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Experimental inoculation of calves with laboratory strains of bovine viral diarrhoea virus

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Abstract

Diarrhoea, erosions and ulcers of the oral mucosa, with conjunctival and nasal discharges, were observed in six calves inoculated with a mixture of two laboratory cytopathic reference strains of bovine viral diarrhoea virus (BVDV)—Oregon C24 V and NADL. The clinical picture was accompanied by biphasic body temperature elevation, transient leukopenia and a decrease in the number of lymphocytes. High dose of viruses and multiple routes of inoculation promoted the development of clinical and hematological changes typical for BVDV infection although laboratory strains were used. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Bovine viral diarrhoea virus; Experimental inoculation; Pathogenesis; Laboratory strains; Clinical course; Leukopenia

Résumé

Chez six veaux inoculés à l'aide d'un mélange de deux souches cytopathiques de laboratoires du virus de la diarrhée bovine (BVDV), on a observé une diarrhée des érosions et des ulcères des muqueuses orales ainsi qu'un écoulement nasal et conjonctival. Une fièvre, une leucopénie et une lymphopénie ont accompagné l'image clinique. La forte dose de virus administrés par différentes voies a permis d'obtenir l'expression clinique et des modifications hématologiques typiques à l'inoculation par virus BVDV, bien que l'on ait utilisé des souches de laboratoires. © 2000 Elsevier Science Ltd. All rights reserved.

Mots-clé: Diarrhée bovine virale; Inoculation expérimentale; Pathogénèse; Souches de laboratoires; Course clinique; Leucopénie

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1. Introduction

Worldwide distribution of bovine viral diarrhoea virus (BVDV) in cattle populations and high economic losses make this virus an important pathogen in modern breeding farms [1–5]. BVDV, along with classical swine fever virus (CSFV) and border disease virus (BDV), belongs to the Pestivirus genus within the Flaviviridae family [6]. Based on the effect in cell culture two biotypes of the virus, cytopathic (cp) and noncytopathic (ncp), can be distinguished [7–9]. In nature ncp strains prevail. From a clinical point of view 70–90% of infections go unnoticed [10]. Subclinical disease is characterized only by a short febrile period and transient leukopenia followed by seroconversion within 3–4 weeks after infection. Problems arise when infection strikes pregnant seronegative cattle within the first 4 months of gestation. The ncp strains of BVDV are able to cross the placenta and infect the fetus. The developing immune system of the fetus acquires tolerance to the virus (infection of fetuses between 58 and 125 days old) and BVDV-immunotolerance develops along with persistent infection [11]. Such a persistently infected animal (PI) becomes the main source of the virus in a herd. Half of the economic losses are due to the presence of PI animals [1].

In recent years the new clinical condition caused by the highly pathogenic ncp strains of BVDV (classified as type II strains or genotype II strains) has been described [12–19]. They were responsible for severe outbreaks of acute bovine viral diarrhoea (BVD) and hemorrhagic syndrome in calves accompanied by a high mortality rate. This form of BVDV infection has not been described in Poland so far. Therefore, we can presume that type I BVDV strains dominate in the field in Poland at the moment. Rare clinical reports of BVD and mucosal disease emphasize problems encountered by veterinarians when diagnosing such syndromes in cattle. On the other hand, the high prevalence of antibodies for BVDV in cattle in Poland, with vaccines being used occasionally, shows that BVDV infection is common in the cattle population in this country [20].

The goal of the study was to induce the clinical disease following BVDV infection of normal healthy farm calves. Calves were chosen from the farm with no prior history of any bacterial or viral infection or disease.

2. Materials and methods

Reference, laboratory strains of BVDV, NADL and Oregon C24 V were titrated in a Madin Darby Bovine Kidney (MDBK) cell line free from pestiviral contamination and grown in Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum (FBS). Calculation of titer was performed according to the Reed and Muench method [21]. The Tissue Culture Infectious Dose 50% (TCID₅₀) of NADL and Oregon C24 V viral mixture used for inoculation of calves was $10^{8.324}$.

