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Serological response against bovine herpesvirus and bovine viral diarrhea virus induced by commercial vaccines in Holstein heifers[1]

Camila C. Baccili[2]*, Camila Cecilia Martin[2], Karen N. Silva[2], Marcílio Nichi[3], Eduardo F. Flores[4], Aníbal E. Vercesi Filho[5], Edviges Maristela Pituco[6] and Viviani Gomes[2]

ABSTRACT.- Baccili C.C., Martin C.C., Silva K.N., Nichi M., Flores E.F., Vercesi Filho A.E., Pituco E.M. & Gomes V. 2019. **Serological response against bovine herpesvirus and bovine viral diarrhea virus induced by commercial vaccines in Holstein heifers.** *Pesquisa Veterinária Brasileira 39(11):870-878.* Departamento de Clínica Médica, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270, Brazil. E mail: camila.rcosta@usp.br

Vaccination is a strategy to the prevention and control of reproductive diseases caused by bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1), however the various compositions of commercial vaccines should be evaluated for their ability to induce protection mediated by antibodies. The objective of this research was to evaluate the production of specific neutralizing Abs against BVDV-1 and 2, and BoHV-1 induced by commercial vaccines composed by different adjuvants. Holstein heifers were vaccinated and distributed in three experimental groups: Group I (G1) was vaccinated with a commercial vaccine containing inactivated BVDV-1, BVDV-2 and BoHV-1 diluted in alum hydroxide as adjuvant (n=9); Group II (G2) was vaccinated with an product containing inactivated strains of BVDV-1, BVDV-2, BoHV-1 and BoHV-5 diluted in oil emulsion as adjuvant (n=10); Group III (G3) was vaccinated with a commercial vaccine containing inactivated BVDV-1 and BVDV-2, besides live modified thermosensitive BoHV-1, diluted in Quil A, amphigen and cholesterol (n=10); A control, non-vaccinated group (n=6) was mock vaccinated with saline. Heifers received two subcutaneous doses of 5mL of each commercial vaccine on the right side of the neck, with 21 days interval. Humoral immune response was assessed by the virus neutralization test (VN) against BVDV-1 (NADL and Singer strains), BVDV-2 (SV253 strain) and BoHV-1 (Los Angeles strain) in serum samples collected on vaccination days zero (D0), 21 (D21) and 42 (D42; 21 days after boosting). Neutralizing Abs against BVDV-1 NADL was detected only in D42, regardless of the vaccine used. Similar geometric mean titers (GMT) for BVDV-1 NADL were observed between G1 (log_2=5.1) and G3 (log_2=5.1). The seroconversion rate (%) was higher in G1 (78%) when compared to G2 (10%) and G3 (40%). For BVDV-1 Singer, it was also possible to detect Abs production in G1 (log_2=5.8, 100% seroconversion rate) and G3 (log_2=3.5, seroconversion rate = 60%), only after the booster dose (D42). Neutralizing Abs to BVDV-2 (SV253) were detected only in G3, observing 90% seroconversion associated with high titers of Abs (log_2=6.7) after the 2nd dose of vaccine (D42). Heifers from G1 and G3 responded to BoHV-1 after the first dose (D21): G1 (log_2=2.5,

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[2] Departamento de Clínica Médica, Universidade de São Paulo (USP), Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270, Brazil. *Corresponding author: camila.rcosta@usp.br

[3] Departamento de Reprodução Animal, USP, Av. Orlando Marques de Paiva 87, Cidade Universitária, Butantã, SP 05508-270.

[4] Graduate Program in Veterinary Medicine, Setor de Virologia, Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria (UFSM), Av. Roraima 1000, Cidade Universitária, Camobi, Santa Maria, RS 97105-900, Brazil.

[5] Agência Paulista e Tecnologia do Agronegócio, APTA Gado de Leite, Rua Heitor Penteado 56, Nova Odessa, SP 13460-000, Brazil.

[6] Instituto Biológico, Laboratório de Viroses de Bovídeos, Av. Conselheiro Rodrigues Alves 1252, Vila Mariana, SP 04014-002.

seroconversion rate = 67%) and G3 (log_2=0.7, seroconversion rate = 80%). In D42, a higher magnitude response was observed in the heifers from G3 (log_2=6.1, 100%) compared with G1 (log_2=4.3, 100%) and G2 (log_2=2.7, 60%). Based on the data obtained, it can be concluded that the commercial vaccine contained aluminum hydroxide (G1) was most effective in the induction of antibodies against BVDV-1. On the other hand, this vaccine did not induce the production of neutralizing Abs against BVDV-2. Only the heifers from G3 (Quil A, amphigen and cholesterol) generated neutralizing Abs against BVDV-2. The animals that received commercial vaccine containing oil emulsion as adjuvant (G2) had a weak/undetectable response against BVDV-1 and BVDV-2. The best protective response against BoHV-1 was observed in heifers vaccinated with the live modified thermosensitive virus.

INDEX TERMS: Vaccine response, serology, bovine viral diarrhea virus, BVDV, bovine herpesvirus type 1, BoHV-1, vaccine, Holstein heifers, cattle.

