

## Research Article

# Pathogenicity of a Non-Cytopathic Bovine Viral Diarrhea Virus 2b Strain Isolated from Cattle in China

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**Abstract**

Bovine Viral Diarrhea Virus (BVDV) type 2 causes Hemorrhagic Syndrome (HS) in cattle and first emerged in 1990 in North America. In 2014, a non-cytopathic BVDV2b strain, SD1301, was isolated and sequenced in China. In order to analyze the virulence of this strain in cattle, five calves were challenged intranasally by spraying 3 ml of virus into each nostril (about  $3 \times 10^6$  TCID<sub>50</sub>/ml). Results showed that all calves developed clinical signs with rectal temperature higher than the base temperature 1°C at least 1 day, and White Blood Cells (WBC) and platelet counts decreased by over 40%. Gross anatomical examination at necropsy found that the lymph node leukopenia, lymphopenia, neutropenia, thrombocytopenia. The virulence of the strain isolated suggested that the virus was like that a moderate virulent strain.

**Keywords:** Bovine viral diarrhea virus type 2; Pathogenicity; China

**Introduction**

Bovine Viral Diarrhea Virus (BVDV), a non-enveloped, single positive-stranded RNA virus in the genus Pestivirus of family Flaviviridae virus, is well known as one of pathogen causing Bovine Respiratory Disease Complex (BRDC) [1].

In nature, BVDV1, BVDV2 and BVDV3 are typed, based on the 5'UTR [2-4]. BVDV2 can be further divided into four genetic subgroups currently [5]. In another type of classification, two biotypes of BVDV 2 were classified as cytopathic (cp) or noncytopathic (ncp) depending upon the effect on cell culture [6]. Infection of BVDV may result in a wide of clinical signs, but most BVDV infections are not accompanied by clinical signs of infection [7]. Highly virulent strains cause acute disease, while low virulent virus may induce a secondary infection as a substantial infection. However, outbreaks of severe hemorrhagic disease were reported in association with BVDV2 [8,9]. Ncp-BVDV infection can result in Persistently Infected (PI) animals which shed the virus throughout its life. This increases the risk of developing Mucosal Disease (MD) when an antigenically similar cp-BVDV and ncp-BVDV were co-infected the same cattle. It is important to understand the virulence of a newly virus to a livestock and tracing the epidemiology of the disease [10].

In China, a BVDV2b isolate was first reported and genetic characterization was analyzed in 2014 [11], while the virulence of the virus was not determined. In this study, we described a further virulence by experimental infection of calves with the BVDV2b strain, based on clinical signs following infection of seronegative, BVDV negative calves.

**Materials and Methods****Samples and virus isolation**

Nasal swabs samples were collected from the cattle that showed mild respiratory clinical signs, such as nasal discharge and cough in Shandong province, China. The nasal swab samples were collected and put into a tube containing 2 ml DMEM (HyClone, USA)

supplemented with 10% horse serum (Hyclone, USA), 150 µg/ml gentamicin sulfate (Sigma, USA), 7.5 µg/ml fungizone (Sigma, USA), and Streptomycin at 100 µg/ml. Three milliliter (3 ml) blood samples were collected using an EDTA vacuum blood tube from the jugular vein. All the samples were kept at 2-8°C and quickly transferred to the laboratory. Nasal swabs were inoculated into MDBK cell monolayers in 24-well tissue culture plates for virus isolation. Briefly, following centrifugation at 1500 rpm for 10 min, the samples were filtered through 0.45 µm membrane (Sigma, USA) and then inoculated onto the MDBK cell monolayer cultured in 0.5 ml DMEM (HyClone, USA) supplemented with 6% horse serum (Hyclone, USA) in 24-well cell culture plates, and incubated at 37°C, with 5% CO<sub>2</sub> for 2 hours. Then, the supernatants were discarded, and plates were rinsed twice with PBS (pH7.2, 0.01 mol/L), and 1 ml DMEM (HyClone, USA), with 3.5% horse serum was added. The infected-MDBK cell plates were checked daily and appearance of Cytopathic Effects (CPE) was observed and recorded. If the CPE was not found, the cultures were frozen and thawed twice and the clarified supernatant was passaged three times in MDBK cells. Un-infected MDBK cultures were included as negative controls and MDBK cells inoculated with BVDV NM01 strain, which was previously isolated and identified by our laboratory, was used as positive control. After 3-4 days of incubation at 37°C, with 5% CO<sub>2</sub> supply, the virus was confirmed by immunofluorescence on cell monolayers. The isolated virus was named BVDV strain SD1301.

