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Simultaneous immunization of cattle with foot-and-mouth disease (FMD) and live anthrax vaccines do not interfere with FMD booster responses

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ABSTRACT

Foot-and-mouth disease (FMD) vaccination in Argentina is compulsory for most of the cattle population and conducted by certified veterinarians. This organized campaign may facilitate the controlled application of other vaccines against endemic diseases, provided immune responses against FMD are not hindered. There is no published information on the interference of immunity against FMD vaccines when applied together with a live bacterial vaccine. In this study we evaluated if the simultaneous application of a *Bacillus anthracis* live vaccine with a commercial tetravalent oil-based FMD vaccine (FMD-vac) used in Argentina, modifies the antibody booster responses against FMD virus (FMDV) in cattle. Two groups of 16 heifers with comparable liquid phase blocking ELISA (LPBE) titers were immunized with the FMD-vac alone or simultaneously with a commercial attenuated bovine anthrax Sterne strain vaccine (ABV). Serum samples were obtained at 0, 25, 60 and 90 days post vaccination (dpv) and specific antibodies against two FMDV vaccine strains were assessed by LPBE, avidity and IgG-isotype ELISAs. Bovines immunized with FMD-vac or FMDV-V + ABV responded with a boost in the LPBE antibody titers and avidity at 25 dpv, and remained within similar levels up to the end of the study. Animals vaccinated with FMD-vac + ABV had significantly higher LPBE titers at 25 dpv, compared to those immunized with FMD-vac alone; which was due to an increase in IgG2 titers. Overall, antibody titers elicited in both groups were similar and followed comparable kinetics over time. We conclude that the simultaneous application of a live anthrax vaccine with the current FMD tetravalent vaccine used in Argentina in cattle previously immunized against FMD, did not counteract the serological response induced by FMD vaccination.

Introduction

Foot and mouth disease (FMD) is a highly contagious acute vesicular viral disease that affects cloven-hoofed animals. FMD virus (FMDV) belongs to the genus *Aphthovirus* in the *Picornaviridae* family, and includes seven serotypes: O, A, Asia, C, and SAT-1, -2, and -3 [1]. The circulation of FMDV in susceptible livestock imposes severe restrictions on the movement and trade of animals and derived products, causing serious economic loss to the affected countries [2]. FMD is endemic in many parts of Asia, Africa, and South America, where vaccination of susceptible populations is widely used as a major control measure. Commercial formulations usually contain more than one virus strain, as immune responses induced by vaccination are strain-specific [3,4].

The vaccine currently used in Argentina is oil-adjuvanted and contains four FMDV strains of latest regional circulation: O1/Campos/Brazil/58 (O1/Campos), A24 Cruzeiro/Brazil/55 (A24/Cruzeiro), A/Argentina/2001 (A/Arg/01) and C3/Indaial/Brazil/71 (C3/Indaial) [5,6]. FMD vaccination is compulsory and rigorously controlled by the local sanitary authority (SENASA). SENASA provides the virus vaccine strains and vaccination is performed by trained and certified personnel [7], animals are properly identified and cold chain is verified and guaranteed. Vaccines are applied to

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the whole cattle population above the 42° South parallel on fixed schedules. Animals older than 2 years are immunized once a year, while calves aged up to 2 years-old are vaccinated every 6 months. Vaccine efficacy as well as surveillance of vaccine immunity is performed by serology. Strain-specific antibody titers obtained with liquid phase blocking ELISA (LPBE) have been statistically correlated to *in vivo* protection to assess vaccine potency and herd immunity through the estimation of a percentage of expected protection (EPP) [8–12].

The controlled and correct application of vaccines is as important as quality control assessments performed to the vaccine itself. Vaccines may fail in inducing protection if cold chain is not preserved or if the vaccine is not properly applied. These side-issues have major impact when working with livestock. However, gathering all the animals, vaccinators and monitoring cold-chain is difficult to achieve, particularly in large regions, areas of difficult access or extensive production systems. In this scenario, the combination or co-administration of vaccines together with the FMD vaccine appears as a practical and efficient option for immunizing livestock, as long as this practice does not interfere with the immunogenicity conferred by those vaccines applied.

