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MICROBIOLOGY

Immunogenicity in sheep of Uruguayan commercial vaccines against bovine alphaherpesvirus 1, 5 and bovine pestiviruses

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ABSTRACT: *The serological responses induced by four commercial inactivated Uruguayan vaccines against bovine alphaherpesviruses (BoHV)-1 and -5 and bovine pestiviruses (BVDV-1, BVDV-2, and HoBiPeV) were evaluated in sheep. Thirty-seven sheep were immunized twice (day 0 and 25) and their serum samples were tested at different intervals (days 0, 25, 40, 60, and 90) post-vaccination (PV). Among the four vaccines tested, only one (G4) could induce the production of moderate neutralizing antibody titers against BoHV-1 and -5 and BVDV-1 and -2. The G3 vaccine showed a neutralizing serological response against the bovine alphaherpesviruses only. The G1 and G2 vaccines produced extremely low levels of antibodies in a few vaccinated animals only (geometric mean titers (GMT) 2.2). Similar levels of immunological responses were induced by the G4 vaccine against BoHV-1 and -5, and titers of neutralizing antibodies induced in approximately 70% of the animals are known to confer protection (GMT > 8). For bovine pestiviruses, the vaccine stimulated response of G4 against BVDV-2 was higher compared to that against BVDV-1, and extremely low for HoBiPeV. The peak of neutralizing antibodies to BoHV-1 and BVDV-1 was observed on days 40 and 60 PV, respectively. Thereafter, a remarkably decrease in neutralizing antibody response was observed at day 90 PV. These results demonstrated that tested commercial Uruguayan vaccines did not induce a serological response of adequate magnitude and duration. Thus, it is important to periodically review formulations and compositions of commercial vaccines against bovine alphaherpesviruses and pestiviruses.*
Key words: *serology, neutralizing antibodies, vaccine response, reproductive disease.*

Imunogenicidade em ovelhas de vacinas comerciais Uruguaias para alfaherpesvírus bovino 1, 5 e pestivírus de bovinos

RESUMO: *A resposta sorológica induzida por quatro vacinas comerciais uruguaias inativadas contra os alfaherpesvírus bovinos (BoHV-1 e -5) e pestivírus de bovinos (BVDV-1, BVDV-2 e HoBiPeV) foi avaliada em ovinos. Os animais foram imunizados duas vezes (dia 0 e dia 25) e o soro testado em diferentes intervalos (dias 0, 25, 40, 60 e 90) após a vacinação (PV). Dentre as quatro vacinas testadas, apenas uma (G4) apresentou títulos de anticorpos neutralizantes moderados para os BoHV-1 e -5, BVDV-1 e 2. A vacina G3 apresentou resposta somente para alfaherpesvírus bovinos. As vacinas G1 e G2 estimularam resposta somente em alguns animais vacinados. Para a vacina G4, observou-se que a resposta imunológica frente ao BoHV-1 e 5 foi semelhante e pelo menos 70% dos animais apresentaram níveis protetivos de anticorpos neutralizantes. Para os pestivírus bovinos, a vacina G4 estimulou resposta para o BVDV-2 mais elevada quando comparada com o BVDV-1, e quase que indetectável para HoBiPeV. O pico de anticorpos neutralizantes para o BoHV-1 foi observado no dia 40 PV e no dia 60 PV para o BVDV-1. Após isso, observou-se um decréscimo considerável na resposta de anticorpos neutralizantes. Os resultados demonstraram que vacinas comerciais uruguaias testadas não induziram resposta sorológica de magnitude e duração adequadas. Assim, ressalva-se a importância de rever periodicamente a formulação e composição das vacinas comerciais para alfaherpesvírus e pestivírus bovinos.*
Palavras-chave: *sorologia, anticorpos neutralizantes, resposta vicinal, doenças reprodutivas.*

INTRODUCTION

Cattle rising has economic importance in the countries of South America, particularly Uruguay, Brazil and Argentina. This region has a large cattle herd, with different purposes, breeds and breeding systems (GUARINO et al., 2008; MAYA et al., 2016). The bovine alphaherpesviruses 1 and 5 (BoHV-1 and -5) and bovine pestiviruses (BVDVs) are associated mainly with productive and reproductive losses (BAKER, 1995; GUARINO et al., 2008). The BoHV-1 and BoHV-5 are two different viral species classified under the family *Herpesviridae*, demonstrating genetic and antigenic similarity, and are characterized by serological cross-reactivity *in vitro* and *in vivo* (BRATANICH et al.,

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1991; DELHON et al., 2003). Bovine pestiviruses (BVDV-1, BVDV-2 and HoBiPeV) belong to the family *Flaviviridae*, which has an RNA genome and is characterized by genetic and antigenic diversity, and variable serological reactivity (BAUERMANN et al., 2013). In the early 2000s, a *HoBi-like* virus was identified as an atypical pestivirus (SCHIRRMEIER et al., 2004). Later, several viruses similar to this were recovered from clinical samples and classified under an emerging group of bovine pestiviruses known as the HoBiPev (BAUERMANN et al., 2013).

