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*Full Length Research Paper*

# Evaluation of immunogenic properties of monovalent and polyvalent inactivated bovine virus diarrhea virus (BVDV) vaccines

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**This study is aimed at evaluating the immunogenicity of two inactivated (mono- and polyvalent) vaccines containing bovine virus diarrhea virus (BVDV) reference and field strains. Three experimental groups were formed: 10 calves vaccinated twice (days 1 and 28) subcutaneously (s/c) with 2 ml of inactivated polyvalent vaccine per animal (Group 1); 10 calves vaccinated twice (days 1 and 28) subcutaneously (s/c) with 2 ml of inactivated monovalent vaccine per animal (Group 2) and 9 unvaccinated calves (Control, Group C). Blood sera were obtained from immunized animals (standard procedure: on days 0, 14, 28, 42 and 56 post-immunization). Geometric mean titer (GMT) values for BVDV neutralizing antibodies were substantially higher in blood sera of calves receiving the inactivated monovalent vaccine. The immune response developed more rapidly in calves immunized with the monovalent vaccine.**

**Key words:** Bovine virus diarrhea virus, immunogenic properties, inactivated vaccines.

## INTRODUCTION

Bovine virus diarrhea virus (BVDV) is a member of the genus *Pestivirus* within the family Flaviviridae. BVDV causes infections of domestic and wild ruminants worldwide and significant economic losses to the cattle industry (Baker, 1995; Brownlie, 1991). BVDV is classified into one of two biotypes, cytopathic (CP) or non-cytopathic (NCP). Based on antigenic and genetic properties, two genotypes of the causative virus have been identified, BVDV-1 and BVDV-2: BVDV-2 makes up about 50% of the isolates in North America, whereas BVDV-1 predominates throughout Europe, with more than 90% (Ridpath, 2005; Vilcek et al., 2005). BVDV-1 and BVDV-2 genotypes have been further subdivided into subgenotypes BVDV-1a, BVDV-1b, BVDV-2a and BVDV 2b in North America (Flores et al., 2002; Ridpath et al., 2000). Another classification describes 11 genetic groups of BVDV (Vilcek 2001). The major economic losses due to BVDV infection include reduced fertility, abortions and the generation of persistent viremic calves, which can develop fatal “mucosal disease” (Bielefeldt, 1995; Brownlie, 1990). Persistently infected (PI) live long-term virus carriers play the key role in BVDV epidemiology. Control of BVDV infection/diseases includes both: 1) biosecurity to prevent entry of PI animals into the herd by testing cattle for BVDV, and 2) vaccines as part of routine immunization programs. These viral vaccines may be modified live virus (MLV), inactivated or chemically altered live virus vaccines (Hjerpe, 1990). The disadvantages of modified live BVDV-containing vaccines include the following: utmost care needed when using and handling this vaccine in order to prevent inactivation, possible contamination with other viruses (Moennig et al.,

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2005), probability of development of postvaccinal mucosal disease (Lambert, 1973), not recommended for pregnant animals (transplacental infections) due to insufficient reliability (Kelling, 1996), possible vaccine strain shedding and introduction of new BVDV strains into herds, and the development of immunosuppression as induced by the vaccinal strain of the virus (Roth and Kaeberle, 1983).

The disadvantages associated with inactivated BVDV vaccines include: higher price, necessity of revaccination (Brock and Chase, 2000), a longer period required to develop immunity (Bolin, 1995), possible anaphylactic and local reactions at the vaccination site, shorter duration of immunity, and the immune response being directed only against certain antigen variants of the virus (Kelling, 1996; Bolin et al., 1991). The purpose of this study is to determine the ability of the experimental inactivated vaccines containing BVDV imunogens to induce neutralizing antibodies to type 1 BVDV strains in vaccinated calves. Two different experimental vaccines (monovalent and polyvalent) were evaluated using detection of virus neutralizing antibodies, as a measure of their immunogenicity in calves.

