IDENTIFICATION OF THE SIBERIAN TYPE TICK-BORNE ENCEPHALITIS VIRUS AND SEROLOGICAL SURVEILLANCE IN MONGOLIA

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ABSTRACT

The goal of this study was to conduct serosurveillance for tick-borne encephalitis virus (TBEV) in Mongolia, to isolate TBEV by in vivo methods and determine subtypes of TBEV. Blood samples were collected from 750 domestic animals in Khuvsgul, Selenge, Bulgan and Tuv aimags in 2012. After sample collection, a total of 184 sera were (horse 43, cattle 41, sheep 44, goat 56 respectivel) randomly chosen and tested for antibodies against TBEV by c-ELISA and virus neutralization (VN) test.The c-ELISA results showed that 17 serum samples were positive and 25 - suspicious. In order to confirm c-ELISA result all these 42 serum samples were checked with VN test. The result of VN test showed 19/42 positive serum samples.

In order to detect of TBEV in Mongolia, Ixodes persulcatus ticks were collected from Eruu sum, Selenge aimag and categorized into pools. Each pool was mixed with sterile PBS and homogenized with a sterile mortar and pestle. The homogenate was centrifuged and the collected supernatant was inoculated into baby hamster kidney (BHK-21) cell line. Cytopathic effects (CPE) in the cell line was observed by light microscopy daily. RNA was extracted from supernatant of cells with CPE and confirmed by RT-PCR using specific primer for the Siberian subtype. The present results were indicated that TBEV in Mongolia was belonged to the Siberian subtype.

KEYWORD: Tick-borne encephalitis virus, *Ixodes persulcatus*, baby hamster kidney cell, ELISA, Virus neutralization test, Tissue Culture Infective Dose ($TCID_{50}$), Siberian type

INTRODUCTION

Tick-borne encephalitis virus (TBEV) is a member of *Flaviviridae*, genus *Flavivirus*, that causes inflammatory infections of the central nervous system, namely severe encephalitis in human and animals [12]. The TBEV positive-sense RNA genome is translated as a polyprotein and subsequently cleaved into 3 structural proteins (capsid protein C, membrane protein M and envelope protein E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4B, NS4B, NS5) [3,7]. The envelope glycoprotein (E protein) is the major structural protein and plays an important role in membrane binding and inducing a protective immune response following virus infection. It carries epitopes detected by neutralization and heamagglutination-inhibition tests that have been used to identify different *Flaviviral* subgroups and species [10]. TBEV has 3 subtypes European, Siberian and Far-Eastern

each of which has its own ecology, clinical presentation and geographic distribution [6, 12]. The European subtype is transmitted by *Ixodes ricinus,* and the Siberian and Far-Eastern subtypes are transmitted by *Ixodes persulcatus* ticks [1, 3, and 8]. *Ixodes persulcatus* ticks, vector of tick-borne encephalitis virus (TBEV) are distributed around Selenge, Bulgan, Orkhon, Tuv, Khentii and Dornod aimags of Mongolia, and it was detected that 3.4% of them carried virus of TBE [2].

Ixodes persulcatus ticks are not only vectors but also excellent reservoir hosts for the viruses they carry [10]. Transmission of TBEV to humans and animals usually occurs by bite of aninfected tick and infected animals are usually clinically asymptomatic, even though the serological result reveals positive [6, 13]. Surveillance of Mongolian livestock was performed and c-ELISA test was used for detecting this disease in 2008. However c-ELISA, VN tests and RT-PCR methods are typically used as modern techniquec to detect of TBEV of human and animals in many countries. Especially serological survey, c-ELISA and VN tests are used commonly [13, 17, 18].

That is why, the goal of this study was to confirm the results of c-ELISA test for TBE using VN test and to determine subtypes of TBEV by molecular biological methods.

MATERIALS AND METHODS

Cell culture

The baby hamster kidney (BHK-21) cell line was grown at 37^0C in Eagle's minimum essential medium (MEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 7% fetal calf serum (FCS).