Six black–white lowland calves 8–10 weeks old and weighing 50–80 kg were used. Clinical examinations and blood tests (body temperature, appetite, mucosal surfaces available for examination, total white blood cell counts, lymphocyte percentage, BVDV serology and virus isolation) were performed for five consecutive days before the inoculation.

Animals were inoculated with a mixture of NADL and Oregon C24 V strains according to the following scheme:

2 × 2 ml intraconjunctivally; 2 × 5 ml intranasally; 5 ml intratracheally; 5 ml intravenously and 10 ml orally.

Clinical observation was initiated 5 days before the inoculation and finished on the 14th day after the inoculation. Rectal temperature, pulse and breathing rates, appetite and accessible mucosal surfaces were inspected twice a day.

Nasal and conjunctival swabs for virus isolation were collected for 14 days starting on the day of inoculation. Blood for hematology was collected from the 3rd to the 14th day after inoculation. Blood for serology was taken on the 5th day before and on the 14th day after inoculation. Micro serum neutralization (MSN) test was performed in MDBK cell line grown in MEM supplemented with 5% FBS. Dilutions of serum (50 µl) were mixed with 50 µl of 100 TCID₅₀ of NADL reference strain. Each serum was tested in triplicate. The titer was read after a 5-day incubation period and expressed as the reciprocal of the highest dilution inhibiting the cytopathic effect in 50% of the wells. The dilution range was from 1:2 to 1:512.

An MDBK cell line free from pestiviral infection and grown in MEM supplemented with 2% FBS was used for virus isolation. Prior to inoculation all calves were tested for persistent infection with BVDV. Three passages of the buffy coats and serum from all calves followed by immunofluorescence (IF) tests for the third passage were performed. For the post inoculation period an MDBK monolayer was inoculated with 0.1 ml of MEM in which nasal and conjunctival swabs were placed. After 1 h of adsorption at 37°C, 4% CO₂, the medium was replaced, followed by two days of incubation in the same conditions. Additionally two blind subpassages were done. Cells from the third subpassage were trypsinized, placed on teflon covered glass slides and allowed to adhere for 3 h followed by fixation in cold acetone for 20 min. VDV Gamakon conjugate (Bioveta-Nitra, Czech Republic) diluted 1:8 was used for 1 h at 37°C, 4% CO₂. Slides were mounted in buffered glycerin pH 8.0 and cytoplasm fluorescence was observed by microscope under UV light. White blood cells suspended in Turk medium were counted in a Burkner chamber. Statistical analysis of body temperature and hematological data was performed using the Student's *t*-test. The mean value estimated for all calves for the period of 5 days before the inoculation was compared with the value of the parameter estimated in each calf on every day after infection. The level of probability has been read at 0.05 or less. Statistical data were expressed as mean values (\bar{x}) with standard deviations (s), *t*-test values (t) and the values significantly different were marked—*P* (the level of probability).

3. Results

The first clinical sign was observed in calf No. 4 on the second day post inoculation (p.i.). This was a deep and dry cough. All calves were depressed and the conjunctival discharge was noticed on the 3rd day p.i. In calf No. 5 the reddening of nasal mucosal surfaces and conjunctivas with an occasional cough was observed. These symptoms were accompanied by a sharp decrease in the white blood cell counts. On the 6th day p.i. serous discharge from the nostrils in calves No. 2 and 3, with lacrimation in the latter, was observed. At the same time in calf No. 4 mucopurulent nasal discharge with spontaneous coughing was noticed. Diarrhea in calves No. 1 and 2 started on the 7th day p.i. It continued until day 14. Other signs in calf No. 1 included: coughing, conjunctival discharge, necrotic focus on cornea, reddening of mucosal surfaces of nostrils, hard palate, cheeks and conjunctivas. In calf No. 2 watery diarrhea started on the 2nd day p.i. and continued through the whole observation period. Two necrotic foci were observed on the hard palate. Bilateral ocular mucopurulent discharge and bilateral purulent discharge from nostrils with reddening of gingival mucosa were noticed. In calf No. 3 diarrhea, coughing and reddening of the mucosal surfaces of conjunctivas, nostrils, cheeks and hard palate were observed.