## MATERIALS AND METHODS

### Animals

Normal healthy beef calves (Simmental race) of mixed sex were used. The calves were 6 to 7 months of age. The vaccinated calves were subjected to cohabitation with the unvaccinated animals (control group) in order to determine the possible transmission of vaccine virus to unvaccinated animals. During the experiment, the calves were kept in a closed building, in free stalls, on slatted floor, under conditions common in intensive calf rearing.

### Vaccine preparation

Monovalent inactivated vaccine - lot 251200. Reference CP strain NADL, infective titer log 10-6 TCID/0.1 ml (CVL Weybridge, provided by Dr. Georgina Ibata) was grown at the MDBK (Madin Darby bovine kidney) cell line. The suspension of the harvested NADL strain of BVDV was heated at 37°C and inactivated by adding ß-propiolactone (diluted in cold distilled water 1:1.000). The inactivation process was followed by rolling (IKA WERKE 200 rpm/min-1) for 3 h at 37°C. The total inactivation of the BVD harvested virus was controlled in accordance with European Pharmacopoeia 5.0., monograph: Bovine diarrhoea vaccine (inactivated) No 01/2005:1952 (Tests, Inactivation, p. 734). The harvested inactivated virus was used in the volume sufficient for 10 doses of vaccine and inoculated in two passages, each of which was observed for 10 days. No live virus was detected in the cell line during the test. The suspension of inactivated virus was stored at - 20°C (the volume of the inactivated virus sample was 65.25 ml). The pH of the suspension following inactivation was 4.57, and was adjusted to 7.4 using 1M NaOH (0.6 ml). The suspension was assayed for sterility using thioglycolate broth, nutrient agar, nutrient broth and Sabouraud broth incubated at 37 and 25°C for 14 days. The assay confirmed sterility of the above suspension. Merthiolate preservative (1:10.000 to 0.7 ml 1% v/v) was added to the inactivated virus sample. At a pH of 7.56, 21.7 ml of sterile 25% (AlOH)3 and 0.29 ml of sterile 20% aqueous solution of saponin (MERCK) were added at a ratio of 1:1.500 to suspension of the inactivated BVDV vaccine strain. The volume of the prepared monovalent vaccine against BVDV infection in cattle was 87.94 ml. The pH of the vaccine - lot 251200 was 8.07 (Cheerer HANA). Sterility assay of the prepared vaccine was conducted in the same manner as that of the suspension.

The assays confirmed sterility of the vaccine sample. Polyvalent inactivated vaccine - lot 270700. Five cp BVDV strains were used for the preparation of the polyvalent inactivated vaccine:

1) W1. - 162903, virus titer log $10^{-6}$ TCID/0.1 ml, 27 ml.
2) W2. - 172984, virus titer log $10^{-5}$ TCID/0.1 ml, 30 ml.
3) W3. – 173481, virus titer log $10^{-5}$ TCID/0.1 ml, 30 ml.
4) W4. - 179725, virus titer log $10^{-5}$ TCID/0.1 ml, 30 ml.
The strains are all BVDV type 1 isolated in the United Kingdom in the 1980s. Monoclonal antibody typing procedure reveals that they are quite distinct.
5) Local BVDV field isolate, derived from a calf suffering from mucosal disease (MD) and identified by standard VN test, with virus titer log $10^{-5}$ TCID/0.1 ml, 30 ml.