Control and prevention programs for these viruses are based on the detection of the infectious agent, elimination of its reservoirs, and adoption of measures that will reduce its further spread in the herd. Vaccination of animals is conducted by producers with the objective of clinical protection, controlling reproductive losses and reducing viral dissemination in the herd (ACKERMANN & ENGELS, 2006; NEWCOMER et al., 2017). The BoHVs and bovine pestiviruses are widespread in Uruguay and cause substantial losses in the production of milk and beef (GUARINO et al., 2008). More than 90% of herds are exposed to BoHV or BVDV and less than 5% of producers use vaccine regularly (GUARINO et al., 2008, MAYA et al., 2016).

The methodology of vaccine production against BoHV-1, BoHV-5 and bovine pestiviruses is not standardized and allows the preparation of immunogens using variable composition of strains and adjuvants (SILVA, et al., 2007a; ANZILIERO et al., 2015). Differences in the immunogenicities of commercial vaccines have already been demonstrated upon their evaluation and, in some cases, also induced inadequate immune responses (ANZILIERO et al., 2015; FULTON & BURGE, 2000; VOGEL et al., 2002a, 2002b). The present study aimed to evaluate the serological response of four Uruguayan commercial vaccines against BoHV-1, BoHV-5, BVDV-1, BVDV-2, and HoBiPeV using sheep as an experimental model.

MATERIALS AND METHODS

Immunization of animals

The humoral immune response of four Uruguayan commercial vaccines (G1, G2, G3 and G4) used for prevention of reproductive diseases in cattle was evaluated. For this purpose, 37 adult (two to four years old) Polwarth female sheep, free of antibodies to bovine alphaherpesviruses 1 and 5 and bovine pestiviruses, were used. The animals were randomly distributed into four groups and kept in natural pastures. All procedures for vaccination and serum collection were carried out as per the recommendations of the Animal Study and Use Committee, Federal University of Pampa (CEUA/UNIPAMPA, registry# 008/2017). Sheep were immunized according to the manufacturer's recommendations for bovids. The timepoint of the first immunization was considered to be day 0 and the booster was administered at day 25 post-vaccination (PV). Their sera were collected on days 0, 25, 40, 60 and 90 PV for the evaluation of serological responses.

Commercial vaccine

All four multivalent vaccines were purchased from local sellers and stored at 4 °C until application. Vaccine compositions, if available, described on the labels are: G1: inactivated suspension BoHV-1 ($\geq 1x10^7$ $TCID_{50}$/mL); BoHV-5 ($\geq 1x10^7$ $TCID_{50}$/mL), BVDV type 1 ($\geq 1x10^5$ $TCID_{50}$/mL), BVDV type 2 ($\geq 1x10^5$ $TCID_{50}$/mL), strains of *Leptospira*, *Campylobacter* and *Haemophilus*, in double oil emulsion adjuvant. G2: inactivated suspension of BoHV-1, BVDV, *Leptospira*, *Campylobacter* and *Histophilus* in double oil emulsion adjuvant. G3: inactivated suspension of BoHV-1 and 5, BVDV 1 and 2, *Leptospira* and *Campylobacter* in aluminum hydroxide adjuvant. G4: inactivated suspension of BoHV-1, BVDV 1 and 2, *Leptospira*, *Campylobacter* and *Histophilus*. The vaccination dose recommended for all four vaccines is 5 mL via subcutaneous route injection and two doses in primo-vaccination.