In calf No. 4 necrotic foci on the mucous membrane of both lips with erosions on the hard palate were observed. The animal lost weight despite the availability of feed.

Body temperature within 5 days before inoculation ranged from 38.5 to 39.5°C. On the 1st day p.i. a significant drop in body temperature was observed ($P < 0.05$). Within 2–8 days p.i. body temperature went up to 40.0–40.1°C in all calves. Biphasic increase of body temperature was observed on the 3rd day and the 6th

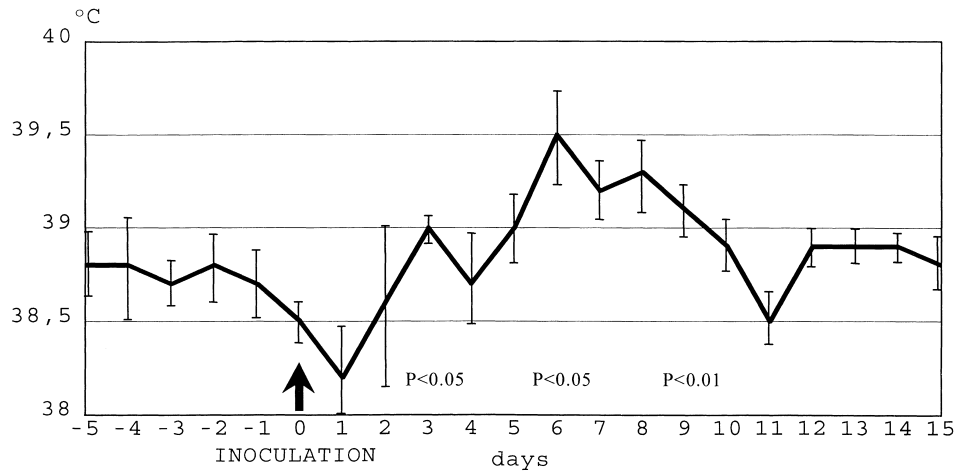


Fig. 1. Body temperature—mean values for all six calves.

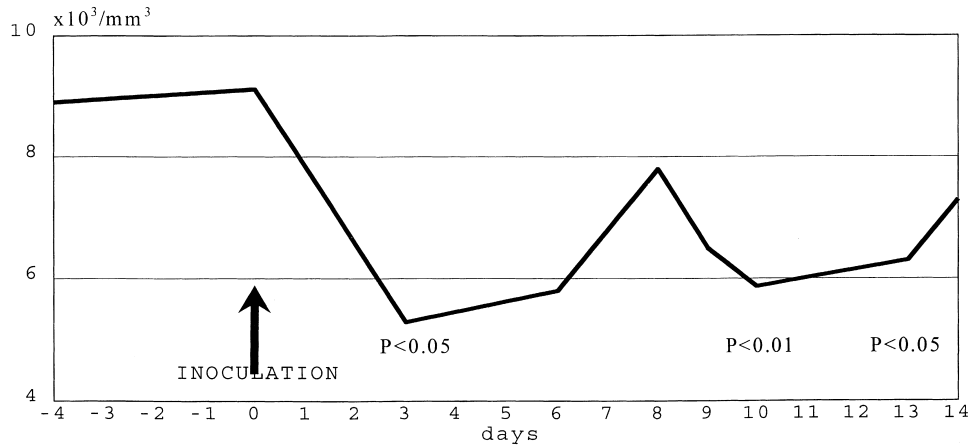


Fig. 2. White blood cell counts—total leukocyte count—mean values for all calves.

day p.i. (Fig. 1). It was statistically significant with $P < 0.05$ and $P < 0.05-0.01$ respectively (the later value refers to the time interval between day 6 and day 9).