All five CP BVDV strains were cultivated at the MDBK cell line. The virus suspension was inactivated by adding ß-propiolactone (final concentration 1:1.000), which was followed by rolling (IKA WERKE 200 rpm/$min^{-1}$) for 3 h at 37°C. The pH of the suspension following inactivation was adjusted to 7.4 using 1M NaOH. Prior to virus inoculation, the MDBK cell line was checked for the presence of the BVD virus. The test was performed following the procedure European Pharmacopoeia 5.0., procedure named: Cell cultures for the production of veterinary vaccines No 01/2005:50204 chapter 5.2.4.; p. 458/459 (detection of specified viruses), using fluorescein conjugated antibodies (BVD) with the presence of a positive control cell line. The MDBK cell line was negative for the BVD virus. The suspension was assayed for sterility using thioglycolate broth, nutrient agar, nutrient broth and Sabouraud broth incubated at 37 and 25°C for 14 days. The assay confirmed sterility of the aforementioned suspension. Upon inactivation of the viral suspension, 1% merthiolate (final concentration 1:10.000) was added to the suspension. Inactivated antigens were adsorbed on sterile $Al(OH)_3$ by adding 25% sterile adjuvant twice, which was followed by continuous rolling on a magnetic mixer. During the process, the vaccine suspension samples were made of 135 ml CP BVDV strains and 33.8 ml $Al(OH)_3$ to obtain a volume of 173 ml. A total of 0.6 ml of the second adjuvant, saponin (MERCK, 20%) was added to the sample, making the final concentration in the vaccine of 1:1.500. The pH of the prepared vaccine - lot 270700 was 7.39. The sterility assay of the prepared vaccine was conducted in the same manner as that of the suspension. The assay confirmed sterility of the vaccine sample.

### Vaccine trials

All 29 calves in vaccine trials were seronegative before the initial vaccination on day 0, as detected by the virus neutralization test. The calves were divided into three experimental groups. Group 1 consisted of 10 male calves vaccinated twice (on days 0 and 28) subcutaneously (s/c) with 2 ml of the inactivated polyvalent vaccine per animal. 10 calves (7 males and 3 females) constituting Group 2 were inoculated twice (on days 0 and 28) subcutaneously (s/c) with 2 ml of the inactivated monovalent vaccine per animal. The control group (C) contained 9 unvaccinated female calves.

### Serum collection

Blood sera were collected, frozen and stored at - 20°C until assayed for antibodies. The collection of the blood sera from the

**Table 1.** Serum antibody titers to BVDV in the calves receiving the polyvalent vaccine (Group 1).

| Calf serial number | VNT Ab titer on day 0/GMT | VNT Ab titer on day 14/GMT | VNT Ab titer on day 28/GMT | VNT Ab titer on day 42/GMT | VNT Ab titer on day 56/GMT |
|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 1:16 4 | 1:32 5 |
| 2 | 0 | 0 | 0 | 0 0 | 1:2 1 |
| 3 | 0 | 0 | 0 | 1:16 4 | 1:4 2 |
| 4 | 0 | 0 | 1:4 2 | 1:64 6 | 1:128 7 |
| 5 | 0 | 0 | 0 | 1:32 5 | 1:32 5 |
| 6 | 0 | 0 | 0 | 1:8 3 | 1:16 4 |
| 7 | 0 | 0 | 0 | 1:16 4 | 1:16 4 |
| 8 | 0 | 0 | 0 | 1:8 3 | 1:8 3 |
| 9 | 0 | 0 | 0 | 1:32 5 | 1:32 5 |
| 10 | 0 | 0 | 0 | 0 0 | 1:16 4 |
| $GMTlog_2$ | 0 | 0 | 0.20 | 3.40 | 4.00 |

calves of both Groups 1 and 2 was performed using standard procedure at bi-weekly intervals (on days 0, 14, 28, 42 and 56 in the vaccine trial). Blood samples for serological testing were taken from the control calves on days 0 and 56 of the experiment.