Virus neutralization

Serum samples were inactivated by being placed in a water bath at 56 °C for 30 min and then subjected to the virus neutralization test, which was carried out in 96-well microplates. Briefly, serum samples collected at each time point were diluted and tested against 100-200 tissue culture infective dose ($TCID_{50}$) of BoHV-1 Cooper (1:2 to 1:256) and BVDV-1 Singer (1:5 to 1:320), as described by ANZILIERO et al. (2015). Thereafter, pestivirus-free MDBK cells (Madin-Darby bovine kidney) maintained in equine serum were added and the microplates were incubated for 96 h. The titer of neutralizing antibodies was considered to be the reciprocal of the highest dilution capable of inhibiting virus replication evidenced by the absence of cytopathic effect. Mean antibody titers were transformed into geometric mean titers (GMT) (THRUSFIELD, 2004). Reactivity of sera from vaccinated sheep with BoHV-5 (SV507/99), BVDV-2 (VS253) and HoBiPeV (SV757/15) was evaluated

using virus neutralization test. With this objective, samples collected on days with the highest levels of antibodies for BoHV-1 and BVDV-1 after vaccinations were selected. The viral strains used in this study were kindly provided by the Virology Sector, Universidade Federal de Santa Maria (SV/UFSM).

RESULTS

The immunogenicity evaluation results of the four commercial vaccines are summarized in Figure 1 and Table 1. Figure 1 demonstrates the curve of the immune response observed for BoHV-1 (Cooper strain) and BVDV-1 (Singer strain). The reactivity for BoHV-5 (strain SV507/99) was evaluated using serum samples collected on day 40 PV and on day 60 PV for BVDV-2 (VS253) and HoBiPeV (SV757/15) (Table 1). Among the four vaccines tested, G4 demonstrated maximum immunogenic activity against BoHV-1 and BVDV-1. The G3 vaccine produced a consistent response against the bovine alphaherpesviruses only. G1 and G2 vaccines stimulated a low, inconsistent and perceptible immune response in a few vaccinated animals only.

On day 25 PV, all animals vaccinated with G4 produced neutralizing antibodies against BoHV-1, the levels of which increased after administration of the second dose. The same response pattern was observed in the case of the G3 vaccine as well, but titer levels were lower. The antibody peak was detected on day 40 PV, after which a level decrease was observed until the last evaluation at day 90 PV. On day 40 PV, G1 and G2 vaccines stimulated responses in only 50% of the animals, but antibodies were reduced to minimal levels in the other two evaluations (Figure 1). Serological evaluation against BoHV-5 demonstrated a reactivity pattern similar to that observed for BoHV-1 (Table 1).

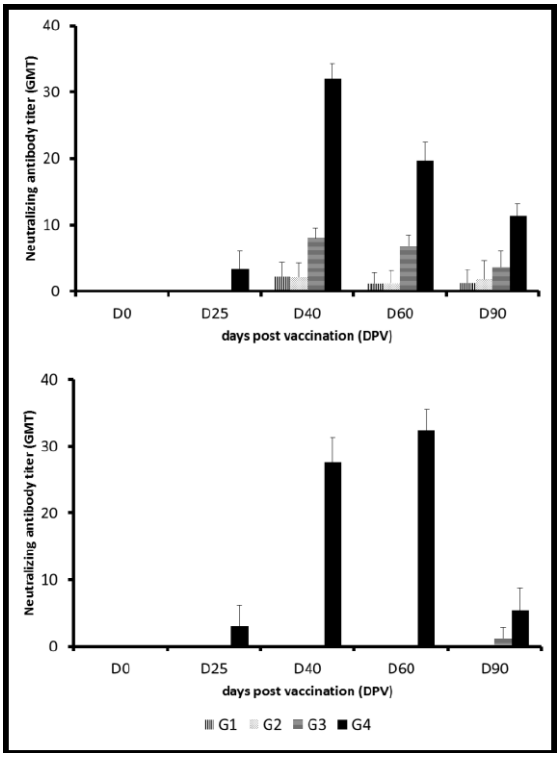

Figure 1 - Evolution of serological response in sheep immunized with four different commercial vaccines against bovine alphaherpesvirus 1 (BoHV-1, Cooper strain) (A) and bovine viral diarrhea virus 1 (BVDV-1, strain Singer) (B) of Uruguayan origin, between days 0 and 90 post-vaccination. The bars represent the GMT and ⊤ indicates standard deviation.

Table 1 – Humoral immune response for bovine alphaherpesviruses (BoHV) 1 and 5, bovine viral diarrhea viruses (BVDV) 1 and 2, and HoBiPev (60 days post vaccination (DPV)) in sheep immunized with four Uruguayan commercial vaccines.

----------Reactivity for anti-BoHV (day 40 PV)----------					
Group	Number	----------BoHV-1----------		----------BoHV-5----------	
		Reagents	GMT [1]	Reagents	GMT
G1	8	4	2.2	1	1.3
G2	10	5	2.1	7	3.7
G3	9	9	8	5	2.7
G4	10	10	32	10	9.2

----------Reactivity for anti-BVDV (day 60 PV)----------							
Group	Number	----------BVDV-1----------		----------BVDV-2----------		----------HoBiPev----------	
		Reagents	GMT	Reagents	GMT	Reagents	GMT
G1	8	0	0	0	0	2	1.4
G2	10	0	0	1	1.2	0	0
G3	9	0	0	0	0	2	1.4
G4	10	10	31.6	10	120.2	2	1.4

[1]GMT - Geometric means titer.