The leukocyte counts ranged from 5500 to 15,100/mm³ before inoculation. Within 3 days p.i. the number of leukocytes dropped rapidly to 3100–6600/mm³ ($P < 0.05$) in calves No. 1, 2, 5 and 6 (Fig. 2; Table 1) and within 5–9 days p.i. it reached the value found in the period before inoculation. Contrary to that, in calf No. 4 the leukocyte count went up from 7500/mm³ on the day of inoculation to 15,100/mm³ on the 6th day p.i. A decrease in leukocyte count from 15,100/mm³ (day “0”) to 6100/mm³ (day 10 p.i.) was observed in calf No. 3 (Table 1).

Table 1
Total leukocyte count for all six inoculated calves

Calf	Days before and after inoculation ^a								
	-4	0	3	6	8	9	10	13	14
1	7.2	7.3	3.3	5.5	7.0	5.3	4.4	5.8	3.9
2	11.5	10.1	6.6	7.0	13.0	8.2	7.6	7.4	10.5
3	8.2	15.1	11.5	10.1	8.4	9.5	6.1	7.8	9.2
4	7.7	7.5	8.2	15.1	7.1	7.1	5.9	7.2	8.8
5	11.6	9.2	3.4	3.1	8.5	4.0	6.8	6.4	7.6
6	7.2	5.5	3.1	2.4	2.8	4.95	4.4	3.2	4.0
Statistics ^b									
\bar{x}	8.9	9.12	5.3	5.8	7.8	6.51	5.87	6.3	7.3
s	2.086	3.34	2.8	2.7	3.28	2.11	1.28	1.68	2.78
t	–	-0.1558	2.6191	2.1328	0.7374	1.9517	6.5811	3.0599	1.6955
P	–	n.s.	0.05	n.s.	n.s.	n.s.	0.01	0.05	n.s.

^a 0—the day of inoculation.

^b \bar{x} —mean values; s —standard deviation; t — t -test values; P —probability.

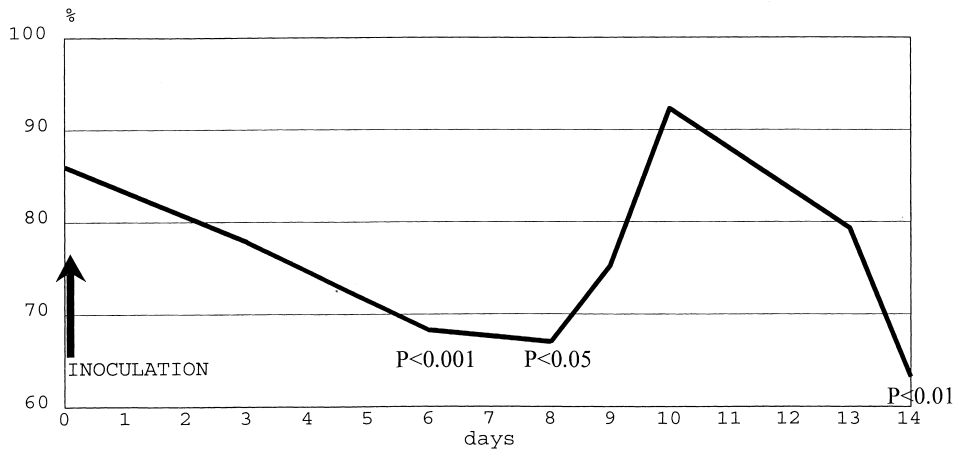


Fig. 3. Percentage of lymphocytes—mean values for all calves.

Generally in four out of the six calves inoculated a sharp decrease in the number of leukocytes was observed within 3 days p.i. Leukopenia has continued for 3 days starting from the 3rd day p.i.

The percentage of lymphocytes dropped in all calves within 6–8 days p.i. ($P < 0.001$, $P < 0.05$) (Fig. 3). The greatest decline was observed in calf No. 2 from 73% (day “0”) to 35% (day 8). In all calves a significant drop in the percentage of lymphocytes in comparison to the values estimated in the period before inoculation was observed on day 14 p.i. ($P < 0.01$) (Fig. 3, Table 2).

