Evaluation of experimental vaccines for bovine viral diarrhea in bovines, ovines and guinea pigs

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ABSTRACT

The bovine viral diarrhea virus (BVDV) infection control should be based on elimination of persistently infected animals and on immunization through vaccination, to prevent fetal infection. However, the efficacy of inactivated BVDV vaccines is variable due to its low immunogenicity. This study evaluated the humoral immune response against homologous and heterologous strains of 7 inactivated BVDV vaccines, in bovines and two experimental models (ovine and guinea pig) which might be used to test candidate vaccines. Vaccines formulated with BVDV Singer, Oregon, NADL, and multivalent, induced seroconversion in the three animal models studied, reaching antibody titres higher than 2. Vaccine containing 125C -genotype 2- only induced a low antibody response in ovine, while VS-115 NCP vaccine was not immunogenic. Furthermore, bovine sera at 60 dpv presented homologous as well as heterologous antibody response, indicating a high degree of cross-reactivity among the strains studied. However, when bovine sera were tested against the Argentine field strain 00-693, they showed the lowest levels of cross-reactivity, suggesting the need of continued surveillance to identify and characterize emerging field BVDV strains. Finally, optimal correlations between bovine-ovine and bovine-guinea pig models were observed, indicating that two alternative species could replace bovines when testing the immunogenicity of BVDV candidate vaccines.

Key words: bovine viral diarrhea virus, BVDV, Argentine field strain, inactivated virus vaccine

RESUMEN

Evaluación de vacunas experimentales para la diarrea viral bovina en bovinos, ovinos y cobayos. El control del virus de la diarrea viral bovina (VDVB) se basa en la eliminación de animales persistentemente infectados, y la inmunización de hembras para prevenir infecciones fetales. La eficiencia de estas vacunas es variable por su baja inmunogenicidad. Se evaluó la respuesta inmune humoral contra virus homólogos y heterólogos de 7 vacunas experimentales inactivadas del VDVB en bovinos y en dos modelos experimentales (ovinos y cobayos). Las vacunas conteniendo VDVB Singer, Oregon, NADL y polivalentes indujeron seroconversión en los tres modelos y se alcanzaron títulos de anticuerpos mayores de 2. La vacuna con VDVB genotipo 2 VS-115, NCP, no resultó inmunogénica. La vacuna genotipo 2 125C sólo indujo baja respuesta humoral en ovinos, mientras que la VS-115, NCP, no resultó inmunogénica. En bovinos se determinó la respuesta a virus homólogos y heterólogos a 60 dpv, lo que indica un alto grado de inmunidad cruzada entre la mayoría de las cepas estudiadas. Cuando los sueros bovinos fueron ensayados con la cepa de campo de Argentina 00-693, los niveles de reacción cruzada fueron más bajos; esto sugiere la necesidad de una vigilancia epidemiológica sostenida a fin de identificar y caracterizar las cepas emergentes del VDVB. La óptima correlación en el modelo bovino-ovino y bovino-cobayo indica su utilidad para evaluar la inmunogenicidad de vacunas inactivadas de VDVB.

Palabras clave: virus de la diarrea viral bovina, VDVB, cepas de campo de Argentina, vacunas inactivadas

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is considered an important cause of economic loss in the cattle industry worldwide (20). BVDV mainly affects young cattle raised under intensive or semi-intensive systems, causing respiratory, reproductive and digestive disorders (20, 23).

In Argentina, where extensive bovine production is prevalent, BVDV is considered one of the main causes of infertility, embryonic death, abortions, teratogenesis and perinatal deaths (15, 22, 25). Calves born from infected cows could became persistently infected (PI). These animals may have reduced viability, but a proportion of them survive, acting as an important reservoir of infection for other cattle (11). Super-infection of PI bovines with BVDV-CP induces a fatal diarrheic disorder called mucosal disease (4), a form of viral infection frequently reported in our country.

The antigenic and genetic diversity of BVDV is increasingly recognized (1, 5), and the differences became more evident using a panel of monoclonal antibodies to char-

acterize the isolates (8, 26). According to its biological behavior, BVDV can be divided into two biotypes, cytopathic (CP) and non-cytopathic (NCP), and the molecular characterization of BVDV isolates allowed its classification into genotype 1 and genotype 2 (27). A wide diversity of circulating BVDV strains has been described in Argentina (12, 16, 25).