**Calves and housing**

Five Mongolian cattle, aged 3-4 months old were purchased from a calf farm in Inner Mongolian Autonomous Region, China. All animals did not vaccinate with BVDV vaccine prior to purchase and were tested BVDV-negative and BVDV-neutralizing-antibodies-negative. All calves were apparently healthy, no clinical signs of depression, cough or other health disorders. The selected animals were transported to an animal facility in Inner Mongolian. All animal experiments were approved by the Animal Care and Use Committee of Chinese Academy of Agricultural Sciences.

## Virus infection and temperature recording

Two days prior to challenge, all calves were transferred to a bio-level 3 safety facility. Calves were randomly allocated into two groups. Three calves were intranasally inoculated with 6 ml (3 mL/nostril) of cell culture grown viral harvest containing 106.5FAID50 per milliliter of the SD1301 strain. Two calves in the control group were inoculated with sterile cell culture medium. The challenge procedure was performed by spraying 3 ml of virus samples into each nostril, using an atomizer (Devillebiss, Somerset, PA, USA). To confirm infectious titers, viral suspensions were back titrated on MDBK cells after inoculation. All calves were monitored for 14 days and rectal temperatures were collected twice a day every day at the regular time by researchers who were blinded to the treatment groups.

## Clinical assessment

Calves were clinically observed daily from day 1 to day 14 post-challenge. Clinical signs including depression, cough, asthma, and other respiratory disease.

## Sample collection

Blood samples were collected from jugular vein into an EDTA vacutainer from 2 days pre-challenge through 14 days post-challenge for automated detection of White Blood Cells (WBC) and thrombocyte counts by a Vetscan HM5 veterinary hematology system (Abaxis, USA).

Deep nasal swab specimens were collected at 1 day prior to challenge through 14 days post-challenge. After collection, swabs were placed in 3 ml of transport DMEM media (Hyclone, USA) supplemented with 10% horse serum (Hyclone, USA), 150 µg/ml gentamicin sulfate (Amresco, USA), 7.5 µg/ml fungi zone (Amresco, USA), and 100 µg/ml streptomycin (Sigma, USA). All swab specimens were stored at -70°C until virus isolation.

## Virus isolation

Viruses were isolated from nasal swabs or blood leukocytes of challenged calves. Samples were inoculated into MDBK for three passages for virus isolation as described by Xue [12].

## Histopathologic study

On day 14 dpi, two virus-infected calves and one control cattle were euthanized. Tissue samples of liver, spleen, lung, heart, kidney, intestine, mandibular lymph node, and mesenteric lymph node were collected and fixed in 10% buffered formalin for histopathological analysis in Inner Mongolia agricultural university.

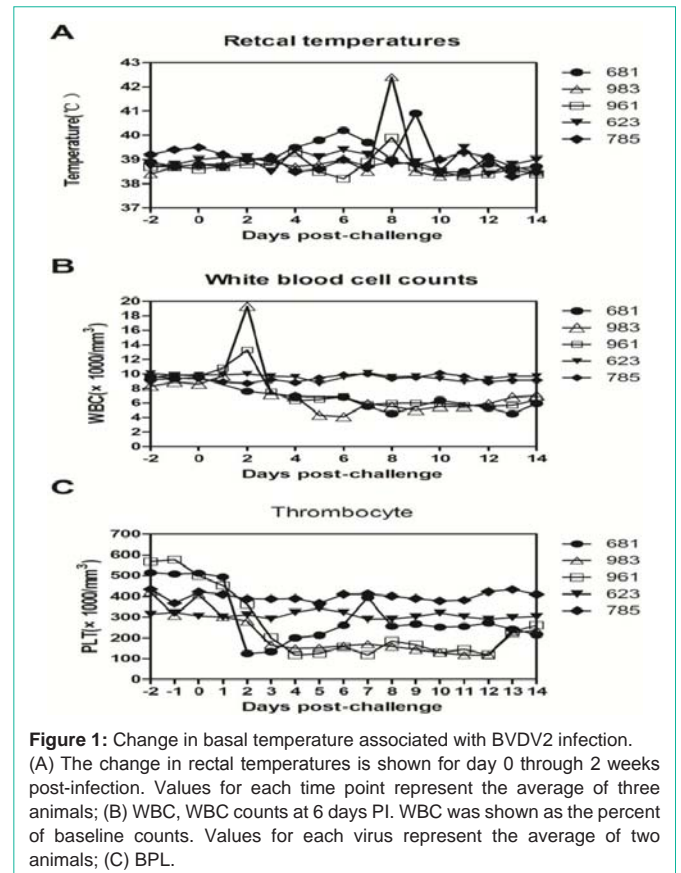
## Data analysis

Results for WBC count and blood platelet count were analyzed using Fisher Exact test of SPSS Version 20 (IBM, China). Statistical significance was set at  $p < 0.05$ .

