phylogenetic tree.

Conclusion: These findings have clearly confirmed RABV in domestic and wild animals of Mongolia. Further molecular characterization indicated that this Mongolian strain is steppe-type virus clade consisting of two sub-clades; the Subclade A might be prevalent in Altai, Khangai, Khentii Mountains as a major genotype, whereas the Subclade B seems to be cosmopolitan in the steppe-type virus clade, is spread in northern central Eurasia.

Keywords: detection, molecular characterization, Mongolia, rabies virus, sub-clade A, subclade B.

Introduction

Rabies is a re-emerging zoonotic viral disease caused by rabies virus (RABV), which belongs to the genus *Lyssavirus*, family *Rabdoviridae*. RABV is a nonsegmented and negative sense 12 kb RNA virus. The genomic RNA consists of five encoding protein genes, including nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) genes [1]. N gene is commonly used for molecular detection and phylogenetic analysis as it is highly conserved among *Lyssavirus* [2].

RABV infects warm blood animals including human, upon bite by rabid carnivorous animals. Human deaths mainly occur in Asia and Africa, especially case occurs in the rural areas [3]. Mongolia is a landlocked country, geographically located in the Eastern central Asia, besides Kazakhstan to the west side, and sandwiched with Russia to the north side, and China to the south side. In Mongolia, the occurrence of rabies has been recorded since 1950, and the first report was associated with a rabid fox in the early 1960s [4]. In 1965, 2175 domestic and 232 wild animal rabies deaths occurred in Sukhbaatar province, and at that time several rabies cases were reported in the neighboring 6 provinces in Mongolia during 1965-1968. Further, during 1970-2005, RABV was responsible for deaths in 21302 animals and 34 human [5]. Botvinkin *et al*. first determined molecular characterization of RABV isolated from 4 rabid animals from Western parts of Mongolia, which are classified within steppe-type virus clade [4]. Furthermore, Boldbaatar *et al*. also determined RABVs within steppe-type virus clades, except one Mongolian RABV that is classified together with arctic-like virus clade in animal rabies cases from Bayan-Olgii, Govi-Altai, Zavkhan, Khovsgol, and Tov provinces during 2005-2008 [6].

In the last few years, Mongolian rabies cases are not available to the public. In Russia and Kazakhstan, rabies is frequently occurring in animals and human, caused by five groups including arctic-like (phylogeny clade B) and steppe-type (phylogeny clade C) virus clades [7]. In China, rabies is commonly reported in the Southern provinces such as Guangxi, Guangdong, Hunan, and Guizhou [8,9]. As for the Northern

provinces in China, rabies is recently reported in Xinjiang and inner Mongolia in China, caused by steppe-type virus and arctic-like rabies clades [10,11]. In the present study, we investigated molecular characterization of Mongolian RABV among wild and domestic animals during 2008-2010 collected from different geographical regions (Ovorkhangai, Dundgovi, Omnogovi, Khentii, and Selenge provinces) [4,6].

Materials and Methods

Ethical approval

The present study was conducted at Laboratory of Molecular Genetics, Institute of Veterinary Medicine, in Mongolia, while brain samples were obtained from dead animals, according to safety precautions in handling procedure.

Samples

Brain tissue samples were collected from 24 animals including wild animals (n=11, 3 wolves, 8 foxes) and domestic animals (n=13, 4 dogs, 4 cattle, 2 camels, 2 sheep, and 1 goat) during 2008-2010 and samples were stored at −30°C until further use. These animals belong to 8 provinces of Mongolia (Govi-Altai, Dundgovi, Zavkhan, Ovorkhangai, Omongovi, Khentii, Selenge and Tov) (Table-1 and Figure-1).

Direct fluorescent antibody test (DFA)

DFA was performed by the manufacturer's instruction using Light diagnostics™ rabies DFA reagent (Millipore Ltd., Livingston, UK, cat. no. 5100). Briefly, brain impressions from the cerebellum and hippocampus were prepared on slides and were fixed by dipping in cold acetone at −20°C for 30 min. Slides were taken out and dried in air. Fluorescein isothiocyanate-labelled anti-rabies monoclonal antibody was added on the marked spot and slides were incubated in a humid chamber at 37°C for 30 min. These slides were rinsed by ×1 phosphate buffered saline at 3 times for 5 min. Mounting fluid was dropped on the spot on the slides and examined under fluorescent microscopy.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) assay

Total RNA was extracted using TRIzol® (Invitrogen, Life Technology, Paisly, UK, cat. no. 15596-018), according to manufacturer's instruction. Briefly, 50 mg brain tissue was homogenized by micro-grinder with disposable pestle. Total RNA was purified by Trizol and chloroform. After isopropyl alcohol precipitation, the RNA was dissolved in 30 µl of double distilled and RNA-free water, and stored at −80°C until use.