Table 2
Percentage of lymphocytes for all six inoculated calves

Calf	Day of inoculation and afterwards ^a							
	0	3	6	8	9	10	13	14
1	89	75	68	68	80	85	85	73
2	73	90	53	35	89	91	75	57
3	87	82	73	88	66	95	85	62
4	89	78	62	70	78	93	61	49
5	84	53	71	55	65	95	75	70
6	94	89	83	86	74	95	95	68
Statistics ^b								
<i>x</i>	86.0	77.8	68.3	67.0	75.3	92.3	79.3	63.2
<i>s</i>	7.15	13.5	10.2	19.9	9.1	3.9	11.7	9.0
<i>t</i>	–	1.2823	7.1465	3.3142	1.8688	–1.9983	2.1721	5.7034
<i>p</i>	–	n.s.	0.001	0.05	n.s.	n.s.	n.s.	0.01

^a 0—the day of inoculation.

^b *x*—mean values; *s*—standard deviation; *t*—*t*-test values; *P*—probability.

Table 3
BVDV shedding in nasal and conjunctival discharges^a

Swabs	Calf	Days after inoculation													
		0	1	2	3	4	5	6	7	8	9	10	11	12	13
Nasal	1		■		■	■	■	■	■	■					
	2		■		■	■	■	■	■				■		
	3		■												
	4		■	■	■	■									
	5		■	■	■	■	■	■	■						
	6		■	■	■	■	■	■	■						
Conjunctival	1		■			■	■	■							
	2		■	■		■	■	■							
	3		■	■	■										
	4		■	■	■	■	■	■							
	5		■	■	■	■	■								
	6		■	■	■	■	■								

^a Shaded areas show viral shedding: grey color — nasal discharge; black color — conjunctival discharge.

All calves were seronegative in respect to BVDV before the inoculation, while on day 14 all the animals except calf No.4 had serum neutralizing antibodies at a titer of 2. Virological testing of the whole blood prior to inoculation was negative in all calves. For the post inoculation period BVDV antigen was detected in 25 conjunctival swabs (69.4%) and 30 nasal swabs (71.4%). Nasal shedding of the virus continued until the 7th day p.i. (also on day 11 in the case of calf No. 2). Conjunctival shedding was 1 day shorter when compared with nasal shedding, and continued until day 6 p.i. (Table 3).

4. Discussion

The object of the experimental inoculation of calves with BVDV was to observe the clinical course of the infection. In nature most of the BVDV infections are subclinical with the ncp strains being the main cause of infection. Besides transient leukopenia there may be no other symptoms of the infection. Castrucci et al. did not observe any clinical signs after inoculation of calves with the ncp strain of the virus [22]. Only pharyngeal swabs and leukocytes containing viral antigen confirmed successful infection. On the other hand inoculation with the cp strain of BVDV led to acute viral infection [22]. Nutall et al. observed only mild clinical signs after inoculation of calves with the NADL strain [25]. Therefore we decided to use two well characterized laboratory strains of BVDV: Oregon C24 V and NADL, looking forward to the results which are described in this paper.

Inoculation of calves with a mixture of these strains proved to be successful based on the typical clinical symptoms, the laboratory results of blood testing (white blood cell counts, percentage of lymphocytes), and viral shedding in nasal and conjunctival discharges. Initial clinical signs were noticed on the 2nd day p.i. and were present throughout the experiment until the 14th day of observation. Diarrhea appeared in all inoculated calves and changes on the mucosal surfaces (reddening, erosions, and necrosis) were observed. Castrucci et al., using the cp strain of BVDV, also observed initial clinical symptoms on the 2nd day p.i. [22]. The symptoms lasted until day 14 p.i. Fever, nasal discharge, diarrhea and leukopenia accompanied the clinical lesions.