One major pathogen affecting livestock, which also has zoonotic impact, is *Bacillus anthracis*. *B. anthracis* is a Gram-positive bacillus that forms spores that are highly resilient, surviving extremes of temperature, low-nutrient environments, and harsh chemical treatment. This bacterium is the etiologic agent of anthrax, an endemic disease in many countries of Southern Europe, South America, Asia and Africa [13]. In Argentina, livestock is concentrated in seven provinces in the center of the country, with 42 million cows and nearly 2 million rural inhabitants which implies a high risk for anthrax transmission [14]. A surveillance performed in Buenos Aires Province, which represents 32% of farming land and 28% of the livestock stock, revealed that 49% of the farms have had at least one outbreak of bovine *B. anthracis* between 1977 and 2013 [15,16].

The anthrax vaccine currently used for adult bovines in Argentina is based on live spores prepared from the attenuated, capsule-deficient *B. anthracis* Sterne strain (Weybridge no. 34F2). The protective effect of a single dose in adult animals is assumed to last for about 1 year [17], and therefore annual booster vaccinations are recommended for livestock. This schedule can be perfectly coupled with the FMD vaccination program, provided that FMDV titers are not reduced due to the simultaneous vaccination, as this could lead to increase the risk of outbreaks in FMDV-free regions.

There is little information in the literature regarding FMD vaccination efficacy when applied together with other vaccines. In fact, only two publications have addressed the simultaneous application of FMD vaccine (FMD-vac) with other veterinary vaccines in bovines. One study showed that immunization of young calves immunized (by subcutaneous route) against FMD and against infectious bovine rhinotracheitis/adenovirus/parainfluenza-3 (by intranasal route) did not interfere with the serological response against the FMD virus strains included in the vaccine [18]. Another study, however, showed interference between FMD-vac and a vesicular stomatitis virus live vaccine [19]. Altogether, available data indicate that the simultaneous application of FMD vaccines, particularly with live vaccines, needs to be evaluated.

In a first attempt to address the possible interference of *B. anthracis* live vaccine (ABV) with FMD-vac we studied the serological response of cattle that received one dose of FMD-vac or FMD-vac simultaneously with ABV. Due to the fact that all adult animals in the region have vaccine-induced anti-FMDV antibodies, and ABV is only applied in adult animals, we evaluated the serological response to a booster FMD-vac dose (4th dose) applied alone or together with the anthrax vaccine. Our data indicate that the simultaneous application of these vaccines do not modify the serological response profiles to FMD booster vaccination. Moreover, higher titers against FMDV were obtained at 25 days post-vaccination (dpv) when both ABV and FMD-vac were applied, mainly due to an increase in IgG2 antibody titers.

Materials and methods

Animals

Heifers used in this study were from the same farm and had four previous FMD vaccinations, corresponding to FMD campaigns of November 2011, March 2012, November 2012 and March 2013. Thirty-two animals were selected from a herd of 120 heifers according to the levels of antibodies against FMDV (O1/Campos strain) measured by ELISA (LPBE, see below) a week before vaccination. Animal handling, vaccination and serum sampling procedures were previously approved by INTA's Animal Welfare Commission (protocol approval No. 025/2011).

Vaccines

Commercial vaccines were used in this study. The FMD vaccine, referred here as "FMD-vac", was an oil-adjuvanted (water-in-oil) vaccine containing inactivated FMDV from four strains: O1/Campos, A24/Cruzeiro, A/Arg/01 and C3/Indaial and produced by a local manufacturer. This vaccine was approved by SENASA according to the current national regulations [6,20].

The bovine anthrax vaccine "PROVIDEAN CARBUNCLO®" (Tecnovax SA, Buenos Aires, Argentina), here called "ABV", contains non-encapsulated, non-virulent spores of *B. anthracis* $F_2$34 Sterne strain with an antigen payload of 1.8×10^7 spores per dose.