RT-PCR was performed in GeneAmp® thermocycler 9700 following previously described method [12]. Briefly, cDNA was synthesized using TaqMan® RT reagent Kit (Applied Biosystems, California, USA, cat. no. N8080234). Briefly, 1 µl total RNA was added in RT-reaction mixture containing ×1 RT buffer, 1.75 mM $MgCl_2$, 0.5 mM each deoxynucleotide triphosphates (dNTPs), 5 mM dithiothreitol, 1 U RNase inhibitor, 0.5 µM JW12 (ATGTAACACC(C/T) CTACAATTG) sense primer, 2.5 U MultiScribe RT, and RNA-free DDW. RT-reaction conditions were as follows: Denaturation of RNA at 96°C for 2 min, RT at 42°C for 45 min, deactivation of RT at 95°C for 5 min, store at 4°C. cDNA was amplified by JW12 sense and JW6 mixture anti-sense primers (JW6DPL: CAATTCGCACACATTTTGTG;

Table-1: Sampling locations, source, and detection of RABV in this study.

GenBank accession number	Hosts	County (Region/province)	Year	DFA	RT-PCR
KT338628	Cattle	Selenge	2008	−	+
KT338631	Cattle	Ovorkhangai	2008	−	+
KT338630	Dog	Dundgovi	2009	+	+
KT338632	Cattle	Dundgovi	2009	+	+
KT338615	Camel	Omnogovi, Mandal-Ovoo	2010	+	+
KT338616	Cattle	Tov, Bayankhangai	2010	+	+
KT338617	Dog	Dundgovi, Erdenedalai	2010	+	+
KT338619	Sheep	Dundgovi, Khuld	2010	+	+
KT338623	Camel	Omnogovi, Mandal-Ovoo	2010	+	+
KT338624	Dog	Zavkhan, Tsagaankhairkhan	2010	+	+
KT338626	Sheep	Tov, Shireet	2010	+	+
KT338627	Goat	Govi-Altai, Khaliun	2010	+	+
KT338637	Dog	Khentii	2010	+	+
KT338629	Fox	Dundgovi	2008	+	+
KT338614	Wolf	Zavkhan, Uliastai	2009	+	+
KT338618	Fox	Dundgovi, Erdenedalai	2010	+	+
KT338620	Fox	Dundgovi, Khuld	2010	+	+
KT338621	Fox	Dundgovi, Ulziit	2010	+	+
KT338622	Fox	Omnogovi, Manlai	2010	+	+
KT338625	Wolf	Zavkhan, Aldarkhaan	2010	+	+
KT338633	Fox	Dundgovi	2010	+	+
KT338634	Fox	Dundgovi	2010	+	+
KT338635	Wolf	Khentii	2010	+	+
KT338636	Fox	Khentii	2010	+	+

DFA=Direct fluorescent antibody, RT-PCR=Reverse transcriptase-polymerase chain reaction

