

## INVESTIGATION ON BOVINE VIRAL DIARRHEA VIRUS AND INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS IN MONGOLIAN DAIRY FARMS

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### ABSTRACT

*In this study, 168 blood sera were collected from dairy cows in Selenge and Tuv aimags during 2013 - 2014. The ELISA was carried out for serological detection of antibodies and antigens to Bovine viral diarrhea virus (BVDV), antibodies to Infectious bovine rhinotracheitis virus (IBRV) in dairy cows in Mongolia. The ELISA results of antibodies of BVDV and antigen of BVDV showed that 86.9% and 3.57%, respectively. The seroprevalence of antibodies against IBRV was found to be 60.7%. In order to confirm of BVDV, One Step RT-PCR was performed in ELISA positive cattle serum samples using specific primer for BVDV. The results showed that 294 bp fragment was successfully amplified. Phylogenetic analysis revealed that the nucleotide sequences 5'UTR gene of the isolates belonged to the BVDV1 subtype. Four isolated virus samples were closely related to China, the another isolate was closely related to the Slovenia BVDV1 isolate.*

**KEY WORDS:** BVDV, IBR, ELISA, One-Step RT-PCR, Phylogenetic analysis, BVDV-1

### INTRODUCTION

The dairy industry in Mongolia was first established in the early 1980s in the central region of Mongolia, through a royal co-operative between the Mongolian and the Russian governments. During the following decades the dairy cattle population and the milk production increased but not sufficiently to meet the increasing market demand for milk and milk products. Therefore, since the middle of the 1990s, the Mongolian government has encouraged farmers to start small holder dairy farms in other parts of the country, including the north-eastern aimags. Initially dairy cattle were introduced from central Mongolia but as the numbers were inadequate, pregnant cows and heifers were imported from

several other countries, mainly Russia, China, Germany and French.

There are about 17000 dairy cattle in 22 herds in these provinces that sell their milk to 9 milk collection centers. Cattle are the natural host of some virus diseases. Bovine viral diarrhea virus (BVDV), Infectious bovine rhinotracheitis virus (IBR-1) are still occur in many countries including Mongolia.

In Mongolia, first time BVDV was diagnosed from dairy cow in 1980 in Central aimag, where dairy cow farm developed. Since that time, dairy cow farms were collapsed because Mongolia transmitted from socialist to democratic system. But since 2000, many dairy cow farms are redeveloping in Mongolia. To

improve the milk product, Mongolia is continuing to import a large number of cows from other countries.

In this regard, there are many viral and bacterial diseases diagnosing in cow population. Nowadays it is observed that prevalence (outbreak) of viral diseases among highly profitable dairy cattle in Mongolia is increasing, so that it is necessary to improve diagnostic, control and preventative methods

## MATERIALS AND METHODS

Seven organized dairy farms located in northern and central regions of Mongolia which development dairy farm were identified for this study during the year 2013-2014 (Figure -1).

For serological investigations, a total of 168 cattle serum samples collected from dairy farms for

for those diseases as quickly as possible.

Due to transition of Mongolian economy to market economy, dairy cattle farms were privatized, furthermore control on those diseases was weakened and study of viral diseases was abandoned.

Therefore, we have involved in this study some Mongolian dairy farms. This study was undertaken to determine the viral diseases in the cow population.

screening against BVDV, IBR-1. Twenty blood serum samples were collected for detection of BVDV. All serum and blood samples were stored at  $-20^{\circ}\text{C}$  until used for testing. Before performing all tests, all serum samples heat inactivated at  $56^{\circ}\text{C}$  for 30 minutes.



Figure 1. Selected Selenge and Tuv aimag are in pink shade

### Bovine viral diarrhea virus

#### Antibody ELISA

All 168 serum samples were screened for antibodies to BVDV by using commercial indirect ELISA kit (IDEXX Europe, B.V., The Netherlands) according to the instructions of the manufacturer. The plates were then read on an automatic plate reader at 450 nm.