RESUMO.- [**Resposta sorológica contra herpesvírus bovino e vírus da diarreia viral bovina induzida por vacinas comerciais em novilhas Holandesas.**] A vacinação é utilizada como estratégia para a prevenção e controle das doenças reprodutivas, causadas pelos vírus da diarreia viral bovina (BVDV) e herpesvírus bovino tipo 1 (BoHV-1), entretanto, as diversas composições de vacinas comerciais devem ser avaliadas quanto a sua eficiência protetiva mediada por anticorpos (Acs). O objetivo desta pesquisa foi avaliar a produção Acs neutralizantes específicos para cepas de BVDV-1 e 2, e BoHV-1 induzida por vacinas comerciais contendo diferentes tipos de adjuvantes. Para tal, novilhas Holandesas foram vacinadas e distribuídas em três grupos experimentais: Grupo I (G1) foi vacinado com uma vacina comercial composta por cepas inativadas de BVDV-1, BVDV-2 e BoHV-1 diluídas em hidróxido de alumínio como adjuvante (n=9); Grupo II (G2) foi vacinado com produto contendo as cepas inativadas de BVDV-1, BVDV-2, BoHV-1 e BoHV-5 em uma emulsão oleosa como adjuvante (n=10); O Grupo III (G3) foi vacinado com uma vacina comercial contendo BVDV-1 e BVDV-2 inativado, além do BoHV-1 vivo modificado e termosensivel, diluídos em adjuvante contendo Quil A, Amphigem e colesterol (n=10); O Grupo Controle não vacinado (n=6) foi inoculado com solução salina. As novilhas receberam duas doses das respectivas vacinas ou solução salina (5mL), com intervalo de 21 dias, por via subcutânea, na tábua do pescoço do lado direito. A resposta imune humoral foi avaliada pelo teste de vírus neutralização (VN) contra o BVDV-1 (cepas NADL e Singer), BVDV-2 (cepa SV253) e BoHV-1 (cepa Los Angeles) em amostras de soro coletadas nos dias (D) de vacinação zero (D0), 21 dias após 1ª dose (D21)e 42 (D42; 21 dias após A 2ª dose). Os anticorpos neutralizantes contra o BVDV-1 NADL foram detectados apenas em D42, independentemente da vacina utilizada. Os títulos médios geométricos (GMT) de anticorpos foram semelhantes entre G1 (log_2=5,1) e G3 (log_2=5,1). A taxa de soroconversão foi maior no G1 (78%) quando comparado ao G2 (10%) e G3 (40%). Para o BVDV-1 Singer, somente após D42 foi observada a produção de Acs no G1 (log_2=5,8; taxa de soroconversão de 100%) e G3 (log_2=3,5; taxa de soroconversão = 60%). Os anticorpos contra BVDV-2 (SV253) foram detectados apenas nas novilhas do G3, observando-se taxa de soroconversão de 90% com altos títulos de anticorpos neutralizantes (log_2=6,7) em D42. Novilhas G1 e G3 responderam ao BoHV-1 após a primeira dose (D21): G1 (log_2=2,5; taxa de seroconversão = 67%) e G3 (log_2=0,7; taxa de seroconversão = 80%). Em contrapartida, foi observada uma maior magnitude de resposta para as novilhas G3 (log_2=6,1; 100%) em D42, em relação aos animais G1 (log_2=4,3; 100%) e G2 (log_2=2,7; 60%). Com base nos dados obtidos, foi possível concluir que a vacina composta por hidróxido de alumínio (G1) foi mais eficaz na produção de anticorpos contra o BVDV-1, em contrapartida esse produto não induziu anticorpos contra o BVDV-2. Apenas as novilhas do G3 (Quil A, amphigen e colesterol) geraram Acs neutralizantes contra o BVDV-2. Os animais que receberam a vacina em emulsão oleosa (G2) como adjuvante apresentaram uma resposta fraca/indetectável contra o BVDV-1 e BVDV-2. A melhor resposta protetiva contra o BoHV-1 foi observada nas novilhas vacinadas com a vacina viva modificada termosensível.

TERMOS DE INDEXAÇÃO: Resposta vacinal, sorologia, vírus da diarreia viral bovina, BVDV, herpesvírus bovino tipo 1, BoHV-1, bovinos, novilha Holandesa, vacina comercial.

INTRODUCTION

Today the world population is growing exponentially and will reach 10 billion inhabitants by 2050 (FAO 2017), increasing the demand for food of animal origin. Brazil is the largest exporter of beef and holds the most significant commercial herd in the world represented by 218 million animals, effectively producing about 5.87 billion liters of milk and slaughtering around 7.37 million heads a year (IBGE 2017). Despite productive efficiency, world demand has put pressure on our country to achieve the best reproductive rates aiming at achieving maximum reproductive efficiency. In this scenario, herd health is a prerequisite for ensuring that investment in genetics and breeding is successful with the birth of healthy calves. Thus, the use of reproductive vaccines to control infectious diseases that act negatively in both the reproductive and productive scenario in the dairy and beef systems has intensified (Weber et al. 2013).

Among the main agents responsible for reproductive losses in cattle are bovine viral diarrhea virus (BVDV) and bovine herpesvirus type 1 (BoHV-1). Studies estimate that the financial losses associated with viral infections generate losses of $ 0.50 to 687.80/animal for BVDV in dairy herds (Richter et al. 2017) and $ 460 to 767 million per year in beef cattle (Givens & Marley 2013). For BoHV-1, it was estimated that an average cost is $ 379 per infected cow (Can et al. 2016).

Bovine Viral Diarrhea (BVD) is cited in the World Animal Health Organization's list of diseases as a worrying virus in cattle production worldwide (OIE 2018). BVDV has a wide antigenic variability, belongs to the family Flaviviridae, genus

Pestivirus, in this genus 11 species are included among them BVDV-1, BVDV-2 and Hobi-like *Pestivirus* (Ridpath et al. 1994, Schirrmeier et al. 2004, ICTV 2019). The impact generated by the BVDV virus is related to its ability to cross the placental barrier, and cause several problems in the embryo or fetus, including fetal resorption, immunosuppression, teratogeny, and mainly cause the birth of persistently infected (IP) calves (Martin et al. 2016, Walz et al. 2017, Jardim et al. 2018). BoHV-1 belongs to the family Herpesviridae, subfamily Alfaherpesviridae, genus *Varicellovirus*, and is associated with the Bovine Respiratory Disease Complex and reproductive disease in bovine females, including abortions (Becker et al. 2015, Costa et al. 2017).