When evaluating the immunogenicity of vaccines against BVDV-1, again, only the G4 vaccine produced a consistently detectable serological response (Figure 1). On day 25 PV, G4 induced the production of neutralizing antibodies in five animals. After the second dose, a secondary response was developed in all animals, which reached the maximum level at day 60 PV, followed by a marked decrease in antibody levels at day 90 PV. The immune responses induced in animals against BVDV-1 by the G1, G2 and G3 vaccines were not consistent and it was possible to detect the presence of neutralizing antibodies for BVDV-2 and HoBiPeV at low titers (5) in five animals only. The sera of animals vaccinated with G4 and collected on day 60 PV showed a higher reactivity against BVDV-2 (Table 1). Only two animals reacted against HoBiPeV upon vaccination, and the titers of antibodies produced by them were the lowest (5).

DISCUSSION

The serological evaluation of animals immunized with four commercial Uruguayan vaccines (G1, G2, G3 and G4) demonstrated a wide variation in immune responses. All collected sera were tested for the presence of neutralizing antibody to BoHV-1 (Cooper strain) and BVDV-1 (Singer strain) (Figure 1). The day PV, at which the highest levels of neutralizing antibodies were observed, was selected to evaluate the occurrence of neutralization against BoHV-5, BVDV-2 and HoBiPeV viruses (Table 1). The G4 vaccine stimulated production of neutralizing antibodies against BoHV-1, BoHV-5 and BVDV-1 and BVDV-2 in 100% of vaccinated animals. The levels of immune responses induced by G4 were superior to those of the other three vaccines. The G3 vaccine induced neutralizing antibodies only against BoHV-1 and BoHV-5. The G1 and G2 vaccines induced weak (GMT ~2) and inconsistent serological responses only against the bovine alphaherpesviruses in few animals (<50%). These two vaccines stimulated the production of neutralizing antibodies only in some animals and their titers (5) were lowest for tested bovine pestiviruses (Table 1). The performance of these vaccines was similar to that observed in other studies as well, in which, higher levels of immune responses against the bovine alphaherpesvirus were detected compared to those of the bovine pestiviruses (ANZILIERO et al., 2015; SILVA et al., 2007a; VOGEL et al., 2002a, 2002b).

Variation in responses observed among vaccines may be associated with the lack of specific parameters outlined for their production, inadequate quality control and/or use of inefficient adjuvants (SILVA et al., 2007b). Inappropriate handling and storage are factors that negatively affect the immunogenicity of vaccines. Although, sheep were used as the experimental model in this study, the exact behavior of the vaccine in cattle would not be reflected here. However, members of this species have been used for experimental purposes and the results

reflected an approximate pattern of response that might occur in cattle, including those of the vaccine protection tests (VOGEL et al., 2001). Another aspect that should be considered is that, after day 60 PV, a marked reduction in neutralizing antibody levels was observed (Figure 1). Thus, the decreases in humoral immune response suggested the need for reduction of revaccination intervals, use of a higher antigen dose or a different adjuvant. Overall, these characteristics indicated that problems exist in vaccine formulations. Antigen concentration and adjuvant can directly impact the immune response of bovines and most probably are the principal problems in vaccine formulation (SILVA et al., 2007b; ANZILIERO et al. 2015).

The serological response against bovine alphaherpesviruses was greater in magnitude in animals vaccinated with G3 and G4 compared to that against bovine pestiviruses. In the other two groups, a low magnitude response was observed in a few animals, and as a result, a more detailed analysis could not be conducted. The neutralizing antibody levels, which are ≥8, for BoHVs are associated with clinical protection (ANZILIERO et al., 2015). The titers of neutralizing antibodies against BoHV-1 induced by the G4 vaccine were between 4 and 16 (GMT 3.4) in 70% of animals after administration of the first dose. After administration of the booster dose, 100% of the animals demonstrated seroconversion, with antibody levels between 8 and 128 (GMT 32) at day 40 PV; subsequently, followed by a reduction up to GMT 11.3 at day 90 PV. In the G3 group, an antibody response was detected in 100% of animals only on days 40 and 60 PV, but with titers ranging from 4 to 16. Previous evaluations of BoHV and BVDV vaccines commercialized in Brazil, Argentina and Uruguay have already demonstrated variations in the resulting immunogenicity (SILVA et al., 2007b; VOGEL et al., 2002b).