#### Antigen ELISA

All 168 serum samples were tested to detect BVDV antigen in cattle sera by a commercial Erns capture ELISA (Herd Check BVDV Ag/Serum plus, IDEXX Laboratories INC.) according to the manufacturers' instructions. The plates were read on an automatic plate reader at 450 nm.

#### One Step RT-PCR

For detection of BVDV, RNA was extracted directly from the antigen positive serum and blood samples using RNA Purification kit (Thermo scientific, USA) according to manufacturer's instructions.

One Step RT-PCR for detection of the BVDV 5'UTR gene was performed with primers (5'-gctagccatgcccttagtagga-3') and (5'-atcaactccatgtgccattacagc-3'). The mixture for One

Step RT-PCR was prepared using RNase-free water 8.5  $\mu\text{l}$ , 2x One Step RT-PCR Buffer III 12.5  $\mu\text{l}$ , Ex Taq (Takara) 0.5  $\mu\text{l}$ , PrimeScript RT Enzyme Mix II 0.5  $\mu\text{l}$ , F and R primers each 0.5  $\mu\text{l}$  and sample RNA 2  $\mu\text{l}$ , (final volume 25  $\mu\text{l}$  l/sample).

The One Step RT-PCR conditions were as follows:  $45^{\circ}\text{C}$  for 10 min,  $95^{\circ}\text{C}$  for 10 min; 35 cycles of denaturation at  $95^{\circ}\text{C}$  for 15 sec, annealing at  $58^{\circ}\text{C}$  for 35 sec, and extension at  $72^{\circ}\text{C}$  for 20 sec; and final extension at  $72^{\circ}\text{C}$  for 10 min. The PCR products were detected in UV light as ethidium bromide-stained 294-bp bands electrophoresed with a marker on 1.5 % agarose gel.

#### Nucleotide sequencing and phylogenetic analysis

Nucleotide sequence of the 5'UTR gene of the BVDV isolates obtained were analyzed. The PCR products for sequencing were separated by 1% agarose gel electrophoresis and purified using QIAquick Gel Extraction Kit according to the company's protocol. The purified products were used as templates in sequencing reactions using a BigDye terminator ver.

3.1 cycle sequencing kit with the same primers as used for amplification. Nucleotide sequencing was performed by Genetic Analyzer 3500 (Applied Biosystems).

The obtained sequences were aligned using CLUSTALW software (Thompson et al., 1994). Phylogenetic analysis was performed using the MEGA software (version 5.2). Bootstrap analysis was carried out on 1000 replicates. The nucleotide sequences were compared with each other using the identity matrix in BioEdit v.7.1.9 software (Hall, 1999) and with the 5'UTR region of the reference

strains available in the GenBank database using blast maximum identity tool (NCBI). Phylogenetic trees were constructed using the neighbor-joining and maximum likelihood methods.

#### **Infectious bovine rhinotracheitis virus**

##### **Antibody ELISA**

Diagnosis of IBR was carried out by detection of antibodies against IBR-1 virus from serum by using CHEKIT- Trachitest Serum- Screening, an ELISA kit from IDEXX Europe, B.V., The Netherlands. All serum samples diluted 1:2 times in dilution buffer.

## **RESULTS**

### **Seroprevalence of Bovine viral diarrhoea virus**

The samples collected area was shown in figure 1. The overall seroprevalence for detection of antibodies against Bovine viral diarrhoea virus was 86.9% (146/168) and each province had positive samples.

Moreover, the prevalence rates varied greatly in different aimags from 60.8% to 100%. However, the overall seroprevalence for detection of antigen for Bovine viral diarrhoea virus was 3.57% (6/168) and five farms had positive samples (Table1).