Given this, reproductive vaccines represent an essential strategy for the control of infections caused by both BVDV and BoHV-1, in an attempt to limit losses related to lower conception rates, early embryonic death, abortions, stillbirths and birth of premature calves caused by viral infections (Walz et al. 2017). Vaccination of cattle against BVDV and BoHV-1 is not yet a widespread practice in our country (Silva et al. 2007b), but a large number of commercial vaccines licensed against these agents in Brazil shows a trend towards continuous use vaccination protocols associated with reproduction. In Brazil, there are around 20 commercial reproductive vaccines, most of them composed of inactivated strains of BVDV (Brasil 2019). To date, only one commercial live modified BVDV (MLV) vaccine has been licensed for use in Brazil by the "Ministério da Agricultura, Pecuária e Abastecimento" (MAPA). Besides, there are no commercial Hobi-like vaccines registered in our country.

Vaccine-induced protection is ensured by its ability to stimulate the production of neutralizing antibodies (Abs), which are capable of binding to epitopes on the surface of antigens. These Abs eliminate invading microorganisms by mechanisms of neutralization, agglutination, complement system activation with opsonization, and pathogen lysis (Chase 2007). The minimum Abs titers needed to protect animals from BVDV challenge suggested by Bolin & Ridpath (1995) are $\log_2$ 4 (1:16), and for BoHV-1, Pospísil et al. (1996) cited $\log_2$ neutralizing Abs titers ≥4 or 5 (≥1:16 or 1:32). However, it is estimated that higher neutralizing Abs titers will provide a greater protective capacity to the host.

Inactivated vaccines are composed of dead viral particles, unable to replicate in host cells, which require greater antigenic mass diluted in adjuvants, and play a role in improving the immunological and consequently protective efficiency of commercial formulations (Vartak & Sucheck 2016, Hogenesch et al. 2018). Adjuvants most commonly used in veterinary medicine are aluminum hydroxide, oily emulsions, saponins, and immune-stimulating complexes (ISCOM). Adjuvants generally act primarily at the site of application by stimulating innate immunity by enhancing antigen recognition and uptake by antigen-presenting cells (APCs) at the injection site, with subsequent migration to the regional lymph nodes for stimulation of a cellular and humoral immune response (Spickler & Roth 2003).

Given the context presented, considering the importance of using efficient vaccines that are capable of generating a protective and lasting immune response in the prevention of reproductive diseases, this research hypothesizes that commercial vaccines containing different types of adjuvants induce different degrees of the humoral response against BVDV and BoHV-1. Our hypothesis is based on the lack of information on the antibody production profile for each type of adjuvant present in commercial vaccines, making it necessary to evaluate the efficacy of these immunogens. Thus, the objective of this research was to evaluate the production of specific Abs for the main strains of BVDV-1 and 2, and BoHV-1 induced by commercial reproductive vaccines available in the Brazilian market containing different adjuvants.

MATERIALS AND METHODS

This research was approved by the Animal Use Ethics Committee of the "Faculdade de Medicina Veterinária e Zootecnia" of USP, protocol number 6229201216. The heifers from this research are part of the experimental farm herd of the "Agência Paulista e Tecnologia do Agronegócio (APTA) Gado de Leite", located in the city of Nova Odessa, São Paulo (22°75'S latitude and 47°27'W longitude). This property was selected for not performing BVDV and BoHV-1 vaccinations. The sanitary protocol adopted by the farm is composed only of FMD and Brucellosis vaccines, required by MAPA.

The field trial was conducted from December 2015 to April 2016. During this period, average temperatures ranged from 17°C to 33°C, according to the "Instituto Nacional de Meteorologia" (INMet). Prior selection of heifers was initiated by animals aged 15-24 months (n=35), seronegative for both BVDV-1 (NADL) and BoHV-1 (Los Angeles) viruses, according to the virus neutralization test (VN), (OIE 2015). Exclusion of persistently infected animals (PIs) was conferred by reverse transcriptase reaction followed by a polymerase chain reaction to BVDV (RT-PCR), according to Basqueira et al. (2017). All heifers remained during the experimental period under extensive management in *Brachiaria Brizantha* cv. *Marandu* with *ad libitum* water and mineral salt supply, the body score of the animals ranged from 2.75 to 4.

Heifers (n=35) were randomly assigned to four experimental groups: Group I (G1) was inoculated with a vaccine composed of inactivated strains of BVDV-1, BVDV-2 and BoHV-1 in aluminum hydroxide as adjuvant (n=9); Group II (G2) was inoculated with inactivated vaccine strains of BVDV-1, BVDV-2, BoHV-1 and BoHV-5 in an oily emulsion as adjuvant (n=10); and Group III (G3) was inoculated with a vaccine containing BVDV-1, inactivated BVDV-2 and thermosensitive modified live BoHV-1 in Quil A, amphigen and cholesterol adjuvant (n=10). The unvaccinated Control Group (n=6) was inoculated with saline, as shown in Table 1. Vaccination protocols followed the manufacturers' recommendations. Heifers received two doses of different commercial vaccines (5mL), 21 days apart, subcutaneously, in the right neck region. Animals in the unvaccinated Control Group received a saline injection (5mL) on the days of administration of the 1st and 2nd doses of vaccines.

The adaptive immune response to BVDV and BoHV-1 was determined at the time of vaccination (D0), the day of revaccination (D21) and 21 days (D42) after the second dose.