#### Virus neutralization test

The collected blood sera were tested by virus neutralization test (VNT) in cell cultures using Nunclon plates. The virus used was CP strain NADL, virus titer log $10^{-6}$ TCID/0.1 ml (CVL, Weybridge). Cell line: MDBK, Brescia. The samples of blood sera were inactivated for 30 min at 56°C. Preliminary testing was performed using twofold dilutions of the blood sera of 1:2 through 1:1024. 25 µl of the blood sera was incubated with 25 µl 100 $TCID_{50}$ BVDV CP strain NADL for 1 h at 37°C. Following incubation, 50 µl of MDBK cell suspension was added at a concentration of $3 \times 10^5$ cells/ml. The inoculated cell line was incubated for 4 days at 37°C, and neutralization of CP strain infectivity was detected by observation of viral cytopathology. On day 4, all blood sera that exhibited neutralizing viral activity in the 1:8 solution were titrated to 1:1024. The endpoint dilution of the blood sera that completely neutralized the BVD virus and prevented the development of the cytopathogenic effect (CPE) was the specific virus-neutralizing antibody titer of the blood sera samples. The results obtained herein were calculated by statistical methods as logarithms of the reciprocal of the serum dilution ($log_{10}$) for VNT expressed as geometric mean titers ($GMTlog_2$) as described earlier by Brugh (1978).

## RESULTS

Prior to vaccination (on the first day of immunization), all animals in each of the three experimental groups were seronegative for BVDV. At the end of the immunization trial (on day 56), all calves of the control group were still seronegative. The samples of blood sera of the seronegative calves receiving the polyvalent inactivated vaccine (Group 1) were tested on the presence and titer of the antibodies to BVDV by a VNT and summarized in Table 1. This study presents individual animals and their respective antibody titers to BVDV NADL strain on blood sera collection on days 0, 14, 28, 42 and 56. In all animals, no neutralization occurred on days 0 and 14 ($GMTlog_2 = 0$). At revaccination (conducted 28 days after vaccination), one animal (1/10) from Group 1 tested seropositive, with BVDV-specific antibody titers of 1:4. Eight of the ten (8/10) calves vaccinated with the polyvalent vaccine on day 0 and revaccinated with same vaccine on day 28 were positive for BVDV specific antibodies on day 42 of blood sera collection. The antibody titers induced by the polyvalent vaccines ranged from 0 to 1:64. At the end of the immunization trial (on day 56), all calves in Group 1 were seropositive. The specific antibody titers against BVDV in the blood sera were within the 1:2 to 1:128 range. From days 28 to 56 of the immunization trial, $GMTlog_2$ values increased continuously (0.2, 3.40 and 4.00). The antibody responses to CP NADL strain BVDV found in the blood sera of the seronegative calves receiving the monovalent vaccine (Group 2) were assessed by the VNT and summarized in Table 2. In all test animals, no neutralization occurred on days 0 and 14 ($GMTlog_2 = 0$). At revaccination, five animals (5/10) from Group 2 tested seropositive, with BVDV-specific antibody titers ranging from 0 to 1:16. All of the ten calves vaccinated with the monovalent vaccine on day 0 and revaccinated with the same vaccine on day 28 showed a BVDV

**Table 2.** Serum antibody titers to BVDV in the calves receiving the monovalent vaccine (Group 2).

| Calf serial number | VNT Ab titer on day 0/GMT | VNT Ab titer on day 14/GMT | VNT Ab titer on day 28/GMT | | VNT Ab titer on day 42/GMT | | VNT Ab titer on day 56/GMT | |
|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 1:32 | 5 | 1:32 | 5 |
| 2 | 0 | 0 | 1:2 | 1 | 1:8 | 3 | 1:4 | 2 |
| 3 | 0 | 0 | 0 | 0 | 1:512 | 9 | 1:128 | 7 |
| 4 | 0 | 0 | 0 | 0 | 1:256 | 8 | 1:64 | 6 |
| 5 | 0 | 0 | 1:4 | 2 | 1:256 | 8 | 1:64 | 6 |
| 6 | 0 | 0 | 1:16 | 4 | 1:128 | 7 | 1:256 | 8 |
| 7 | 0 | 0 | 1:4 | 2 | 1:128 | 7 | 1:128 | 7 |
| 8 | 0 | 0 | 1:2 | 1 | 1:512 | 9 | 1:128 | 7 |
| 9 | 0 | 0 | 0 | 0 | 1:256 | 8 | 1:256 | 8 |
| 10 | 0 | 0 | 0 | 0 | 1:128 | 7 | 1:64 | 6 |
| $GMTlog_2$ | 0 | 0 | 1.00 | | 7.10 | | 6.20 | |