Table 1

Seroprevalence of Bovine viral diarrhoea virus								
Aimags	Name of Soum	Name of farms	No. of sera tested (heads)	No. of sera positive antibody	Positive rates (%)	No. of sera positive antigen	Positive rates (%)	
Selenge	Bayangol	Altangerel	20	14	70	-	-	
		Sukhbat	33	27	81.8	-	-	
		Bayanmunkh	17	17	100	1	5.8	
Tuv	Bornuur	Altai	25	25	100	1	4	
		Tsetsgee	25	25	100	1	4	
	Batsumber	Lkhagvaa	25	24	96	2	8	
	Arhust	Nuudelchin	23	14	60.8	1	4.3	
Total			168	146	86.9	6	3.57	

### **One Step RT-PCR**

After extraction of RNA from the antigen positive serum and blood samples, One Step RT-PCR was amplified using primer sets targeting the protein 5'UTR gene of BDVD, which produced 294 bp amplicon. Result of the PCR was shown in figure 2.

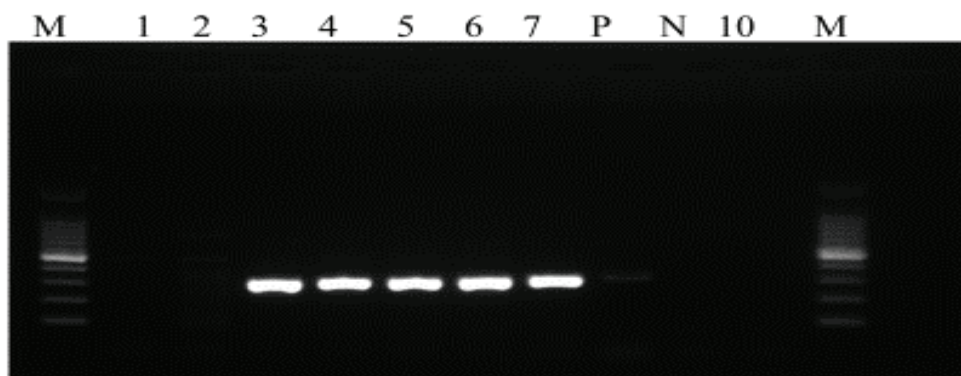


Figure 2. Result of PCR

*Agarose gel electrophoresis of PCR product. M-100 bp ladder; 1, 2 and 10 samples were negative, samples 3-7 positive, P-positive control, N-negative control for BVDV*

Nucleotide sequencing and phylogenetic analysis  
 All 5 BVDV samples were positive in One Step RT-PCR using 5'UTR gene targeting protein primers which produced 294 bp amplicon. The nucleotide of these 5 BVDV isolates were successfully sequenced, and all data were deposited in Genbank under accession numbers LC043217-LC043221.

Phylogenetic analysis of the fragments from 5'UTR region revealed that all 5 Mongolian viruses belonged to BVDV species-1. The 5'UTR sequence analysis confirmed completely this classification. The phylogenetic tree of BVDV isolates based on the 5'UTR region is presented in figure 3.