Virus isolation and identification

For the detection of TBEV, ticks were collected from Eruu sum, Selenge aimag. Ticks were stored alive in 50 ml Falcon tubes with saturated humidity at room temperature until testing. All collected ticks were categorized into pools according to regions where they were sampled. The size of pools ranged from 5 to 20 ticks per pool. Each pool was suspended with sterile PBS and homogenized with a sterile mortar and pestle. The homogenate was centrifuged and the collected supernatant was inoculated into monolayer BHK cell line [16]. Cell cytopathic effects (CPE) were observed by light microscopy daily. RNA was extracted from supernatant of cells with CPE and confirmed by RT-PCR.

RNA extraction and preparation of cDNA for PCR

RNA was extracted from supernatant of cells with CPE using RNeasy mini kit (Qiagen, Germany) according to manufacturer's instructions. The cDNA synthesis was performed using random hexamer primers (Takara, Japan) and MMLV reverse transcriptase (Invitrogen) according to manufacturer's instructions.

RT-PCR with TBEV E gene specific primers

RT-PCR for detection of the TBEV E gene was performed with primers EncE-L (5'-GACCAGAGTGATCGAGGCTG-3') and 1643-R (5'-GCCAGATCATTRAACCAGTC-3') [9]. The mixture for RT-PCR was prepared using RNase-free water 13.3 мкл, dNTP mixture 1.6 мкл l, 10x buffer 2 мкл l, F and R primers each 1 мкл l, Ex Taq 0.1 мкл l, and sample cDNA 1 мкл l (final volume 20 мкл l/sample). The PCR conditions were as follows: 94°C for 5 min;40 cycles of denaturation at 94°C for1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min; and final extension at 72°C for 7 min. The PCR products were detected in UV light as ethidium bromide-stained 388-bp bands electrophoresed with a marker on 1.5 % agarose gel.

RT-PCR with TBEV specific primer for the Siberian subtype

In order to allow the identification of the TBEV subtypes, cDNA samples that were found to be positive by previous screening were selected for a second PCR assay with the following specific primers for the Siberian subtype TBEV (TBEV-E-f 5'-GGTTGCCGTTGTGTGGTTGAC-3' and TBEV-E-r 5'-TTCCGGATAGTGTGCGTAGTTG-3').

Titration of virus /$TCID_{50}$/ of isolated virus

BHK cell monolayers were formed in 96-well plates and were inoculated with the 10 fold serially diluted virus solutions. Inoculated cells were incubated in MEM containing 2% FCS at 37^0C. CPE was observed by light microscopy daily and virus titers were calculated by the Behrens-Karber method [4].

Serum samples

Blood samples were collected from 750 domestic animals in Khuvsgul, Selenge, Bulgan and Tuv aimags in 2012. After collection, a total of 184 serum samples were (horse 43, cattle 41, sheep 44, goat 56 respectively) randomly chosen and tested for antibodies against tick-borne encephalitis virus.

ELISA

Serum samples were tested in commercially available EIA TBEV-Ig kit (Test-Line, Ltd., Czech Republic) according to the manufacturer's instructions. Native serum samples were inactivated at 56^0C for 30 min. Results were expressed as a ratio of average OD value of the negative control / OD value of the sample. The cut-off value for positive sera were ≥2.00, negative sera were <1.50 and suspected sera were 1.50-1.99. All these positive and suspected sera were then tested in confirmatory assay, the virus neutralization test (VN).

Virus Neutralization (VN) test

A virus isolate with100$TCID_{50}$/0.1 ml was used for VN test. A virus titer has been adjusted at $10^{-1.5}$-$10^{-3}TCID_{50}$/0.1 ml, which is a commonly used titer in VN test. For the VN test were used positive (17) and suspicious (25) serum samples which were diluted from 1:2 to 1:64 and inactivated at 56^0C for 30 min.

RESULTS

Virus isolation

When inoculated TBEV into monolayer BHK cell line, 80% of cell cytopathic effect was observed after 96 hours. Light microscopic picture of CPE and non CFE of cells are shown in figure 1. CPE and specific cell abnormality during TBEV infection were presented such as anisocytosis (various cell sizes), spindle shape of fibroblasts become round, and altered cells detached from flask wall and suspend in medium. There were no signs mentioned above in control group cells, all cells attached to the flask wall, cell spindle shape wasn't lost and cells were in similar size (fig.1).