The anti-BoHV-5 responses induced by the four vaccines have a similar pattern to that observed for BoHV-1, but with lower GMT. Both BoHV-5 and BoHV-1 are antigenically similar and confer cross-reactivity *in vitro* and *in vivo*, including cross-protection (ANZILIERO et al., 2011; BRATANICH et al., 1991; SPILKI et al., 2004). BoHV-1 and BoHV-5 are found in Uruguay and at least two vaccines (G1 and G3) have both viruses in their compositions. Similar to BoHV-1, the G4 vaccine stimulated the production of higher levels of anti-BoHV-5 antibodies compared to others.

The neutralizing antibody response to bovine pestiviruses is unsatisfactory. Only the response induced by the G4 vaccine was consistently detectable and of a moderate magnitude against BVDV-1 (GMT 31.6) and BVDV-2 (GMT 120.2). The maximal level of neutralizing antibody response was observed at day 60 PV, and was subsequently followed by a marked decline (day 90 PV). This characteristic suggested that a low intensity immunogenic stimulation was induced upon the vaccination (Figure 1). The cross-reactivity was evaluated at day 60 PV and it was demonstrated that the response against BVDV-2 was higher than that against BVDV-1 and HoBiPeV. Reactivity to HoBiPeV was observed in two animals and can be considered as a cross-serology phenomenon since the G4 vaccine is composed only of BVDV-1 and BVDV-2 strains. The G1, G2, and G3 vaccines stimulated neutralizing antibodies in a few vaccinated animals and their titers were found to be minimal. The low immunogenicity of commercial vaccines for BVDV is alarming and this fact has already been demonstrated previously (ANZILIERO et al., 2015; VOGEL et al., 2001; VOGEL et al., 2002a). The cause of low vaccine performance may be associated with the variability of the immunogens, antigen concentration and type of adjuvant (FULTON, 2015).

Neutralizing antibody titers above 80 and 160 are known to be associated with clinical protection against BVDV (FULTON & BURGE, 2000). Neutralizing antibody levels equal to or greater than 80 were observed in 70% of animals vaccinated with G4 between days 40 and 60 PV (Figure 1 and Table 1). These data suggested that only some animals would be protected in the field challenge. Low immunogenicity and antigenic variability of bovine pestiviruses can contribute to vaccine failures (NEWCOMER et al., 2017; VOGEL et al., 2001).

Producers and technicians have a goal of controlling reproductive and productive losses caused by alphaherpesviruses and bovine pestiviruses. The increased demand for vaccines should be accompanied by the introduction of periodic programs to evaluate immunogenicity of vaccine strains, detection of circulating viral variants in herds, and suitability of adjuvants used (FULTON, 2015; NEWCOMER et al., 2017). The use of replicative (live) vaccines is also an option that should be considered, since they stimulate the immune system in a broader and prolonged fashion compared to non-replicative (inactivated) vaccines. However, the use of replicative vaccines requires more care, since they may produce immunosuppression or gestational losses (FULTON & BURGE, 2000).

CONCLUSION

Based on the results, it can be concluded that the tested commercial Uruguayan vaccines produced a variable serological response. Only one vaccine (G4) could induce the production of neutralizing antibodies to BoHV-1, BoHV-5, BVDV-1, and BVDV-2 with moderate titer values. The G3 vaccine stimulated responses against the alphaherpesvirus only. The G1 and G2 vaccines stimulated the production of neutralizing antibodies at low or undetectable levels. As expected, none of the four vaccines stimulated a response against HoBiPeV, which is considered as an emerging pestivirus in South America. The neutralizing immune response produced in vaccinated animals was short-term. Therefore, none of the tested vaccines can be employed for in herd control programs. Furthermore, there is an urgent need to re-evaluate the formulation and composition of commercial vaccines, as well as to test the immunogenicity periodically and continuously.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

ETHICS COMMITTEE

All procedures for vaccination and serum collection were conducted as per the recommendations of the Animal Study and Use Committee, (registry # 008/2017).

AUTHORS' CONTRIBUTIONS

IM, CKT and MCSB conceived and designed experiments. IM, AAA, JMV, NVS and IJR performed the experiments, IM and AAA carried out the lab analyses. IM and JMV supervised and coordinated the animal experiments and provided clinical data. IM performed statistical analyses of experimental data. IM, CKT and MCSB prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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