Experimental design

The day of vaccination, 32 animals that had received three previous vaccinations and showing LPBE titers against FMDV O1/Campos ranging from 3.37 to 3.96, were selected from a herd of 120 animals, and randomly distributed in two groups of 16 animals. One group received 2 mL of FMD-vac applied subcutaneously in the left side of the neck (Group FMD-vac). The other remaining 16 animals received the same vaccine and also 2 mL of ABV (also subcutaneously) in the right side of the neck (Group FMD-vac + ABV). Serum samples (2 aliquots of 2 mL each per animal) obtained at 0, 25, 55 and 90 dpv were stored at −20 °C for further serological assessments.

Liquid phase blocking ELISA (LPBE)

Total anti-FMDV O1/Campos and anti-FMDV A24/Cruzeiro antibody responses were assessed in serum samples by LPBE performed as stated by the OIE Manual using a rabbit antiserum to capture inactivated whole 140S viral particles, and a guinea-pig antiserum as detector antibody, both of them strain-specific as described before [21]. Antibody titers were expressed as the reciprocal Log10 of serum dilutions giving the 50% of the absorbance recorded in the virus control wells without serum.

Single dilution avidity ELISA

Avidity assessment of specific antibodies was performed as described before [22]. The Avidity Index (AI) was calculated as the percentage of residual activity of the serum sample after a 20 min urea washing step, relative to that of untreated sample: AI% = (OD sample with urea/OD sample without urea) × 100.

Isotype ELISAs

Isotype ELISAs were performed as reported before [21,22] using HRP-conjugated antibodies anti IgG1 (1:750), IgG2 (1:750) and IgM (1:500) (AbD Serotec, Oxford, UK). Serum samples were run in twofold serial dilutions starting at 1:50. Titers were expressed as Log10 of the dilution factor reaching the cut-off value OD = 0.2 [22].

Data analysis

The "expected protection percentage" (EPP) was used as a reference to protective vaccine-induced responses. The EPP relates antibody titers measured by LPBE at 60 dpv, with the percentages of protection achieved for the same groups of animals after *in vivo* challenge experiments performed at 90 dpv following the "protection against generalized foot infection" (PGP) test. LPBE titers corresponding to EPP values = 75% (EPP-75%) are 2.11 for the O1/Campos strain [23,24] and 1.90 for A24/Cruzeiro strain [24].

Time-course titers obtained by LPBE, AI and IgG-subtype ELISAs were plotted and results between the two experimental groups were compared by ANOVA 2-factor repeated measures followed by Bonferroni multiple comparisons test. Mann–Whitney test was used when data from two groups were compared. The confidence interval was 95%. Statistical analyses were carried out using GraphPad Prism v5.0 (GraphPad Software).

Results

Total antibodies against FMDV

Thirty-two calves with LPBE titers (against FMDV O1/Campos strain) ranging from 3.37 to 3.96 were randomly grouped to receive either FMD-vac alone (mean LPBE titer at 0 dpv = 3.69; range 3.37–3.94) or both FMD-vac and ABV (mean LPBE titer at 0 dpv = 3.68; range 3.39–3.96). Serum samples were obtained the day of vaccination and at 25, 55 and 90 dpv and analyzed by LPBE and avidity ELISA against two of the strains included in the vaccine, O1/Campos and A24/Cruzeiro.

LPBE kinetics curves were similar between both groups, for both tested strains (Fig. 1). Total antibody titers increased after vaccination in all the animals, and were maintained within high levels [23] ($\geqslant$3.7) up to the end of the experiment. At 25 dpv, antibody titers against O1/Campos where significantly higher from those observed at 0 dpv for the group immunized with FMD-vac + ABV, compared to FMD-vac (Fig. 1). The increase in antibody titers at 25 dpv for animals immunized with FMD-vac alone compared to FMD-vac + ABV was significant for O1/Campos ($p = 0.04$) but marginally significant for A24/Cruzeiro ($p = 0.052$).