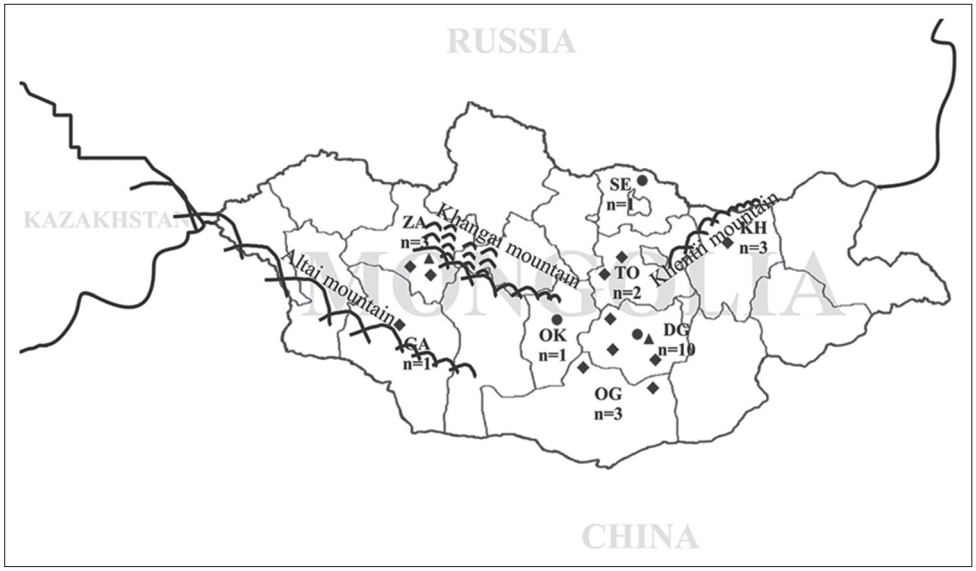

Figure-1: Sampling locations and sources of brain tissue samples in this study. Black circles, triangles, and diamonds indicate sampling year 2008 (●), 2009 (▲), and 2010 (◆), respectively. The name of provinces was abbreviated by GA (Govi-Altai), Dundgovi (DG), ZA (Zavkhan), OK (Ovorkhangai), OG (Omnogovi), KH (Khentii), SE (Selenge), and TO (Tov). Number of samples were indicated by abbreviation (n=).

JW6E: CAGTTGGCACACATCTTGTG; JW6M; CAGTTAGCGCACATCTTATG) using AmpliTaq Gold® PCR Master Mix (Applied Biosystems, California, USA, cat. no. 4327058), and 1 µl cDNA was added in the PCR-mixture containing ×1 PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM each dNTPs, 1 µM each sense and anti-sense primers and 0.5 U Taq polymerase, and distilled water. PCR-reaction conditions were: Denaturation at 95°C for 5 min, amplification 40 cycles denaturation at 95°C for 30 s, annealing at 45°C for 30 s, elongation at 72°C for 30 s, with a final elongation at 72°C for 10 min. The PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide. The amplified PCR products were visualized under ultraviolet light.

Sequencing and phylogenetic analysis

RT-PCR amplicons were directly sequenced on 3130xl Genetic Analyzer using BigDye® Terminator V 3.1 cycle sequencing kit (Applied Biosystems, California, USA, cat. no. 4337454) following instructions as per manual. Institute of Veterinary Medicine, Mongolia has sequencing facilities. The sequences were initially analyzed by basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and then analyzed by MatGAT 2.02 software [13] so as to calculate their identity scores. The phylogenetic tree was constructed by MEGA software version 6.06 [14] using a maximum likelihood method based on the Tamura 3-parameter model [15].

Results and Discussion

In the present study, the 2008-2010 reports of rabies in animals were updated to public knowledge. We collected brain tissue samples from 24 rabid wild and domestic animals. Out of 24 samples, RABV were detected from 22 samples by DFA (Table-1), whereas RT-PCR detected nucleoprotein gene fragment (606 bp) from all of the 24 samples. RT-PCR was found to be more sensitive than DFA in the detection of RABV positive samples. RT-PCR amplified minute quantity specific RNA isolated form the tissue samples, whereas DFA is qualitative and relative test [16]. The nucleotide sequence was initially analyzed to align from raw sequences of forward and reverse primers in each PCR amplicon, and then a total length of 442 bp nucleotide sequence was determined from each PCR amplicon. This sequence is located between 71 and 512 positions in the genome of RABV. Identity scores of nucleotide sequences were shared 96.2% and 100% among Mongolian RABVs in the present study (Table-2). In the phylogenetic tree, Mongolian RABVs are classified within steppe-type virus clade in the Figure-2a. The steppe-type virus clade spreaded in a wide geographical region such as European parts, Kazakhstan, Russia, and Mongolia [5,7]. In contrast, this steppe-type virus clade was clearly divided by two sub-clades in the phylogenetic tree; Sub-clade A and B are supported with 76% and 25% of bootstrap values, respectively (Figure-2b). The most of Mongolian RABVs are classified within Sub-clade A, except a sequence from Buryatia in Russia, which is nearly bordered with the northern region of Mongolia. Probably, this Sub-clade A is only prevalent in regions of Altai, Khangai, Khentii Mountains, which are located in Mongolia (Figure-1). However, small parts of Altai Mountain are located along Kazakhstan, Russia, and China. As for the subclade B consisted of sequences of RABV viruses isolated from Mongolia, Russia, and Kazakhstan. It seems that subclade B has a wide range of prevalence in the northern part of Eurasia including Mongolia, Kazakhstan, Russia and some European regions [7]. A few number of Mongolian RABVs

Table-2: Identity scores among N gene sequences in this study.