Control of BVDV infections may be achieved by eliminating the carriers (PI animals) and preventing fetal infection through vaccination (1, 2, 10, 14, 30). Furthermore, protection against severe post-natal infections has become essential since the emergence of more virulent strains, i.e genotype 2 viruses (24, 27, 30).

Since post-vaccination accidents in other parts of the world have been reported when using live-virus vaccines (2), Argentina only allows the use of inactivated vaccines. Efficacy of commercially available inactivated vaccines is a controversial issue due to the diversity of circulating strains, and the absence of experimental evidence on protection levels during different outcomes of the infection (1, 2, 8, 9, 13, 14, 30). Therefore, the introduction of more efficacious vaccines is being demanded by veterinarians and farmers. In this regard, it is also essential to develop alternative animal models to allow the evaluation of vaccine immunogenicity by laboratories and control organizations.

This study evaluates the humoral immune response of experimental inactivated monovalent and multivalent BVDV vaccines tested against homologous and heterologous strains in the natural host (bovines). Additionally, this study compares the immune response obtained in bovine with two experimental animal models, ovines and guinea pigs that could be used as alternative in vivo models to evaluate the immunogenicity of BVDV Vaccines.

MATERIALS AND METHODS

Virus
The following BVDV reference strains were included in the formulation of vaccines and/or cross-seroneutralization studies: Singer; Oregon; NADL; 125 C, genotype 2 (kindly provided by Dr. J. Ridpath, Ames, Iowa, USA and Dr. L. Weber, INTA, Castelar) and strains VS 115 NCP, VS 87 CP, and field strain 00-693 INTA Balcarce.

Vaccines
Roller flasks containing Madin Darby Bovine Kidney (MDBK) cells were inoculated with the following BVDV strains: Singer, Oregon, NADL, 125 C (genotype 2) and VS 115 NCP. The viruses were harvested at 72 h post-inoculation, when a 80% cytopathogenic effect (CPE) was observed. Viral suspensions were clarified at 3,500 rpm for 15 min, and viral titre was assayed by $TCID_{50}$ in primary culture of bovine fetal testicle (BFT).

Viral suspensions were inactivated with 0.05% formaldehyde for 24 h at room temperature. Additionally, a volume of the Singer strain was pelleted at 4 °C with 7% polyethyleneglycol (PEG) 6,000, in 2.3% NaCl for 24 h, and then concentrated by centrifugation at 10,000 rpm for 1 hour. The pellet was suspended in buffer Tris-HCl EDTA, pH: 7.6. Finally, PEG was removed by centrifugation at 12,000 rpm for 10 min.

Vaccines were formulated in oil adjuvant (30:70, water:oil), as follows: Vaccine 1, Singer strain, $10^{7.33}$ $TCID_{50}$/ml; Vaccine 2, concentrated Singer strain, 10^8 $TCID_{50}$/ml; Vaccine 3, Oregon strain, 10^7 $TCID_{50}$/ml; Vaccine 4, NADL strain, $10^{7.50}$ $TCID_{50}$/ml; Vaccine 5, 125 C strain 10^6 $TCID_{50}$/ml; Vaccine 6, VS 115 NCP strain, $10^{6.33}$ $TCID_{50}$/ml; Vaccine 7, a combination of 5 strains mentioned above; and Vaccine 8, placebo.

Animals and vaccination scheme
Eight groups of four bovines each, of approximately 1 year of age and seronegative to BVDV, were inoculated intramuscularly (IM) with 2 doses, 30 days apart, of 5 ml of each vaccine. Eight groups of four ovines older than 1 year of age and eight groups of four guinea pigs of 500 g weight received 2 doses of 2 ml and 1 ml of each vaccine IM, respectively. All animals were bled at time 0 and every 30 days until 120 days post vaccination (dpv) for bovines and 180 dpv for ovines and guinea pigs.

Serum neutralization test
A serum neutralization test was used to monitor the humoral response, as previously described (18). Neutralizing titres (NT) were expressed as the logarithm of the reciprocal of the highest serum dilution that inhibited 100% of CPE. For VS 115 NCP, the virus presence was determined by direct immunofluorescence (DIF), using a fluorescein-labeled polyclonal anti-serum, following manufacturer's instructions (American BioResearch, TN, USA). In all cases, sera were tested against the Singer strain, while bovine sera at 60 dpv were also evaluated against all reference strains, including an Argentine field strain, 00-693.