Avidity indexes kinetic curves were comparable for both groups (Fig. 2). Interestingly, multi-vaccinated animals had lower level of avidity against O1/Campos than against A24/Cruzeiro at 0 dpv. Antibody avidity was boosted after vaccination, though differences were not significant. High AI levels were maintained thereafter until the end of the experiment for both tested strains.

Isotype responses

We hypothesized that the higher titers observed for O1/Campos strain at 25 dpv might be due to an increase in a particular isotype titers, probably biased by the cytokine environment induced by the live bacteria. To test this hypothesis, IgG1 and IgG2 titers against O1/Campos were determined in serum samples from both experimental groups obtained at 0, 25 and 90 dpv (Fig. 3). An increase in IgG1 and IgG2 anti-FMDV serum titers were observed for all vaccinated animals against both virus strains. IgG1 titers and kinetics were similar in both groups (Fig. 3A). However, IgG2 titers were higher for those bovines immunized with FMD-vac + ABV compared to FMD-vac alone at 25 dpv (Fig. 3C and D). At longer time-points, however, no significant differences were detected.

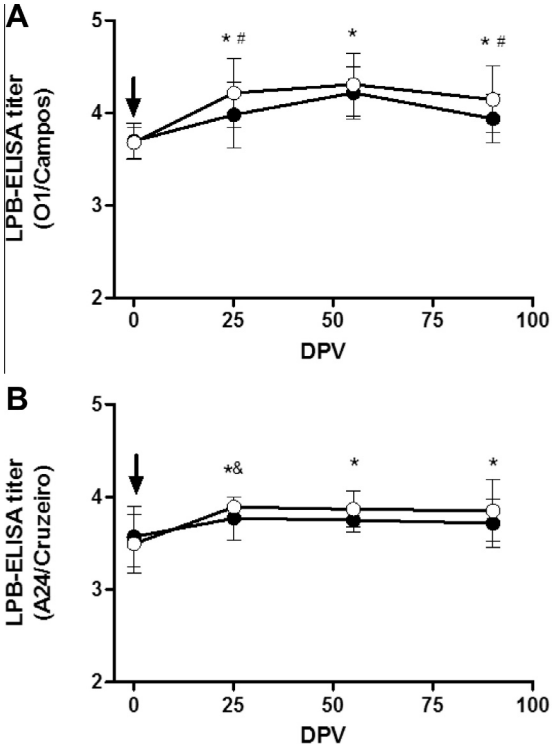

Fig. 1. Kinetics of total antibody titers against O1/Campos (A) and A24/Cruzeiro (B) measured by LPBE. Group FMD-vac ($n = 16$; black circles) correspond to bovines that received one dose of a commercial tetravalent FMD vaccine. Animals from group FMD-vac + ABV ($n = 16$; white circles) were simultaneously immunized with FMD-vac and a commercial bovine anthrax vaccine. Mean titers standard errors (SEM) are depicted. Vaccination is indicated with an arrow. A titer of 2.11 is considered to be related to an EEP $\geqslant$ 75% for O1/Campos strain. [#]Titers against O1/Campos were significantly higher in FMD-vac + ABV compared to FMD-vac at 25 dpv. [&]Titers against A24/Cruzeiro were significantly higher in FMD-vac + ABV compared to FMD-vac at 25 dpv. [*]Titers are significantly higher than those measured at 0 dpv.

Discussion

FMD has global consequences, costing an estimated USD $6–$21 billion each year in prevention expenditures and agricultural damage [2]. A significant portion of this cost is shouldered by the world's poorest countries, which experience major economic losses from trade restrictions. Argentina is free from the disease, but situated in a territory under a constant threat of incursions of the virus. Local sanitary authorities carry on an active compulsory vaccination program in large regions of the country, which assures the maintenance of the FMD-free status granted by the World Organization for Animal Health [25].

In this scenario, other disease control programs may also benefit from the complex logistics deployed for the FMD vaccination campaigns, by the simultaneous application of their corresponding vaccines. Argentina conducts such combined vaccination programs for Brucellosis in 3–8 months-old calves [26] and recently a pilot plan has been launched in certain sanitary districts of the country for simultaneous vaccination against FMD and anthrax.