GenBank accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
KT338614	100	100	99.8	98.9	100	98.6	100	98.6	98.9	100	99.8	99.8	100	99.3	97.5	98.6	99.5	98	97.7	98.4	98.4	99.1	99.5	98
KT338615		100	99.8	98.9	100	98.6	100	98.6	98.9	100	99.8	99.8	100	99.3	97.5	98.6	99.5	98	97.7	98.4	98.4	99.1	99.5	98
KT338616			100	99.1	99.8	98.9	99.8	98.9	99.1	99.8	99.5	99.5	99.8	99.1	97.7	98.6	99.3	98.2	98	98.6	98.6	98.9	99.3	98.2
KT338617				100	98.9	99.8	98.9	99.8	100	98.9	98.6	98.6	98.9	98.2	98.6	98.2	98.4	97.5	97.3	98.6	99.5	98	98.4	97.5
KT338618					100	98.6	100	98.6	98.9	100	99.8	99.8	100	99.3	97.5	98.6	99.5	98	97.7	98.4	98.4	99.1	99.5	98
KT338619						100	98.6	99.5	99.8	98.6	98.4	98.4	98.6	98	98.4	98	98.2	97.3	97.1	98.4	99.3	97.7	98.2	97.3
KT338620							100	98.6	98.9	100	99.8	99.8	100	99.3	97.5	98.6	99.5	98	97.7	98.4	98.4	99.1	99.5	98
KT338621								100	99.8	98.6	98.4	98.4	98.6	98	98.4	98	98.2	97.3	97.1	98.4	99.8	97.7	98.2	97.3
KT338622									100	98.9	98.6	98.6	98.9	98.2	98.6	98.2	98.4	97.5	97.3	98.6	99.5	98	98.4	97.5
KT338623										100	99.8	99.8	100	99.3	97.5	98.6	99.5	98	97.7	98.4	98.4	99.1	99.5	98
KT338624											100	99.5	99.8	99.1	97.5	98.6	99.5	97.7	97.7	98.2	98.2	98.9	99.3	97.7
KT338625												100	99.8	99.1	97.3	98.4	99.3	97.7	97.5	98.2	98.2	98.9	99.3	97.7
KT338626													100	99.3	97.5	98.6	99.5	98	97.7	98.4	98.4	99.1	99.5	98
KT338627														100	96.8	98	98.9	97.3	97.1	97.7	97.7	98.4	98.9	97.3
KT338628															100	97.3	97.3	96.2	96.2	97.7	98.2	96.6	97.1	96.2
KT338629																100	98.4	96.8	96.8	97.7	97.7	98	98.2	96.8
KT338630																	100	97.5	97.7	98	98	98.6	99.1	97.5
KT338631																		100	99.8	97.1	97.1	97.1	97.5	100
KT338632																			100	96.8	96.8	96.8	97.3	99.8
KT338633																				100	98.2	98	98.4	97.1
KT338634																					100	97.5	98	97.1
KT338635																						100	99.1	97.1
KT338636																							100	97.5
KT338637																								100