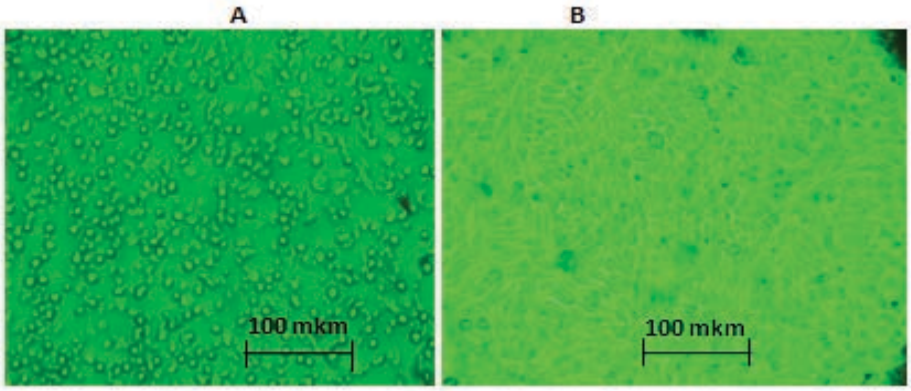

Fig.1. A. Cell cytopathic effect, B. non cell cytopathic effect

RT-PCR

After reverse transcription, the E gene of the synthesized cDNA of isolated virus was amplified by PCR using primer sets targeting the protein E gene of TBEV, which produced 388 bp amplicon. Result of the PCR was shown in figure 2.

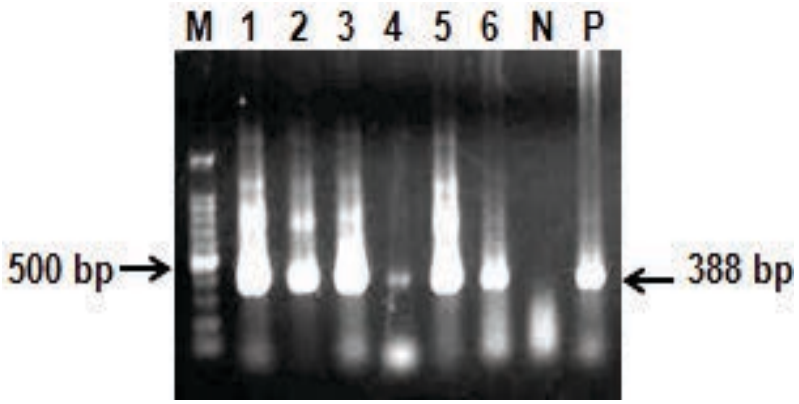

Fig. 2. Agarose gel electrophoresis of PCR product. M-100 bp ladder; 1-6 samples were positive for TBEV, N-negative control, P-positive control**.**

PCR for TBEV subtyping

For the second PCR, the positive samples for TBEV were amplified using Siberian subtypes primers of the TBEV. All samples were amplified 1500 base pair. Result was shown in figure 3

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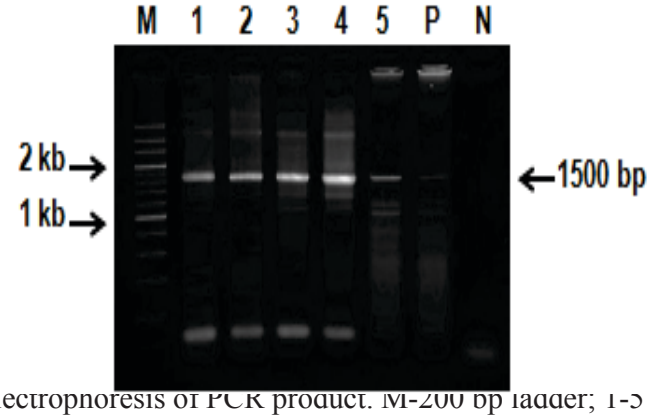

Fig. 3. Agarose gel electrophoresis of PCR product. M-200 bp ladder; 1-5 samples were positive for TBEV, P-positive control (Vasilchenko strain), N-negative control**.**

The result of RT-PCR for identification of TBEV subtypes using specific primers for the Siberian subtype showed that the subtype was close to the Siberian Vasilchenko strain, butdifferent from Far Eastern Oshima strain. Details are in table 1.