The research of neutralizing Abs against BVDV-1 (NADL) and BoHV-1 (Los Angeles) were performed according to the recommendations of the World Organization for Animal Health (OIE 2015) at the Bovine Virus Laboratory, Biological Institute. In 96-well flat-bottom polystyrene microtiter plates, column 1 of the test plate was intended for cell control, column two for toxicity control of each serum, and in columns three through 12, samples were diluted in logarithmic base 2 ($\log_2$) from dilutions 1:10 (BVDV-NADL) and 1:2 (BoHV-1-Los Angeles) in eight replications using the minimum essential medium cell culture medium as diluent (MEM) containing 1% antibiotics

Table 1. Distribution of experimental groups, the composition of commercial vaccines according to adjuvants and strains for BVDV-1, BVDV-2, and BoHV-1

Groups (G)	Strains
Group 1 (n=9)	BVDV-1 (Singer) and BVDV-2** - inactivated, strains provided by INTA and CEVAN BoHV-1 (Los Angeles) inactivated
Group 2 (n=10)	**BVDV-1 and BVDV 2 (inactivated) **BoHV-1 and BoHV-5 (inactivated)
Group 3 (n=10)	BVDV-1 (5960) and BVDV-2 (53637) - inactivated BoHV-1 (Cooper) - chemically altered thermosensitive
Control Group (n=6)	Saline inoculation

** Strains not provided by the manufacturer; CEVAN = Center for Animal Virology, Argentina, INTA = National Institute of Agricultural Technology, Argentina.

and 5% BVDV antibody-free fetal bovine serum. Duplicate serum 50μL was added to the wells of the plates, after which 50μL of the respective virus solution containing $TCID_{50}$/100μL (50% tissue culture infective doses) was added. The plates were incubated for 18-24h for BoHV-1 and one hour for BVDV in an oven at 37°C with 5% CO_2. After incubation an MDBK (Madin-Darby bovine kidney) cell suspension was added to each well of the plates. After this process, the plate was incubated in an oven at 37°C with 5% CO_2 for 4-5 days. The search for neutralizing antibodies against BVDV-1 Singer and BVDV-2 VS253 was performed in the Virology Sector of the "Universidade Federal de Santa Maria", following the protocol described by OIE (2015). Serum dilutions were 1:5, with 100 infective doses for 50% of the cell cultures (TCID50) of each virus used.

Plates were read after 96h of incubation by observing the visible cytopathic effect (CPE) on the cell monolayer under an inverted microscope. The neutralizing antibody titer was considered to be the reciprocal of the highest serum dilution capable of neutralizing viral replication. Samples that did not show neutralization at the lowest dilution were considered negative (Reed & Muench 1938).

The statistical analysis of neutralizing antibody titers of each animal and the frequency of seroconversion was performed using the SAS statistical program (version 9.4, SAS Institute Inc., Cary/NC). Data that did not present parametric distribution were transformed following two approaches. First, the data were ranked; after this step, the data were submitted to a log_{10}, square root, or inverse transformation, this methodology was described by Templeton (2011). In the case where the transformations occurred, the P presented refers to the tests related to the transformed values, while the described values are real.

All variables were evaluated for distribution concerning the gaussian curve by the guided data analysis function. The variables were tested for fixed effects of treatments (G1, G2, G3, and Control) and days (0.21 and 42), as well as the interaction of treatment and day effects by MIXED procedure (PROC-mixed, SAS), with post hoc test "least significant difference" (LSD). The models were tested according to covariance structures, using the "Akaike information criterion" (AIC).

The seroconversion rate was presented in frequency (%) because it is a quantitative data, and the comparison between groups was performed by Chi-square test and between times by Cochran's Q test. Analyses were considered significant when P≤0.05 (*).

RESULTS

The mean geometric titers (GMT log_2) of Abs and the seroconversion frequencies (%) for BVDV (type 1 and 2) and BoHV-1 strains produced by vaccinated heifers, as well as the differences between groups and times are presented in Table 2 and Figure 1.

It was possible to observe differences for the fixed effects detected by PROC-mixed in relation to BVDV-1 (NADL and Singer), BVDV-2 and BoHV-1 specific Abs, regarding groups (P=0.001), days (P=0.001) and Trt x day (P=0.001).

Regarding BVDV-1 NADL, none of the heifers belonging to vaccinated Groups 1, 2, and 3 seroconverted after the 1st dose of commercial vaccines (D21). In D42, the average of Abs (GMT-log_2) were similar between heifers G1 (GMT-log_2=5.1) and G3 (GMT-log_2=5.1), but the seroconversion rate was higher in G1 (78%, 7/9) compared to G3 (40%, 4/10). The G2 heifers had similar mean Abs titers to the unvaccinated Control Group, and only one animal (10%) responded to vaccination.

Regarding the BVDV-1 Singer strain, G1 heifers presented 22% (2/9) and GMT log_2=0.1 seroconversions after primo vaccination (D21), but this group did not differ statistically from G2 and G3. In D42, heifers belonging to G1 had higher mean titers of Abs (GMT-log_2=5.8) and 100% seroconversion when compared to G3 heifers (GMT-log_2=3.5; seroconversion rate 60%). The G2 animals did not respond to the BVDV-1 Singer strain.

The BVDV-2 VN test (VS253) showed that only Group 3 produced Abs after the first dose (GMT-log_2=1.0) and the 2nd vaccine dose (GMT-log_2=6.7), seroconversion presented was 10% (1/10) at D21 and 90% (9/10) at D42. The Groups 1 and 2 showed undetectable production of neutralizing Abs against BVDV type 2.

Regarding BoHV-1, specific Abs were detected in D21 only for G1 (GMT log_2=2.5) and G3 (GMT log_2=0.7), the observed seroconversion was 67% (6/9) and 80% (8/10). In D42 revaccination, higher response intensity was observed in G3 heifers (GMT-log_2=6.1; 100% seroconversion rate), followed by G1 (GMT-log_2=4.3; 100% seroconversion rate), G2 (GMT-log_2=2.7; seroconversion rate 70%) and negative control.