## Results

### Clinical signs and rectal temperature

Clinical signs were scored according to the severity of clinical symptoms, including elevated rectal temperatures, depression, loss of appetite, nasal discharge, respiratory distress, excessive salivation. Two calves infected with the virus developed significant clinical signs with the characteristic nasal discharge and depression at 2 day post-inoculated (dpi). All challenged calves showed asthma and



**Figure 1:** Change in basal temperature associated with BVDV2 infection. (A) The change in rectal temperatures is shown for day 0 through 2 weeks post-infection. Values for each time point represent the average of three animals; (B) WBC, WBC counts at 6 days PI. WBC was shown as the percent of baseline counts. Values for each virus represent the average of two animals; (C) BPL.

excessive salivation. Calves from the control group had no clinical signs throughout all experiment courses. The body temperature of calf #681 was elevated 39.8°C at 5dpi and 40.2°C at 6dpi, with the highest temperature of 40.8°C at 9 dpi. Calf #983 was 42.4°C at 8 dpi. Calf of #961 was reached 39.4°C at 3 dpi and 39.9°C at 8 dpi, respectively (Figure 1A). Calves of control group were within the normal temperature (Table 1).

### Leukopenia

Baseline of WBC counts and blood platelet of all calves were within normal scopes. WBC counts of challenged calves started decreasing from 2 dpi. The WBC counts of calf #681 dropped significantly from 9.3 (1000 per mm<sup>3</sup>) at day 0 to 4.5 (1000 per mm<sup>3</sup>) at day 8 dpi, decreased by 51.6%. The WBC counts of calf #983 decreased from 8.9 (0 day) to 4.1 (6 dpi), decreased by 53.9%. The WBC counts of calf #961 decreased from 11.3 (0 day) to 5.77 (7 dpi), decreased by 48.9%. The mean WBC counts in the control group did not decrease, and were significantly higher ( $P < 0.05$ ) than those of the challenge group (Table 2,3 and Figure 1B).

### Thrombocytopenia

Blood platelet count of all the calves declined significantly ( $p < 0.05$ ) compared the challenged day with post-challenged day (from 2 to 14). As a single day 8 dpi, the platelet count of calf #681 reduced consistently from 514×10<sup>9</sup>/L at challenge day to 257×10<sup>9</sup>/L. The platelet count of calf #983 decreased from 422×10<sup>9</sup>/L to 159×10<sup>9</sup>/L. The platelet count of calf #961 dropped from 577×10<sup>9</sup>/L to 185×10<sup>9</sup>/L. All the challenged cattle decreased by 50%, 62.3%, 67.9%, respectively.

**Table 1:** Temperature of post challenge virus inoculation.

Group	Animal #	Day post challenge																	
		-2	-1	0	Base temperature	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Challenge	681	38.9	38.7	38.8	38.8	38.7	39.0	39.0	39.5	39.8	<b>40.2</b>	39.7	39.0	<b>40.9</b>	38.5	38.5	38.8	38.6	38.7
	983	38.4	38.7	38.8	38.6	38.8	39.1	38.9	38.7	38.8	39.0	38.5	<b>42.4</b>	38.5	38.3	38.4	39.1	38.7	38.5
	961	38.7	38.7	38.6	38.6	38.7	38.8	38.8	39.3	38.5	38.2	38.9	<b>40.1</b>	38.7	38.5	38.3	38.4	38.6	38.4
Control	623	38.7	38.8	39.0	38.8	39.1	39.1	38.5	39.4	39.1	39.5	39.2	38.8	38.9	38.5	39.5	38.4	38.8	39.0
	785	38.6	38.4	38.5	38.5	39.0	39.0	38.9	38.5	38.6	39.0	38.7	38.9	38.8	39.0	38.8	38.6	38.3	38.5