Statistical analysis
The serology results were statistically evaluated by analysis of variance (ANOVA $P < 0.05$), followed by Bonferroni's averages comparison test. The correlation between the guinea pig or ovine models versus the bovine one was evaluated using Pearson's correlation test and linear regression analysis. Evaluation was performed with SAS/STAT (version 6.03, Int. Inc., Cary, NC, USA).

RESULTS

Evaluation of immunogenicity of experimental BVDV vaccines in bovines, ovines and guinea pigs:

Bovines
All animals immunized with Vaccines 1, 2, 3, 4, and 7 seroconverted at 60 dpv, showing significantly higher antibody titres compared to placebo ($p<0.05$) (Figure 1A). Table 1 shows that antibody titres means ranged from 2.3 to 2.8 at 60 dpv, showing no significant differences among them. This was also observed at 90 and 120 dpv. No seroconversion was observed in animals immunized with Vaccines 5 and 6.

Ovines
Figure 1B shows that ovines seroconverted after administration of all vaccines, except Vaccine 6. Statistical analysis at 60 dpv demonstrated that Vaccine 2 was the most immunogenic, followed by Vaccines 1, 3, 7, and 4. In addition, Vaccine 5 showed a short-term antibody response, which was not observed in the bovine model (Table 1).
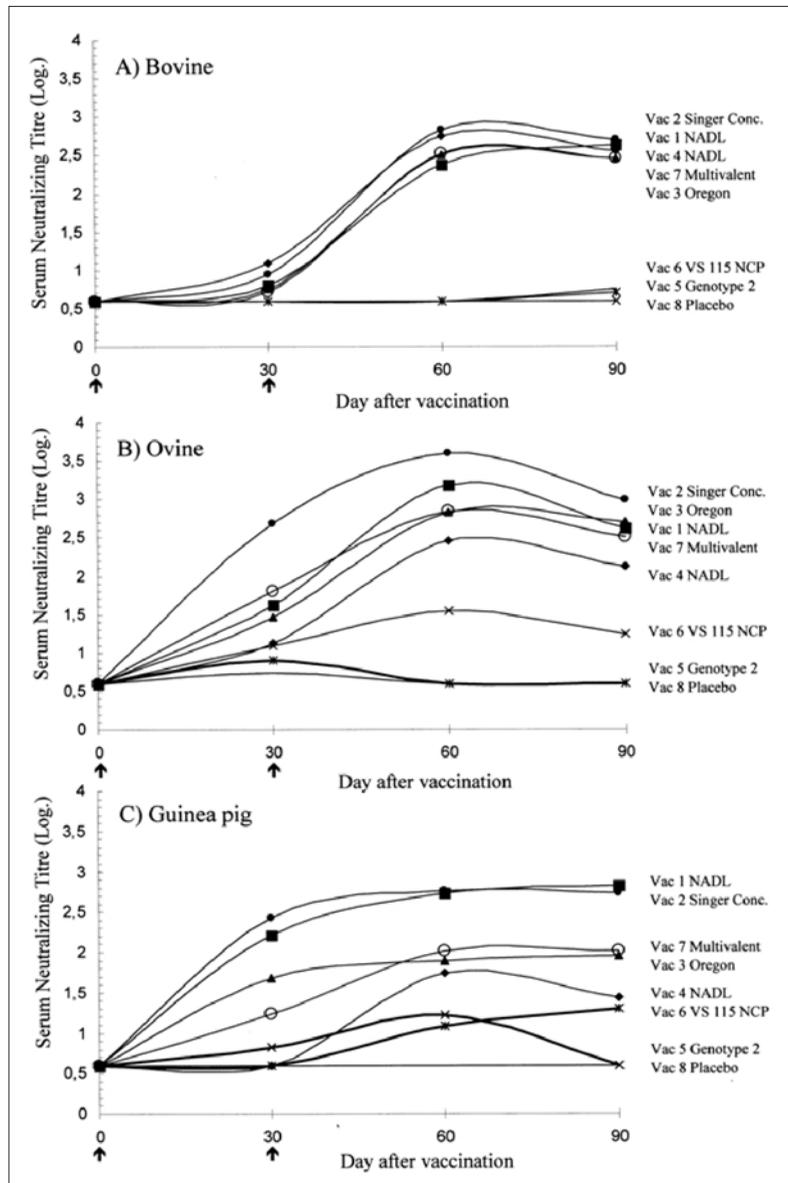
Figure 1. Kinetics of the serum immune response to BVDV-Singer strain in bovines (A), ovines (B) and guinea pigs (C), after immunization with 7 inactivated BVDV experimental vaccines. Arrows indicate vaccination time.