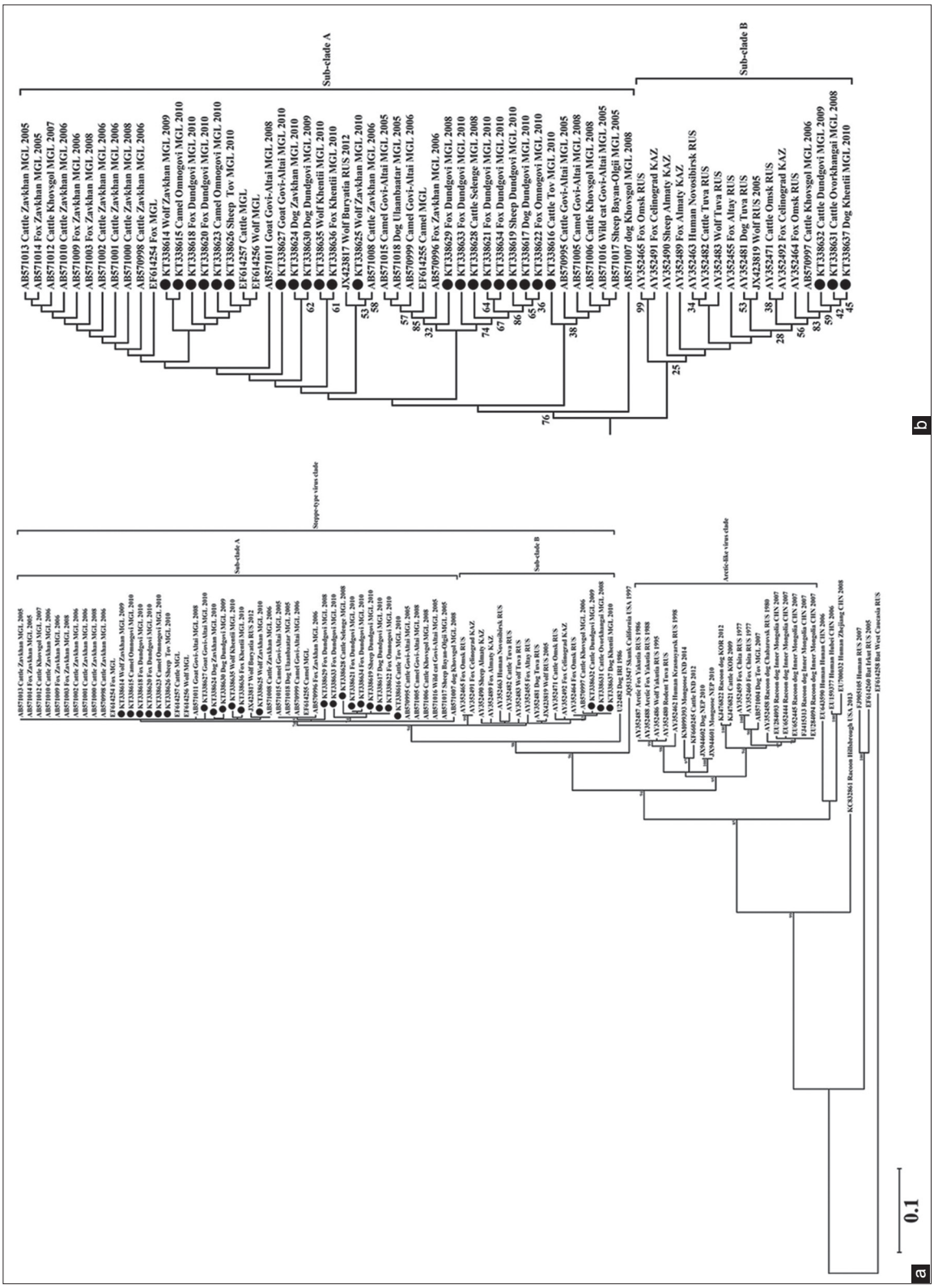

Figure-2: The phylogenetic tree involves 94 sequences including detected Mongolian rabies virus and retrieved sequences from GenBank. The percentage of phylogenetic tree in which the associated taxa clustered together is shown next to the branches. The reliability of the taxa groupings was evaluated using bootstrap with 1000 replicates. The phylogenetic tree is drawn to scale, with branch lengths measured in the number of substitutions per site. A total of 442 positions were analyzed for the phylogenetic tree, except all the gaps and missing data were eliminated, and <70% bootstrap values were hidden, (a) Steppe-type virus clade was topological viewed from initial reconstructed phylogenetic tree by sub-tree (b).

belong to Sub-clade B (Figure-2b). In the previous study (cases during 2005-2008), Mongolian RABVs were classified within steppe-type virus clade, except only an RABV, which belongs to arctic-like virus clade [6]. However, we cannot isolate arctic-like virus in the present study.

In summary, Mongolian RABVs mainly belongs to the Sub-clade A and B within steppe-type clade. The steppe-type viruses are widely distributed in Mongolia and mainly cause the incidence of rabies in wild and domestic animals. Particularly, the Sub-clade A might be a major dominant genotype of RABV in Mongolia.

Authors' Contributions

In the present study, BT has designed and performed all experiments under supervision of BB . EBat, EB, PD, and SS have contributed in sample preparation, experimental procedure and English language editing. All authors participated in draft and revision of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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