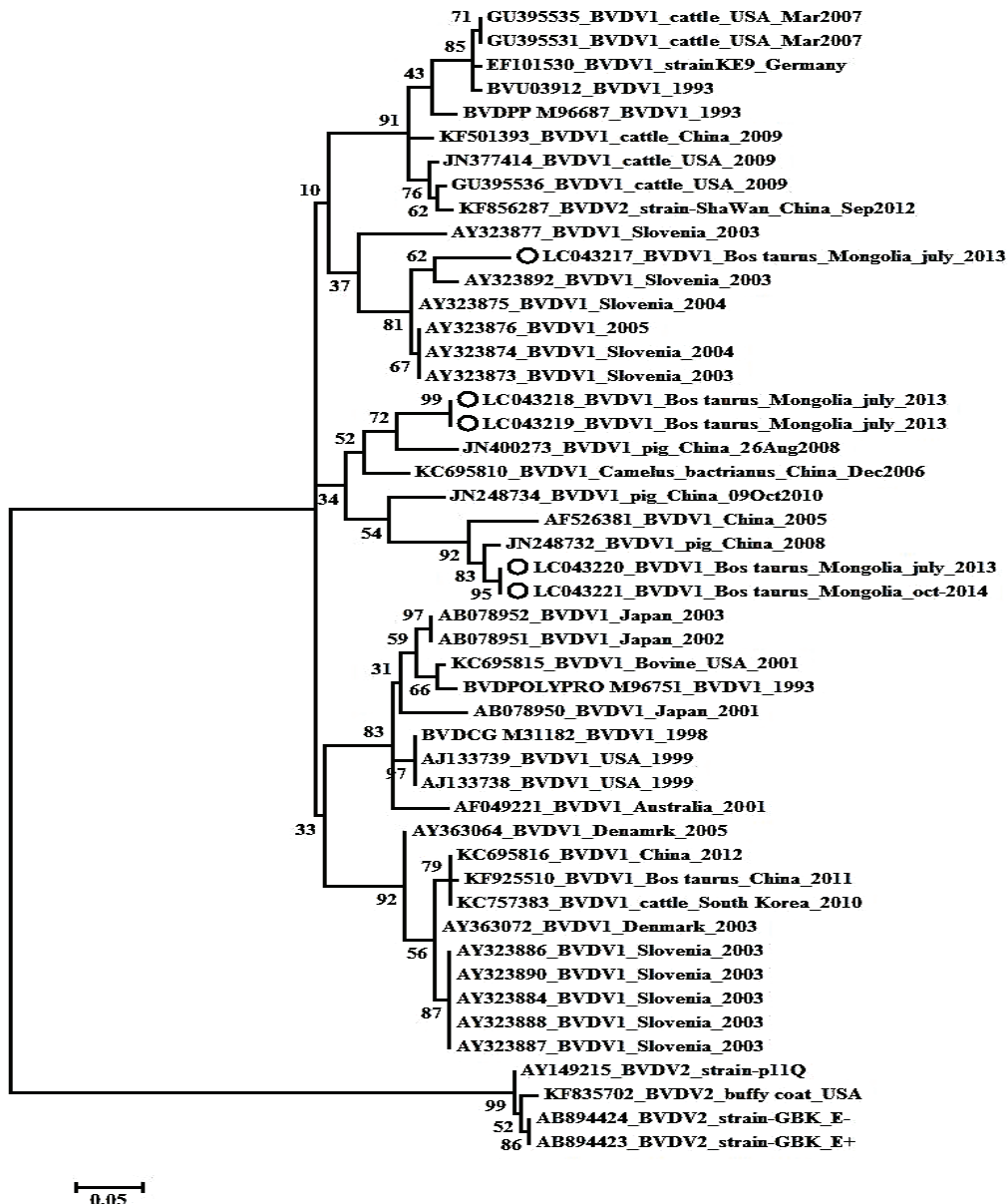


Figure 3. Phylogenetic tree of 5 Mongolian field isolates of BVDV within 5'UTR region, created with neighbor-joining method and bootstrap test (n = 1000) using Mega ver. 5.2. Reference strains from GeneBank are indicated in red circles.

Comparison of the 5'UTR products at the herd level revealed high nucleotide sequence homology ranging from 94% to 98% sequence homology were identified. No relationship between the geographic distribution and the BVDV subtype was observed. This is the first report describing genetic variability of the field isolates of bovine viral diarrhea virus in

Mongolia. In order to minimize the possibility of biased genotyping results (Vilcek et al., 2005), total RNA was extracted directly from serum without prior virus multiplication in cell culture to avoid any possible contamination from the cell culture what has been discussed previously (Polak et al., 2008). Samples from positive animals came from seven

herds located in central parts of Mongolia. In the herd, there was no vaccination of BVDV-1 were identified, the owner implemented vaccination just after the identification of the first detection of BVDV symptoms clinically showed and confirmed by molecular biology's techniques above described.

### Seroprevalence of Infectious bovine

### rhinotracheitis virus

The overall seroprevalence for detection of antibodies against Infectious bovine rhinotracheitis virus was 60.7% (102/168) and six farms had positive samples. Moreover, the prevalence rates varied greatly in different provinces from 40% to 94.1% (Table 2).