The intensity of antibody production in each group is shown in Table 3. The frequency of heifers with minimum protective titers (≥16) for BVDV-1 strains was detected only at D42. Regarding BVDV-1 (NADL), Groups 1 and 3 showed frequencies of 67% (6/9) and 20% (2/10), while for BVDV-1 (Singer) it was 89% (8/9) and 40% (4/10). Group 2 heifers had lower titers than 16. Regarding BVDV-2 (VS-253), only G3 presented Abs titers, being 80% (8/10) protective. Regarding BoHV-1 (Los Angeles) protective Abs (≥16) in D21 were detected only in 1/9 (11%) in G1. In D42, the vaccine used in G3 induced Abs in 90% (9/10) of animals, followed by G1 with 44% (6/9) and G2 with 20% (2/10).

Table 2. Geometric mean titers (GMT-log2) of neutralizing antibodies and seroconversion rates (%) for BVDV-1 (NADL), BVDV-1 (Singer), BVDV-2 (VS253) and BoHV-1 (Los Angeles) induced by commercial vaccines containing different types of adjuvants in Holstein heifers

Strains	Day	GMT (log_2)				Seroconversion (n/%)			
		Group 1 (n=9)	Group 2 (n=10)	Group 3 (n=10)	Control (n=6)	Group 1 (n=9)	Group 2 (n=10)	Group 3 (n=10)	Control (n=6)
BVDV-1 (NADL)	D0	0.0 ± 0^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0 ± 0^{a}	0% (0/9)^{b}	0% (0/10)^{a}	0% (0/10)^{b}	0%(0/6)^{a}
	D21	0.0 ± 0^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0 ± 0^{a}	0% (0/9)^{b}	0% (0/10)^{a}	0% (0/10)^{b}	0%(0/6)^{a}
	D42	5.1 ± 5.6^{Aa}	0.0 ± 0^{Ba}	5.1 ± 6.6^{Aa}	0.0 ± 0^{Ba}	78% (7/9)^{Aa}	10%(1/10)^{Ba}	40%(4/10)^{Aa}	0%(0/6)^{Ba}
BVDV-1 (Singer)	D0	0.0 ± 0^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0±0	0% (0/9)	0% (0/10)	0% (0/10)	0%(0/6)
	D21	0.1 ± 1.1^{b}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0±0	22% (2/9)	0% (0/10)	0% (0/10)	0%(0/6)
	D42	5.8 ± 5.5^{Aa}	0.0 ± 0^{Ca}	3.5 ± 3.7^{Ba}	0.0±0	100% (9/9)	0% (0/10)	60%(6/10)	0%(0/6)
BVDV-2 (VS253)	D0	0.0 ± 0^{a}	0.0 ± 0^{a}	0.0 ± 0^{b}	0.0±0	0% (0/9)	0% (0/10)	0% (0/10)	0%(0/6)
	D21	0.0 ± 0^{a}	0.0 ± 0^{a}	1.0 ± 2.6^{b}	0.0±0	0% (0/9)	0% (0/10)	10%(1/10)	0%(0/6)
	D42	0.0 ± 0^{Ba}	0.0 ± 0^{Ba}	6.7 ± 6.9^{Aa}	0.0±0	0% (0/9)	0% (0/10)	90%(9/10)	0%(0/6)
BoHV-1 (Los Angeles)	D0	0.0 ± 0^{c}	0.0 ± 0^{b}	0.0 ± 0^{c}	0.0 ± 0^{a}	0% (0/9)^{c}	0% (0/10)^{b}	0% (0/10)^{c}	0%(0/6)^{a}
	D21	2.5 ± 3.3^{Ab}	0.0 ± 0^{Bb}	0.7 ± 0^{ABb}	0.0 ± 0^{Ba}	100% (9/9)^{Ab}	10%(1/10)^{Bb}	80%(8/10)^{ABb}	0%(0/6)^{Ba}
	D42	4.3 ± 4.0^{Ba}	2.7 ± 2.0^{Ca}	6.1 ± 5.6^{Aa}	0.0 ± 0^{Da}	100% (9/9)^{Ba}	70%(7/10)^{Ca}	100%(10/10)^{Aa}	0%(0/6)^{Da}

Group 1 = commercial vaccine containing aluminum hydroxide, Group 2 = commercial vaccine containing oily emulsion, Group 3 = commercial vaccine containing Quil A, amphigen and cholesterol; ^{A,B,C} uppercase letters in the same row demonstrate difference between groups, ^{a,b,c} lowercase letters in the same column demonstrate difference between times, data without letters showed no differences. For GMT differences were detected by the PROC MIXED test; seroconversion rate was tested between groups by Chi-square test and between times by Cochran's Q test; the analyzes were considered significant when P≤0.05.