specific antibody response on collection day 42. The antibody titers induced by the monovalent vaccine ranged from 1:8 to 1:512. At the end of the immunization trial (on day 56), all calves were seropositive. The specific antibody titers against BVDV in the blood sera obtained from Group 2 animals ranged from 1:4 to 1:256. The $GMTlog_2$ values highly increased between 28 and 42 days – reaching their peak values of 1.00 to 7.10 $log_2$) and slightly declined 56 days after vaccination (6.20 $log_2$).

Statistical evaluation of the results obtained included the initial formation of statistical base which composed of 3 variables - trial day, experimental group and log-transformed values of specific antibody titers to BVDV ($GMTlog_2$) - that was used in an individual LSD test - the least significant difference test, performed by means of a commercial statistical software (SPS statistical software ver. 5.0). The LSD test results are given in tabular form (Table 3). The geometric means titers ($GMTlog_2$) for each treatment group were compared for significant differences between the vaccine groups (1 and 2) at given dates of blood sera collection. The results on the log-transformed values of specific antibody titers to BVDV ($GMTlog_2$) for the Groups 1 and 2 animals as analyzed by the LSD test suggest that no significant difference was found between Groups 1 and 2 calves on day 28 post-vaccination, nor between days 42 and 56 in both Groups 1 and 2 animals. Statistically very significant differences of up to 99% were recorded between all other groups (Table 3).

## DISSCUSION

Regarding the mentioned hazards, research activities have become more focused on the development of effective inactivated vaccines. In our study we opted for inactivated (mono and polyvalent) vaccines due to their greater safety and ability to provide protection to cattle of almost all ages, bred for different purposes. Previously published observations (Howard et al., 1994) suggested that it is not necessary to use live avirulent virus to achieve protection of the respiratory tract and that immunity is not dependent on a local immune response. Considering the large volume of literature available on this subject, the personal experience acquired during our research and the identified research tasks that are associated with testing the immunogenicity of mono and polyvalent vaccines against BVDV in beef cattle, special importance was given to adequate selection of BVDV immunogens and CP vaccinal strains. We opted for MDBK cell line because comparison of the five cell cultures for viral antigen production showed that four of the cell cultures (calf testis cells, nasal mucosa line, embryo lung cells and lung-wash cell line) yielded similar amounts of antigen while MDBK cells gave a titer of antigen that was about 16-fold higher than that of the other four (Howard et al., 1994). β-Propiolacton was used for chemical inactivation which was in line with the O.I.E. recommendations (O.I.E., 2000: Manual of Standards for Diagnostic Tests and Vaccines, Chapter X.5., Bovine viral diarrhea). In order to stimulate a stronger immune response, $Al(OH)_3$ was used as an adjuvant and saponin as the second adjuvant, according to the

**Table 3.** Values of specific antibody titers against BVDV in blood sera of the test animals as obtained by VNT method and analyzed by LSD test.

| Groups | $\bar{x}$ | 42 - II | 56 - II | 56 - I | 42 - I | 28 - II | 28 - I |
|---|---|---|---|---|---|---|---|
| 28 - I | 0.2 | 6.9** | 6.0** | 3.8** | 3.2** | $0.8^{ns}$ | / |
| 28 - II | 1.0 | 6.1** | 5.2** | 3.0** | 2.4** | / | |
| 42 - I | 3.4 | 3.7** | 2.8** | $0.6^{ns}$ | / | | |
| 56 - I | 4.0 | 3.1** | 2.2** | / | | | |
| 56 - II | 6.2 | $0.9^{ns}$ | / | | | | |
| 42 - II | 7.1 | / | | | | | |