**Table 2:** WBC counts after challenge virus inoculation ( $\times 10^9/L$ ).

Group	Animal #	Day post challenge												
		-2	-1	0	$W_0$	1	2	3	4	5	6	7	8	
Challenge	681	9.2	9.3	9.5	9.3	10.1	7.6	6.8	6.9	6.6	6.8	5.5	4.5	
	983	8.3	8.9	8.6	8.6	9.7	19.3	7.2	6.9	4.3	4.1	5.9	5.5	
	961	9.5	9.9	9.7	9.7	10.8	13.3	7.5	6.3	6.5	6.9	5.8	6.0	
Control	623	10.1	9.8	9.9	9.9	9.9	10	9.7	9.9	8.7	9.5	10.2	9.6	
	785	9.7	9.4	9.3	9.5	8.9	8.7	9.2	8.8	9.5	9.9	10	9.3	

**Table 3:** Percentage of decrease of WBC counts.

Group	Animal #	Day post challenge								
		1	2	3	4	5	6	7	8	
Challenge	681	8.6%	-18.3%	-26.9%	-25.8%	-29.0%	-26.9%	<b>-40.9%</b>	<b>-51.6%</b>	
	983	12.8%	124.4%	-16.3%	-19.8%	<b>-50.0%</b>	<b>-52.3%</b>	<b>-31.4%</b>	<b>-36.0%</b>	
	961	11.3%	37.1%	-22.7%	<b>-35.1%</b>	<b>-33.0%</b>	<b>-28.9%</b>	<b>-40.2%</b>	<b>-38.1%</b>	
Control	623	0.0%	1.0%	-2.0%	0.0%	-12.1%	-4.0%	3.0%	-3.0%	
	785	-6.3%	-8.4%	-3.2%	-7.4%	0.0%	4.2%	5.3%	-2.1%	

**Table 4:** Blood platelet counts of calves after challenge virus ( $\times 10^9/L$ ).

Group	Animal #	Day post challenge											
		-2	-1	0	$W_0$	1	2	3	4	5	6	7	8
Challenge	681	514	508	512	511	493	125	134	200	213	261	395	257
	983	422	306	410	379	300	280	165	149	152	162	170	159
	961	568	577	500	548	453	361	201	118	125	158	118	185
Control	623	314	322	304	313	300	311	290	323	343	322	289	290
	785	434	367	422	407	410	389	388	390	367	411	412	401

The average greatest decline in circulating platelet observed after inoculation with the virus was significantly different from control groups (Table 4,5 and Figure 1C).

### Viremia

The extent of viremia caused by the BVDV challenge, as detected from 2 dpi to 14 dpi in blood samples or nasal swabs was summarized in Table 6. Three infected cattle had viral shedding as early as 2 dpi. The virus was detected in blood 10 days after inoculation and up to 6 days post inoculation (dpi) in nasal swabs. Peak shedding was detected at 4-8 dpi both in blood or nasal swab.

### Histopathology

Two randomly challenged calves (#675 and #642) and one control calf (#683) were euthanized at 14 dpi for histopathology. Gross pathological findings in the inoculated calves were in the respiratory and digestive system. Hemorrhages in the spleen and

mesenteric lymph nodes were observed. Significant microscopic lesions were present in the trachea, characterized with coagulation necrosis of epithelial cells and submucosal hemorrhages with minimal lymphocytic infiltrate. Moderate or severe edema was present in the intestinal mesenteric lymph nodes in all inoculated calves and they showed mild to moderate follicular lymphohyptolysis. None of the samples from the control cattle showed any pathological changes (Figure 2).

### Discussion

In this report, virulence of BVDV2b strain SD1301 was characterized for the first time in China. Severe disease characterized with pyrexia, leukopenia, thrombocytopenia, lymphopenia, and asthma were induced in calves on upon challenge with BVDV-SD1301. A previous study showed that the most clinically severe form of acute BVDV infection was associated with ncp BVDV-2 strains

**Table 5:** Percentage of decrease of blood platelet counts after challenge virus inoculation.

Group	Animal #	Day post challenge							
		1	2	3	4	5	6	7	8
Challenge	681	-3.5%	<b>-75.5%</b>	-73.8%	-60.9%	-58.3%	-48.9%	-22.7%	-49.7%
	983	-20.8%	-26.1%	-56.5%	<b>-60.7%</b>	-59.9%	-57.3%	-55.1%	-58.0%
	961	-17.3%	-34.1%	-63.3%	<b>-78.5%</b>	-77.2%	-71.2%	<b>-78.5%</b>	-66.2%
Control	623	-4.2%	-0.6%	-7.3%	3.2%	9.6%	2.9%	-7.7%	-7.3%
	785	0.7%	-4.4%	-4.7%	-4.2%	-9.8%	1.0%	1.2%	-1.5%