In this study, fever in calves appeared on the 3rd day p.i. Then the body temperature dropped and rose again with maximal values on the 6th day p.i. From the 8th day p.i. the body temperature started to decrease. Statistically significant increases in body temperature were observed on the 3rd, 6th, 7th, 8th and 9th day p.i. Also, Traven et al. observed biphasic fever with maximum values on the 8th and 9th days p.i. in calves infected with BVDV by natural contact with a persistently infected animal [23]. In this study fever reached its maximum on the 6th day p.i.

Leukopenia typical for BVDV infection appeared in four calves within 3–6 days p.i. In two other calves at the same time an increase of the number of leukocytes with a drop to the values observed before inoculation was noticed. Statistically significant differences in the number of leukocytes when compared with pre-inoculation values were observed on the 3rd, 10th and 13th day p.i. The decrease of the leukocyte count in all calves ranged from 21 to 66% in the p.i. period when compared to the value before inoculation. Phillip observed a 25% and higher decrease of leukocyte counts in 12 out of 15 calves inoculated with the cp strain of BVDV [24]. Transient leukopenia lasting for a few days along with seroconversion were the typical features for the BVDV infection. Nutall et al. did not detect leukopenia in calves inoculated with the NADL strain, while clinical signs were mild [25]. Traven et al. also observed a biphasic drop in the leukocyte count after infection with the ncp strain of BVDV [23].

In all calves inoculated with BVDV we observed a drop in the percentage of lymphocytes. Statistically significant decreases were observed on day 6, 8 and 14 p.i. ($P < 0.001$, $P < 0.05$ and $P < 0.01$ respectively). Bolin et al. and Ellis et al. showed that BVDV caused a decrease in the absolute numbers of B and T lymphocytes and in the percentage of T lymphocytes along with a decrease in the number of neutrophils [26,27]. The typical course of the clinical picture, e.g. biphasic body temperature rise with characteristic changes in the white blood cell counts, which we observed in the experimental calves, has restrained us from any bacteriological testing. Clinical examination (body temperature, appetite, pulse and breath rate, mucosal surfaces available for examination, white blood cell counts, serological testing and virus isolation) performed during the 5 days before the inoculation did not reveal any clinical disorders. Therefore, we considered that there was no reasonable cause for additional laboratory tests to be done on these animals (i.e. bacteriological testing).

All calves were seronegative against BVDV before inoculation. Also, virus isolation tests were negative in all animals before experimental inoculation. At the end of the experiment (14 days p.i.) seroconversion was observed in five calves at the titer of 2 in the virus neutralization test. Other authors have found that the seroconversion appears within 10–21 days after BVDV infection [23,25,28,29].

Brownlie et al. found that the acute phase of shedding of BVDV starts on the 4th day and continues until the 10th day post infection although it can last even up to 2 weeks [30]. In this study nasal shedding of BVDV continued until the 7th day p.i. Only in calf No. 2 BVDV was also present in nasal swabs on the 11th day p.i. It is possible that we have isolated the strain or strains inoculated into the calves. Our understanding of the shedding of the virus and the time span of the shedding is that the clearance of the virus we put into the mucosal surfaces was no longer than 1–2 days p.i. This is probably evidenced by the biphasic pattern of virus shedding we observed in calves No. 1, 2 (nasal cavity) and in calves No. 1, 2, 5 (conjunctival sacks). The second turn of virus shedding has probably occurred as a result of virus replication on the spot. Lacoste et al. detected BVDV antigen in nasal swabs between the 4th and the 7th day p.i. [31]. In Traven's study calves were infected in a natural way by direct contact with a persistently infected calf [23]. They observed diarrhea, reddening of oral and nasal membranes, biphasic fever, transient leucopenia and lymphopenia. In our experiment the results were similar to those observed by Traven et al. although cp laboratory strains of BVDV were used.

The original outcome of the experiment is that the two laboratory strains Oregon C24 V and NADL applied in the same animal at the same time at high doses have caused a clinical picture similar to that observed in the course of BVDV infection.

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