28 - I = Trial day 28, experimental Group 1
28 - II = Trial day 28, experimental Group 2
42 - I = Trial day 42, experimental Group 1
56 - I = Trial day 56, experimental Group 1
56 - II = Trial day 56, experimental Group 2
42 - II = Trial day 42, experimental Group 2

$^{ns}$- non significant; **- $p<0.01$ - statistically very significant differences.

modified method of Howard et al. (1994). 2 ml of the mono- and polyvalent vaccines used in this study were subcutaneously (s/c) administered to each test animal; whereas available literature data had previously reported the use of higher doses of similar inactivated vaccines to inoculate animals. The standard dose of inactivated vaccine prepared by Howard et al. (1994) was 4 ml. Describing the effectiveness of the BOVIDEC (C-VET) vaccine, Brownlie et al. (1995) reported that the vaccine dose administered was 4 ml per animal. The dose of inactivated vaccine developed from the “258” BVD CP strain isolated from a case of acute MD in a herd of young cows in Denmark was 3 ml (Meyling et al., 1985). Fulton et al. (1995) compared the geometric mean titer ($GMTlog_2$) values of virus-neutralizing antibodies to BVDV in the animals immunized with four different commercial types of vaccine. The $GMTlog_2$ ratio for the test calves receiving either mono- or polyvalent inactivated vaccine against BVDV infection in this study was 5.00 on days 28, 2.08 on day 42, and 1.55 on day 56 of blood sera collection for serological testing. This result suggests that the $GMTlog_2$ values obtained were higher in animals receiving the monovalent vaccine. It is the opinion of the present authors that the immune response of the vaccinated animals to a single BVDV strain was stimulated more easily than the polyclonal response to five strains of the same virus. However, the effect of possible interactions between the BVDV antigens that may have led to reduced immunogenicity of certain strains used in this study antigens or weaker immunogenicity of the same vaccine should not be eliminated from consideration.

The results of this study comply with those of the following authors: Fulton and Burge (2001) reported that the highest virus-serum neutralizing antibody titer (after a single MLV dose or two doses of inactivated vaccine) was detected on day 42 following primo-vaccination. Brownlie et al. (1995) determined that the humoral immune response to the first dose of inactivated vaccine was weaker, turning into a typical anamnestic response stimulated by the second dose involving a much faster increase in antibody concentrations and higher antibody titers than after the primo-inoculation. Bolin (1993) reported some data as regarding the testing of the protective capacity of inactivated vaccines in accordance with the United States Code of Federal Regulations 546-547, 1990.

The data suggested that the virus-neutralizing antibody titer should be >/= 8 in four out of five test calves, which can be achieved 14 or more days after revaccination. Even with this highly stringent regulation applied, the inactivated vaccines produced in this study conform to established standards. The vaccines used in this study gave satisfactory results with regard to the rate of development of the BVDV specific hummoral immune response in vaccinated animals. Highly significant differences were determined between Groups 1 and 2 in antibody titer level on days 42 and 56 of study. Although a small increase in antibody titer was observed during this period, very significant differences were determined in BVDV antibody specific titers depending on the type of vaccine used (monovalent or polyvalent).

The results obtained suggest the necessity to conduct further investigations aimed at increasing the immunogenicity of the vaccines by using higher-quality antigens and adjuvants and defining optimum doses and immunogen inoculation methods for test animals. The need to induce long-lasting protection of animals against BVDV infections requires both the use of more immunogenic vaccines and conducting trials in artificial infestation of pre-vaccinated animals with virulent BVD strains to provide more long-lasting immunity. This would enable immunoprophylaxis not only for respiratory infections in cattle but also for *in utero* infections as the greatest concern to BVD/MD epidemiology and pathogenesis.

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