**Table 6:** Leukopenia, thrombocytopenia, viraemia and temperature of vaccinated and control animal groups.

Group	Animal #	Leukopenia (%) <sup>a</sup>	Blood platelet <sup>b</sup> (%)	Virus isolation <sup>c</sup>	Highest <sup>d</sup> temperature	Total days temperature >39.7°C <sup>e</sup>
Challenge	681	51.6	50	7/5	40.9	4
	983	53.9	62.3	6/3	42.4	1
	961	48.9	67	6/5	39.9	1
Control	683	10.2	4.5	0/0	39.5	0
	668	18.3	22.3	0/0	39.0	0

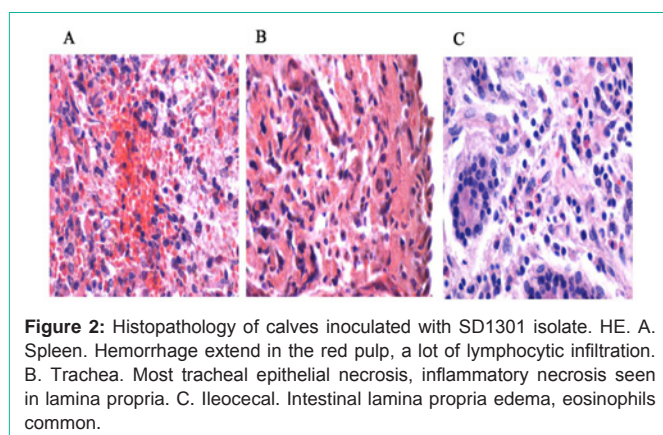
<sup>a</sup>Maximum percent reduction in cell count (3-8 dpi), compared to an average of two pre-inoculation values.

<sup>b</sup>Maximum percent reduction in blood platelet count (3-8 dpi), compared to an average of two pre-inoculation values.

<sup>c</sup>Number of days with positive virus isolation from blood/nasal swab 1 to 8 dpi.

<sup>d</sup>The highest rectal temperature during 1 to 14 dpi.

<sup>e</sup>Total days of >39.7°C during 1 to 14 dpi.



[13]. However, not all BVDV-2 are highly virulent [14]. Thus, the wide range of clinical presentations following acute BVDV infections depends on the viral strain and immune status of the animal [15,16]. The characteristics of infection with high and low virulence BVDV2 strains have been confirmed already [17-22]. Cattle infected with a low virulent virus showed mild clinical signs, accompanied by with a light fever (rectal temperatures between 39.2 and 40.0°C) less than three days and a decrease of white blood cells less than 40%. Comparatively, high virulent virus-infected cattle showed a clinical presentation with a high fever than 40.0°C, even to 41.7°C for 3 or more days, the WBC and platelets were both dropped greater than 40%. In some field examples mortality rates ranged from 20% to 50% in related with a highly virulent BVDV2 strains [23,24]. On the basis of fever exceeding 40.8°C, WBC drops reaching 50% and platelet drops exceeding 40%, the virus would be categorized as high virulence, but the calves did not develop severe clinical signs. So we grouped the strain as a moderate virulent virus according to the clinical signs severity, which was similar with the previous report, which showed that not all BVDV2 isolates cause clinically severe

disease. The highest observed temperatures were not always positively associated with the greatest drops in lymphocytes and platelets. Virus was isolated from WBC of all inoculated animals between days 3 and 10 post-inoculation. This indicates that all animals developed viremia during the course of infection. In generally, a low virulence strain was used as a vaccine strain, while a high virulence BVDV2 strains is used as challenge strains to assess the efficacy of protection induced by vaccination. Even though the data was different for each cattle, all calves clearly developed viremia and clinically symptoms following inoculation. The strain can be used as a challenge strain to evaluate the efficacy of protection of a vaccine.

With the rapid development of livestock farm, the number of pigs reached 451 million, cattle for 100 million [25]. Emergence of the new ncp BVDV 2b was more complex and need more highlight the infection of BVDV. This new found give us a support to pay more attention to the BVDV in China. Despite the mild clinical signs of the disease, BVDV can affect the immune system to increase the risk of second infection. We who should take appropriate measures to control the disease and take surveillance program in a larger livestock population in vast geographical areas.

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