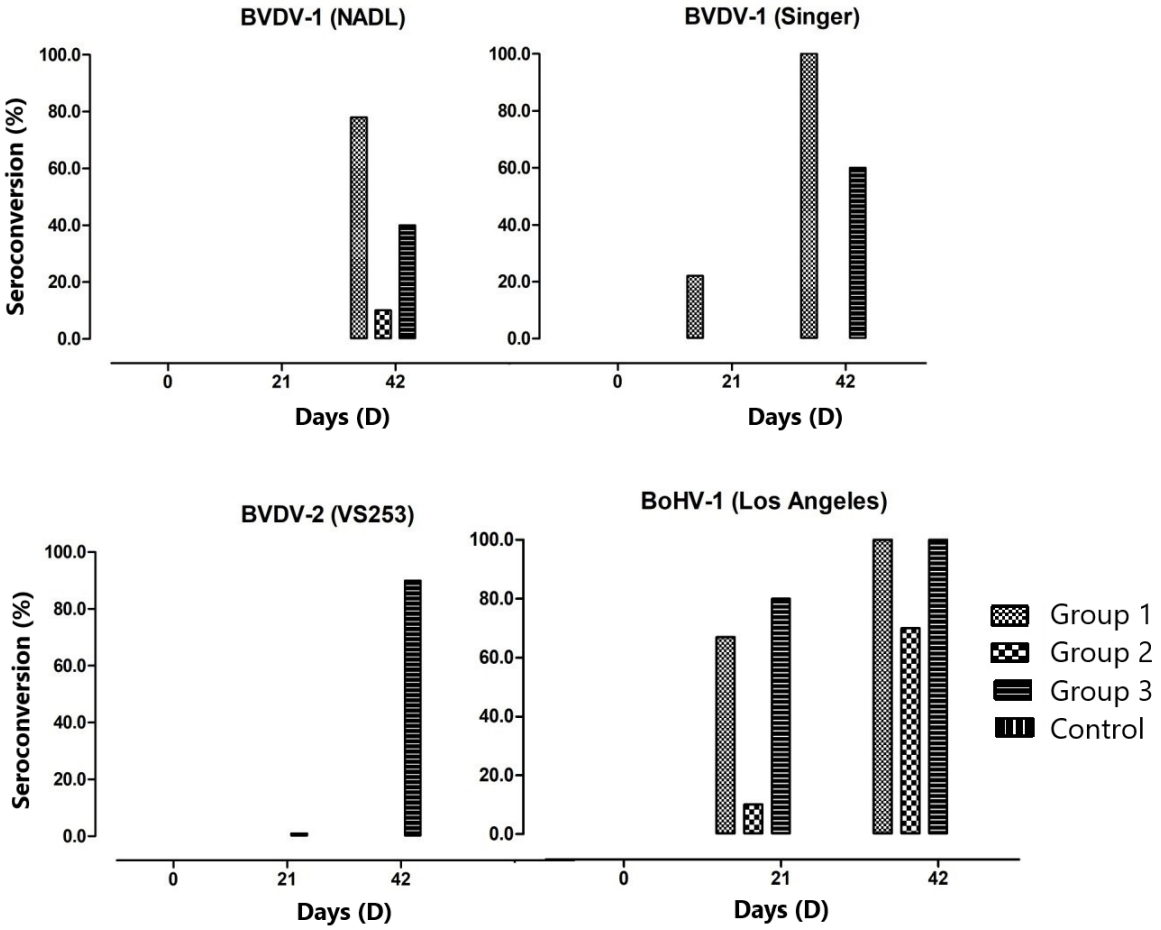

Fig.1. Seroconversion frequencies (%) of Group 1, 2 and 3 heifers inoculated with commercial vaccines containing different adjuvant types for BVDV-1 (NADL), BVDV-1 (Singer), BVDV-2 (VS253) and BoHV-1 (Los Angeles) viruses.

Table 3. Frequency distribution (%) according to antibody production intensity against BVDV-1 (NADL), BVDV-1 (Singer), BVDV-2 (VS253) and BoHV-1 (Los Angeles), induced by commercial vaccines containing different types of adjuvants in Holstein heifers

Virus (strains)	Neutralizing Ab titers	Group 1 (n=9)		Group 2 (n=10)		Group 3 (n=10)	
		D21	D42	D21	D42	D21	D42
BVDV-1 (NADL)	2	0% (0/9)	0% (0/9)	0% (0/10)	10% (1/10)	0% (0/10)	0% (0/10)
	10	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	20	0% (0/9)	33% (3/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	40	0% (0/9)	22% (2/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	160	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	316	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	Titers ≥16	0% (0/9)	67% (6/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
BVDV-1 (Singer)	5	22% (2/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	10	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	20	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	30% (3/10)
	40	0% (0/9)	44% (4/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	80	0% (0/9)	22% (2/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	160	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	Titers ≥16	0% (0/9)	89% (8/9)	0% (0/10)	0% (0/10)	0% (0/10)	40% (4/10)
BVDV-2 (VS-253)	10	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	20	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	10% (1/10)	20% (2/10)
	40	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	160	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	10% (1/10)
	≥320	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	Titers ≥16	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	10% (1/10)	80% (8/10)
BoHV-1 (Los Angeles)	2	0% (0/9)	11% (1/9)	10% (1/10)	10% (1/10)	80% (8/10)	0% (0/10)
	4	55% (5/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	0% (0/10)
	8	0% (0/9)	44% (4/9)	0% (0/10)	40% (4/10)	0% (0/10)	20% (2/10)
	16	0% (0/9)	11% (1/9)	0% (0/10)	20% (2/10)	0% (0/10)	0% (0/10)
	32	11% (1/9)	22% (2/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	64	0% (0/9)	11% (1/9)	0% (0/10)	0% (0/10)	0% (0/10)	20% (2/10)
	126	0% (0/9)	0% (0/9)	0% (0/10)	0% (0/10)	0% (0/10)	40% (4/10)
	Titers ≥16	11% (1/9	44% (4/9)	0% (0/10)	20% (2/10)	0% (0/10)	90% (9/10)

D = day, Group 1 = commercial vaccine containing aluminum hydroxide, Group 2 = commercial vaccine containing oily emulsion, Group 3 = commercial vaccine containing Quil A, amphigen and cholesterol.

DISCUSSIONS

The results obtained in this research vary in the magnitude of the humoral response to strains of BVDV-1, BVDV-2, and BoHV-1.