Table 1

Base pair mismatches between primers and primer-binding regions of different TBEVstrains

Primer and strain names	***Primer base pair mismatches***
Forward primer TBEV-E-f	***GGTTGCCGTTGTGTGGTTGAC***
Vasilchenko (TBEV Siberian)	***---A----------------***
Oshima (TBEV Far-Eastern)	***-C-------A------A----***
Reverse primer TBEV-E-r	***TTCCGGATAGTGTGCGTAGTTG***
Vasilchenko (TBEV Siberian)	***---G--T---------------***
Oshima (TBEV Far-Eastern)	***----C-T-CACACGC-------***

Titration of virus /$TCID_{50}$/

The determined titer of TBEV was 5.7 $TCID_{50}$/0.1ml by Behrens-Karber method.

Serological tests

From 184 serum samples, 17 samples were positive and 25 - suspicious by c-ELISA. All these 42 serum samples were checked with VN test in order to confirm the c-ELISA result. For the VN test, 17 serum samples were positive which also were positive by c-ELISA. The neutralizing antibody titers were from 1:8 to 1:32. For the suspicious by C-ELISA serum samples, 2 sera were positive by the VN test and the neutralizing antibody titers were 1:4. The remaining suspected 23 serum samples were negative by the VN test (Table2-A).

Statistical analysis

According to T.S.Saiduldin's methodology [11], quantitative interpretation was performed on the results of ELISA and VN test and correlation between those methods was $p \leq 0.05$.

Table 2-A

The result of serological survey of livestock using c-ELISA and VN tests

No. tested sera	Province	Specific	Result of C-ELISA	Result of VN tests/ titer	No. tested sera	Province	Specific	Result of C-ELISA	Result of VN tests/ titer
1	Selenge	horse	+	1:8	23	Tuv	sheep	+	1:8
2	Selenge	horse	±	negative	24	Tuv	sheep	±	negative
3	Selenge	horse	±	negative	25	Tuv	sheep	±	negative
4	Selenge	Cattle	+	1:16	26	Tuv	goat	+	1:32
5	Selenge	Cattle	±	negative	27	Tuv	goat	+	1:8
6	Selenge	Cattle	±	negative	28	Tuv	goat	±	negative
7	Selenge	sheep	+	1:32	29	Tuv	goat	±	negative
8	Selenge	sheep	+	1:8	30	Tuv	goat	±	negative
9	Selenge	sheep	±	negative	31	Khuvsgul	horse	+	1:8
10	Selenge	sheep	±	negative	32	Khuvsgul	horse	+	1:8
11	Selenge	goat	+	1:32	33	Khuvsgul	horse	+	1:8
12	Selenge	goat	±	negative	34	Khuvsgul	horse	±	negative
13	Selenge	goat	±	1:4	35	Khuvsgul	horse	±	negative
14	Selenge	goat	±	negative	36	Khuvsgul	Cattle	+	1:8
15	Selenge	goat	±	negative	37	Khuvsgul	Cattle	+	1:8
16	Selenge	goat	±	1:4	38	Khuvsgul	Cattle	±	negative

17	Bulgan	sheep	±	negative	39	Khuvsgul	sheep	+	1:16
18	Bulgan	goat	±	negative	40	Khuvsgul	goat	+	1:16
19	Bulgan	goat	±	negative	41	Khuvsgul	goat	+	1:8
20	Tuv	horse	+	1:16	42	Khuvsgul	goat	±	negative
21	Tuv	Cattle	±	negative	43	Control +sera		+	1:64
22	Tuv	Cattle	±	negative	44	Control - sera		-	negative