Table 2

Seroprevalence of Infectious bovine rhinotracheitis						
Aimags	Name of Sum	Name of farms	No. of sera tested (heads)	No. of sera positive antibody	Positive rates (%)	
Selenge	Bayangol	Altangerel	20	9	40	
		Sukhbat	33	25	75.7	
		Bayanmunkh	17	16	94.1	
Tuv	Bornuur	Altai	25	20	80	
		Tsetsgee	25	15	60	
		Lkhagvaa	25	17	68	
		Nuudelchin	23	-	-	
Total			168	102	60.7	

## DISCUSSION

In organized dairy farms, high economic losses are attributed mainly due to reproductive disorders, caused by infectious agents like BVDV, IBR etc. Hence, in the present of abortions were screened against the diseases, BVDV, bovine brucellosis and IBR, so as to pinpoint a disease control strategy.

Study of BVDV in Mongolia was performed by Mongolian scientist Perenlei. L in 1980. Since 1982, he had produced BVDV cell culture vaccine as experiment to prevent dairy farms according to technology of Institute of Pilavski, Hungarian [Perenlei et al.]. Since that period, dairy cattle haven't been vaccinated with BVDV and disease surveillance hasn't been done yet. As a result of our study, prevalence of BVDV is high in dairy cattle.

Serological tests performed in the present study for diagnosis of BVDV are antibody ELISA and antigen ELISA. An overall sero-prevalance of BVDV was recorded to be 86.9% (146/168) and 3.57% (6/168) by antibody ELISA and antigen ELISA, respectively. As seen in our study, result of BVDV antibody detection analysis is higher than the result of antigen detection test. This indicates that dairy cattle have chronic or latent infection of BVDV. Although, antigen detection test result indicates that BVDV is sexually transmitted among cattle of same farm.

BVDV-1 is the most prevalent species in worldwide, especially in United Kingdom (Strong et al., 2013), in other countries like France, Spain, Austria, Slovenia, Slovakia, Russia and China only single BVDV-1 strains were identified (Graham et al., 2001; Giangaspero et al., 2001; Tajima et al., 2001; Vilcek

et al., 2001; Drew et al., 2002; Vilcek et al., 2002). The import of live animals into Mongolia after political system changes 1990 showed increasing trend and the number of imported calves was growing. The main exporting countries, responsible for over 90% of all imported animals were and are Russia, France, Poland, Slovenia, Slovakia, Czech Republic and the especially China.

We isolated 5 strain of BVDV virus from cattle in 2013-2014. This is the first isolation of the BVDV virus in Mongolia. The 5'UTR gene of all the 5 isolates belonged to the BVDV1 subtype.

Our first 2 isolates acc. no. LC043218 and LC043219 of BVDV-1 the genotype relation is closely related with Chinese strain 2008 but in pig species with nucleotide sequence homology of 94%, while other 2 isolates acc. no. LC043220 and LC043221 of BVDV-1 the genotype relation is closely related with Chinese strain 2008 but in pig species with nucleotide sequence homology of 98% and those isolates strain are related with Chinese strain 2008. However, last isolate acc. no. LC043217 of BVDV-1 the genotype relation is closely related with Slovenian strain 2003 with nucleotide sequence homology of 96%. That is surprising situation. It's may indicate the possible transmission between the countries. Our studies carried out to the first isolation of BVDV and their subtyping in central part of Mongolia, it's probably due to the expand of cattle trade over decades. Similar observations were made in China.

As a neighboring countries China and Russia, the increasing import of live cattle in recent years may

lead to the appearance of BVDV-1 in Mongolia. According to the authors, the introduction of BVDV types was possibly caused by the import of live animals from continental Europe as a result of mass culling of animals during foot-and-mouth disease outbreak in 2001 and the elimination of infected animals during the eradication of bovine tuberculosis. For the IBR (Infectious bovine rhinotracheitis), In 1988-1989, antibody detection test (Virus

neutralization assay) for Bovine rhinotracheitis was carried out on 1713 bovine blood samples from 22 farms and 614 (35.8%) of them were positive [Purevtseren et al.]. Since that time, study on IBR hasn't been done. As a result of our study, antibody detection test result for Bovine rhinotracheitis is 60.7% (102/168) which is higher than previous study, and it shows that prevalence of this disease seems to be increased.

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