The mean neutralizing antibody titers against the BVDV-1 NADL strain were similar between vaccinated heifers G1 and G3. On the other hand, G2 vaccinated animals had similar antibody titers to the control. Seroconversion rates were observed only in the secondary vaccine response, with 78% reagents in G1, 40% G3, and 10% G2. Similar response pattern was observed for BVDV-1 Singer strain. The cutoff point for protective antibody titers was considered above 16 (Bolin & Ridpath 1995), so in this study, we found that the protective response profile for BVDV-1 (NADL and Singer) was 67% and 89% (G1), 20% and 40% for G3 in D42. Bolin & Ridpath (1995) found that animals with neutralizing antibody titers below 1:16 and challenged with the non-cytopathic BVDV virus (strain 890) showed acute disease with hyperthermia, leukopenia, thrombocytopenia, and diarrhea.

Vaccines used in cattle are generally multivalent, immunizing animals against several pathogens in a single administration. However, mixing bacterial and viral strains can present a significant immune challenge, as it requires a simultaneous response to immunodominant antigens and less immunogenic antigens, in addition to the possible inclusion of immunosuppressive agents and preparations containing both live virus and inactivated virus (Kreutz 2012).

The low immunogenicity of national BVDV vaccines has been reported in previous studies by Brazilian research teams. A study by Lima et al. (2005) verified effective humoral response in cattle after 30 days of vaccination with an experimental formulation containing attenuated BVDV-1 (GMT=1612.7) and BVDV-2 (GMT=151.0), while the other inactivated commercial vaccines showed weak response and partial. In the same research, two vaccines containing aluminum hydroxide induced BVDV-1 seroconversion in 32/36 (GMT=14.3) and 22/28 (GMT=25.1) animals. Regarding BVDV-2, seroconversion was observed in 27/36 (GMT=10.0) and 12/28 cattle (GMT=11.5) after vaccination with formulations containing aluminum hydroxide. The only oily vaccine in the study showed that 16/30 (GMT=40.0) and 10/30 (GMT=10.0) seroconverted to BVDV-1 and BVDV-2, respectively. Gomes et al.

(2014), reported low passive antibody transfer against BVDV after vaccination of commercially formulated prepartum cows containing BVDV-1 (NADL and Singer), BVDV-2 and BoHV-1 (Los Angeles) diluted in hydroxide aluminum as an adjuvant. The authors state that only 14.28% (1/7) of calves in the group of vaccinated mothers had neutralizing antibodies against BVDV in colostrum and blood serum from birth at 15 days of age. Baccili et al. (2018) evaluated the vaccine response of prepartum Holstein cows and their influence on passive immunity transfer, and it was found that only 33% (2/6) of vaccinated cows seroconverted after two doses of BVDV 1 polyvalent vaccine (strain 5960) and BVDV 2 (strain 53637) inactivated; live and heat-sensitive; BoHV-1 (strain RBL 106) and BBPI3-V (strain RLB 103); live attenuated; BRSV (strain 375), diluted in ISCOM adjuvant.

Inactivated vaccines contain inert viral particles, unable to replicate in the animal, abolishing the risk of fetal infection in pregnant cows; they are more stable under field conditions and are less costly to produce. The predominant response profile in this vaccine is the B-lymphocyte-mediated Th2 (humoral) type, which is critical for the production of antibodies that neutralize infectious viral particles before they infect host target cells. Despite these advantages, the search for vaccines that are also capable of stimulating cellular response (Th1) is essential, as this is the primary mechanism for host defense after the establishment of infection caused by viral agents (Kelling 2004, Silva et al. 2007b, Platt et al. 2008, Kreutz 2012). Given this context, this type of vaccine requires the addition of adjuvants in order to increase immunogenicity, improving antigen presentation, and thus amplifying both humoral and cellular immune response. The addition of adjuvants, on the other hand, imposes vaccine reactions on the animal, especially after multiple applications (Shams 2005, Newcomer et al. 2017).

In this research, it was found that vaccinated animals produced neutralizing antibody titers only at D42, regardless of the vaccine composition. Development of the humoral immune response after the first dose of a vaccine may take three to four weeks to occur, they should be driven by revaccination. At the time of the first vaccination, the antibody concentration does not increase until 10-14 days from the date of application of the first vaccine dose, and then the antibody concentration slowly increases. Therefore, the importance of booster should be reinforced with the application of the second dose of commercial formulations to boost the increase in antibody concentration. After the second dose, antibody concentration increases rapidly within 24 hours and peaks within a few days, with this response maintained for weeks or months (Chase et al. 2008).

A study by Anziliero et al. (2015) found different results from our research regarding seroconversion of animals vaccinated with a vaccine containing adjuvant ISCOM (G3). The authors analyzed eight commercial BVDV vaccines containing BVDV-1 and BVDV-2 strains. Animals immunized with the vaccine containing ISCOM adjuvant showed 100% seroconversion, with moderate to high titers for BVDV-types 1 and 2. In the present study, we observed that only 60% of animals seroconverted to BVDV-1 Singer. However, the work performed by Anziliero et al. (2015) used beef cattle of mixed breeds, aged 8 to 12 months, belonging to properties located in the central region of Rio Grande do Sul. These methodological differences may explain our divergent results since we use cattle with different aptitudes, breed, and age, as well as climatic variations between the south and southeast regions. Regarding the other seven vaccines researched by Anziliero et al. (2015), the authors detected partial seroconversion to BVDV-1 in animals receiving oily adjuvant, aluminum hydroxide, and Selenium Max adjuvant vaccine. Three other vaccines tested containing aluminum hydroxide as adjuvant did not induce the production of detectable antibodies against BVDV-1 in no vaccinated animals. Regarding BVDV-2, only two commercial vaccines induced partial responses against BVDV-2.