+=positive
- =negative
±=suspected
Control +sera= c-ELISA positive sera
Control - sera = c-ELISA negativa sera

Table 2-B

Dilution of the virus control used for the VN test

Dilution of the virus					Titration of the virus
10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	
+	+	-	-	-	$10^{-1.5}$
100 $TCID_{50}$	10 $TCID_{50}$	1 $TCID_{50}$	0.1 $TCID_{50}$	0.01 $TCID_{50}$	

DISCUSSION

Study of tick-borne encephalitis virus in Mongolia was performed by Russian scientist B.A.Kraminskii in 1979 and the virus was first isolated from the liver of the marmot hunted in Dornod aimag. As well, serological testing of some permanent residents in forests and taiga of Khangai and Khentii mountains revealed that detection of antibody against tick-borne encephalitis virus accounts for 9.5% [2]. Tick, which transmits TBE (*Ixodes persulcatus*), is distributed in Selenge, Bulgan, Orkhon, Tuv, Khentii and Dornod aimags [2]. Result of study by S.Sugir and others revealed that the prevalence of TBE is higher according to serological testing in horse, cattle, sheep and goat populations in Selenge, Bulgan and Dornod aimags [5]. Also, results of our study of the virus isolation and serological testing are in agreement with those reported by above authors.

In order to isolate causative agent of TBE, the method for inoculation of pathologic specimen in BHK-21 cell line described by T.Tsutomu et al [17], and their results are consistent with those of the present study. When cells were infected with positive control of the virus diluted from100 $TCID_{50}$/0.1ml to 0.01 $TCID_{50}$/0.1 ml, cytopathic effect was detected in both 100 $TCID_{50}$/0.1 ml and 10 $TCID_{50}$/0.1 ml titrations or equal to $10^{-1.5}$ $TCID_{50}$/0.1 ml. This result is in agreement with the information that titration of a virus equal to $10^{-1.5}$-$10^{-3}$$TCID_{50}$/0.1 ml means the virus neutralization test is seen to be true as reported by Brain. W. J [4] and according to documents such as Diagnosis of animal influenza released from WHO (Table 2 B).

Because, the structure of TBE virus is similar to that of Japanese encephalitis virus, there is a case of cross-reactivity [15]. A total of 184 serum samples were used in our testing, and competitive ELISA testing for detection of antibodies specific to TBE causative agent demonstrated 17 samples are positive and 25 samples are doubtful. With the purpose of confirmation relied on above information, we tested a total of both ELISA positive and doubtful 42 samples by VNT, and ELISA positive 17 samples were tested as positive by VNT. However, 2 samples of 25 ELISA doubtful samples were positive by VNT. The results of our study reveal that the virus neutralization test is highly sensitive reaction, which is capable of measuring neutralizing

antibodies contained in the serum. Therefore, we are in agreement with the authors who used broadly VNT in serology and mentioned its sensitivity is higher than ELISA [17, 18]. Above mentioned serological testing results demonstrate that use of TBE virus isolated by us can be used in a serological method - virus neutralization test for diagnostic purpose.

Detection of ticks *Ixodes persulcatus* infected with the causative agent of this disease as a result of the testing and typing of collected ticks, which transmit TBE, is also consistent with the results of the study performed by J.Bataa et al. that causative agent of the disease is present in Selenge aimag [11]. As well it reveals the result is similar to the study that ticks *Ixodes persulcatus* transmit the causative agent of TBE in both Far East and Siberia and spreads in East Europe and Asia [13].

Causative agent of TBE divided into 3 types [2, 6] and it is demonstrated that nucleotide sequence from causative agent isolated by us is closer to those from Vasilchenko strain isolated from Russian Novosibirsk belonging to the Siberia type. Difference of the causative agent isolated by us with nucleotide sequence from type of Far East reveals it is in agreement with the study of Stepan and Prey that the Siberian type is in our country [6, 3]. Currently, according to the results of our study, it is probably that the causative agent of the Siberian type in our country is slightly different from that type in Russian Federation and therefore it is necessary to perform more detailed study in the future.

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