Guinea pigs

In this case, vaccines induced different levels of seroconversion (Figure 1C). The statistical analysis at 60 dpv, grouped the vaccines into 3 categories: Vaccine 1 and 2 were, significantly, the most immunogenic, followed by Vaccines 7, 3, and 4, which also showed significant differences compared to placebo. Finally, guinea pigs immunized with Vaccines 5 and 6 did not seroconvert.

Correlation between animal models

Optimal linear correlation indexes were found between bovines and ovines (R = 0.925, p = 0.0004), and between bovines and guinea pigs (R = 0.845, p = 0.0041, Pearson's

test). The following regression coefficients were obtained after linear regression analysis: bovine-ovine R^2 = 0.85, p = 0.0004; bovine-guinea pig R^2 = 0.71, p = 0.0002. The highest correlation was observed between the bovine and ovine models.

Comparative study of immunogenicity after challenge with different strains of BVDV

Due to the diversity of circulating strains, this study evaluated the immune response of bovines at 60 dpv, against the following BVDV strains: Singer, Oregon, NADL, 125 C genotype 2, VS 87 CP, VS 115 NCP, and a field strain 00-693 isolated during a BVDV outbreak in

Table 1. Comparison of neutralizing antibody titres to BVDV Singer strain in bovine, ovine and guinea pig at 60 dpv.

Group	Immunogen	Neutralizing antibody titre against DVB Singer (60 dpv)		
		Bovine[1]	Ovine[1]	Guinea pig[1]
1	Singer	2.3A[2] (±0.62) [3]	3.2AB(±0.67)	2.7A (±0.23)
2	Singer Conc	2.8A (±0.51)	3.6A (±0.37)	2.7A (±0.25)
3	Oregon	2.5A (±0.59)	2.8AB (±0.28)	1.9B (±0.22)
4	NADL	2.7A (±0.33)	2.4B (±0.33)	1.7B (±0.39)
5	Genotype 2	<0.6B (±0.0)	1.5C (±0.59)	1.2C (±0.04)
6	VS 115 NCP	<0.6B (±0.0)	1.5C (±0.0)	1.2C (±0.04)
7	Multivalent	2.6A (±0.59)	2.8AB (±0.30)	2.0B (±0.30)
8	Placebo	<0.6B (±0.01)	<0.6D (±0.01)	1.1C (±0.01)

[1] Correlation index for the bovine-ovine model, R = 0.9742 (p = 0.001). Bovine-guinea pig, R = 0.7613 (p = 0.0047), (Pearson's correlation test, $p < 0.05$).
[2] Arithmetic mean in the same column with different letters indicates significant difference among groups (One-Way ANOVA-Bonferroni, $p < 0.05$).
[3] Values in parenthesis indicate the standard deviation.

Table 2. Cross reactivity of bovine sera obtained at 60 dpv.

Groups	Immunogens	Neutralizing Antibody Titre						
		Singer	Oregon	NADL	Genotype 2	VS 115 CP	VS 115 NCP	BVDV 00-693
1	Singer	2.3	2.8	2.7	2.4	2.4	2.7	2.0
2	Singer conc	2.8	2.8	3.0	2.2	2.9	2.9	2.1
3	Oregon	2.5	2.6	2.8	2.3	1.9	2.8	1.9
4	NADL	2.7	2.8	3.0	2.5	2.2	3.1	2.2
5	Genotype 2	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
6	VS 115 NCP	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
7	Multivalent	2.5	2.6	3.0	1.8	2.3	2.3	2.0
8	Placebo	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6

Argentina. Animals immunized with Vaccines 1, 2, 3, 4, and 7, which presented high NT, showed similar antibody responses to both homologous as well as heterologous strains, indicating a high degree of cross-reactivity among these virus strains (Table 2). However, lower antibody titres were observed in all vaccine groups when tested against the 00-693 BVDV strain.