In general, the aluminum hydroxide-based adjuvant-containing vaccine stood out in this research in protecting against BVDV-1 NADL and BVDV-1 Singer, presenting higher antibody titers, seroconversion rate and neutralizing antibody production. Three main mechanisms are described in an attempt to explain the ability of aluminum hydroxide to stimulate the immune system. The first is its ability to adsorb antigenic particles, which retain the antigen at the injection site, increasing the recruitment time of antigen-presenting cells and the uptake of viral particles by the cells dendritic. Secondly, aluminum hydroxide generates cell necrosis at the injection site resulting in the release of molecular patterns, including DNA, uric acid, ATP, IL-1α, and IL-33, which induce APCs recruitment at the site injection (Hogenesch et al. 2018). Finally, aluminum hydroxide converts soluble antigen into particulate form (in association with adjuvant), facilitating its phagocytosis by macrophages and dendritic cells, which will migrate to local drainage lymph nodes, with the induction of a predominant immune response Th2 type (Lambrecht et al. 2009, Ghimire 2015).

Evaluation of response against BVDV-2 VS253 revealed that only G3 heifers showed a response against this viral strain. One hypothesis for this finding is that the vaccine used for G3 contains immunostimulating complexes (ISCOM) as an adjuvant, which is known to be a more potent adjuvant than the others. The ISCOMs form nanoparticles, similar to spherical capsules composed of cholesterol, amphigen (phospholipids and glycolipids) and saponin derivatives (Quil A). At nanoparticles formed by the mixture are approximately 30-40nm in diameter, which allows greater mobility for drainage to the lymph nodes, as well as local stability, thus increasing antigen absorption and presentation to dendritic cells, this set of factors increase the chances of cellular (Th1) and humoral (Th2) immune response induction (Sjölander et al. 1998, Saliba et al. 2017).

BVDV has high antigenic variability with low cross-serological reactivity between BVDV-1 and BVDV-2 species, which represents an obstacle to vaccination protocols (Ridpath et al. 1994). The frequencies of subgenotypes found in Brazilian herds according to Silveira et al. (2017) are approximately 35.9% for BVDV-type 1a, 31.4% BVDV-2b, 10.1% BVDV-1b, 6.7% BVDV-1d, 2.2% BVDV-2c and 1, 1% BVDV-1e. Flores et al. (2000) identified genotypes and subgenera through phylogenetic analysis of 17 BVDV isolates, with 23.5% of genotype 1a (BVDV-1a), 52.9% of genotype 1b (BVDV-1b) and 23.5% genotype 2 (BVDV-2). It is noteworthy that herds that use commercial formulations containing only BVDV-1 are more susceptible to BVDV-2 infections (Basqueira et al. 2017).

Regarding BoHV-1, a higher magnitude was observed for G3 of ($\log_2$=6.1), the mean titers in secondary response were $\log_2$=4.3 in G1 and $\log_2$=2.7 for G2. Probably this phenomenon

by the inclusion of thermosensitive modified live BoHV-1 in the commercial formulation used for vaccination of G3 heifers. Replicative vaccines generate a more potent immune response than those composed of inactivated viruses due to the ability of the vaccine virus to replicate in animal cells (Vartak & Sucheck 2016). Thermosensitive vaccines contain a chemically altered living virus that can replicate only at lower temperatures (30-33ºC) than in the body (37ºC), which precludes the development of systemic infections following vaccination with this type of antigen (Patterson et al. 2012).

Silva et al. (2007a) used calves aged 10 to 14 months to evaluate the immunogenicity of six commercial vaccines containing inactivated BoHV-1 antigens: one Brazilian (BR), one North American (US), two Uruguayan (UR1 and UR2) and two Argentine (ARG1 and ARG2). Only the US vaccine presented 87.5% of the animals with minimum titers (≥16 or 32), (Pospísil et al. 1996). The other vaccines had titers below 16 in 62.5% (5/8, BR), 33.3% (4/9, UR1), 75% (6/8, UR2) and 83.3% (5/6, ARG2) of calves. The ARG1 vaccine presented even lower performance, in which only three animals (37.5%) seroconverted. Considering the minimum protective antibody titer for BoHV-1 (≥16), we found in this research that 90% of G3 heifers had protective titer, while only 44% and 20% of G1 and G2 females were protected. Pospísil et al. (1996) found that vaccinated pregnant cows with neutralizing antibody titers above 1:16 and 1:32 challenged with BoHV-1 (TD strain) did not show respiratory disease and maintained their pregnancies without any abnormality.

Care must be taken when adjusting a vaccination protocol. In this research, the vaccine containing aluminum hydroxide (G1) presented better antibody production against BVDV-1, a result compatible with the higher capacity of the adjuvant aluminum hydroxide modular Th2 type immune response. The inoculum of G3 generated higher titers for BVDV-2 and BoHV-1. Importantly, this research did not evaluate the Th1 immune response mediated by cytotoxic lymphocytes. Theoretically, vaccines containing ISCOM complexes have a more exceptional ability to induce Th1 cellular immune response. Vaccination should induce both immune (humoral and cellular) responses to the protection and defense of cattle against viral agents, so further research is needed to associate the effect of adjuvants on both humoral and cellular responses.

CONCLUSION

Based on the data obtained, it was possible to conclude that the aluminum hydroxide (G1) vaccine was more effective in producing antibodies against BVDV-1, whereas this product did not induce antibodies against BVDV-2. Only G3 heifers (Quil A, amphigen and cholesterol) generated neutralizing Abs against BVDV-2. Animals receiving the oily emulsion (G2) vaccine as adjuvant showed a poor/undetectable response against BVDV-1 and BVDV-2. The best protective response against BoHV-1 was observed in heifers vaccinated with the modified thermosensitive live vaccine.

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