DISCUSSION

Control of BVDV infection should be based on detection and elimination of PI animals and on immunization through vaccination to prevent fetal infection (6, 14, 10, 21). This study evaluated several inactivated BVDV vaccines, and compared the natural host with two models, ovines and guinea pig, which might be used to test vaccine potency.

The immunogenicity of BVDV inactivated vaccines directly correlates with antigenic concentration. Furthermore, efficacy of a vaccine depends on the selection of the vaccine strain, the antigen concentration and the adjuvant selected (13, 19, 29, 30). Our results confirmed some of these observations, since only the animals immunized with vaccines containing a concentration of antigen = 10^7 $TCID_{50}$/ml seroconverted. In contrast, formulations with lower virus titres did not produce significant NT even against homologous strains (except Vaccine 5, which presented a low titre and short duration response in the ovine model). Therefore, 10^7 $TCID_{50}$/ml seems to be the critical concentration, and any given BVDV inactivated vaccine below that level should be discarded. On the other hand, an increase of virus concentration (10^8 $TCID_{50}$/ml for Vaccine 2), did not result in higher immunogenicity in all the models studied. Complemen-

tary tests using higher concentrations of antigens are needed to confirm this observation.

Neutralizing antibodies titres, particularly IgG1 levels, are critical to control the viral infection, showing a direct correlation between NT and severity of the disease (5). Moreover, passive antibodies acquired through colostrum, presenting titres ranging from 0.3 to 2.4, were not enough to protect calves after challenge with homologous virus strains, but effectively protected against severe disease (5). BVDV isolation from nasal swab samples also demonstrated continued transmission of the virus even in immunized calves (5). In view of this, the high post-vaccination neutralizing titres described in this study would result in significant levels of protection. Our results also suggest that vaccines formulated using oily adjuvant would ensure increased antibody levels and persistence of antibodies over time. Additional studies are required to determine the most suitable vaccination scheme.

Selection of animal models to evaluate vaccines directed against BVDV is a controversial issue, especially in our country where high prevalences of BVDV are found in bovines, thus, increasing the probability of becoming naturally infected. The high correlation observed between bovine-ovine and bovine-guinea pig models indicated that both models (ovine and guinea pig) might be useful to monitor BVDV vaccines. Given that the highest correlations were found between bovine and ovine models, and the fact that ovines presented the highest NT (3.6 NT at 60 dpv, Figure 1), pregnant sheep should be the model of choice for testing BVDV vaccines in our country, as previously reported in The Netherlands (7, 30). In addition, given its low cost and easy implementation, the guinea pig model would be useful to test immunogenicity of BVDV vaccines at production laboratories, or to be used as evaluation method by the control agencies.

Due to the diversity of BVDV strains, induction of cross protection is a desirable characteristic of a vaccine (9, 13, 30). The lack of cross protection could explain the birth of BVDV infected calves from cows immunized prior to service with inactivated BVDV vaccines (1). In contrast, cattle infection with BVDV induced high titres of neutralizing antibodies, which cross-reacted with other biotypes. This study showed that bovine sera at 60 dpv presented homologous as well as heterologous antibody response, indicating a high degree of cross-reactivity among the strains studied. Homotypic and heterotypic protection using three different reference strains was also previously reported during a study that evaluated an inactivated BVDV vaccine (5, 2, 3, 28, 29). Although those studies indicated that formulation of multivalent vaccines could be replaced by vaccines containing one strain with higher infectious titre, their results using the field strain 00-693 indicate the presence of major antigenic differences and the need of incorporating several strains into the vaccine formulation. Interestingly, sera from naturally infected animals in Argentina neutralized that strain, which

differed from vaccinated animals (16). These results also suggest the need of continued surveillance to identify and characterize emerging field BVDV strains, and eventually update vaccine composition.

This study evaluated different formulations of BVDV vaccines, and determined the level of neutralizing response, against homologous as well as heterologous strains. However, complementary studies are needed to further evaluate the efficacy of these formulations.

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