There is little information, however, on how the co-application of FMD-vac and other vaccines may interfere with the immune

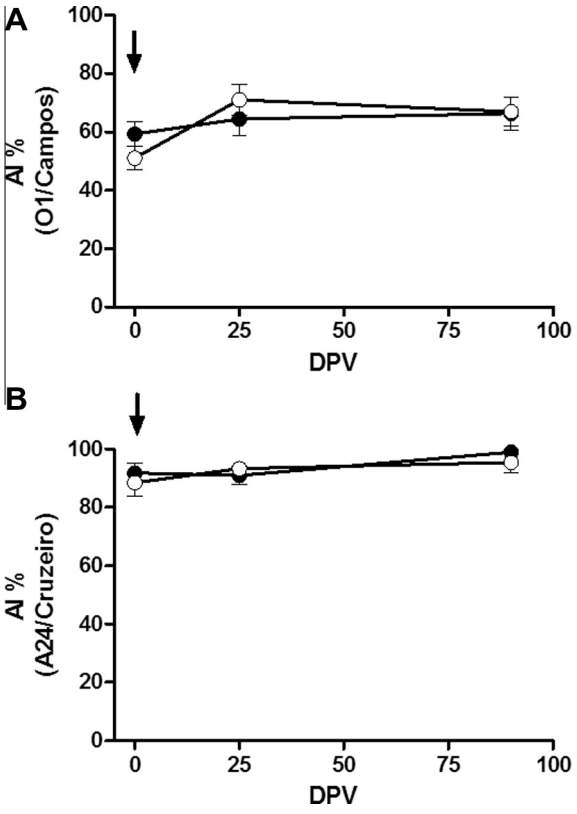

Fig. 2. Kinetics of the avidity index of antibodies against O1/Campos (A) and A/24 Cruzeiro (B) measured by ELISA in serum from animals immunized with one dose of a commercial tetravalent FMD vaccine applied alone (FMD-vac, black circles) or simultaneously with bovine anthrax vaccine (FMD-vac + ABV, white circles). Mean values ± SEM were shown. Vaccination is indicated with an arrow. Values AI ⩾ 24.5% are considered protective for an A strain.

response against FMDV. Here we evaluated if the simultaneous application of a tetravalent FMD vaccine and a bacterial live vaccine modify booster humoral responses against two of the strains included in the FMD vaccine. The study was designed to evaluate booster responses to match the practical approach applied in the field, as adult animals that should be immunized with the anthrax vaccine would have already undergone at least three FMD official vaccination campaigns.

FMD immune responses to vaccination are evaluated by SENASA using LPBE, and there are published curves relating LPBE titers with protection. An EPP of 75% has been estimated to correspond to LPBE titers equal to 1.90 and 2.11 for A24/Cruzeiro and O1/Campos strains, respectively [23,24]. The animals included in this study presented antibody titers corresponding to EPP values above 99% for both strains, at all-time points and regardless the vaccination they received. The kinetics curves obtained with a booster FMD vaccination are similar to those measured before, using also FMD commercial vaccines [22]. These results indicate that the simultaneous vaccination with FMD-vac and ABV did not modify the levels of total circulating anti-FMDV antibodies induced by FMD-vac, for the two tested strains.

Avidity of specific antibodies has also been associated to protection. Although explored as an indirect assessment of cross-protection, avidity of antibodies is related to their ability to neutralize the virus and probably to favor its clearance *in vivo*. At the beginning of the experiment, avidity levels were over the 24.5% AI threshold established before for A strains [22,27]. Avidity indexes were lower at 0 dpv for O1/Campos strain, compared to A24/Cruzeiro. This might probably be related to the higher structural stability of A24/Cruzeiro 140S particles compared to O1/Campos, which may impact on the induced immune responses [28]. However, as no AI curves have been validated yet for O1/Campos strain, we cannot rule out the possibility of a technical artifact in this assessment. Overall, antibody avidity was equally boosted after applying FMD-vac or FMD-vac + ABV, producing

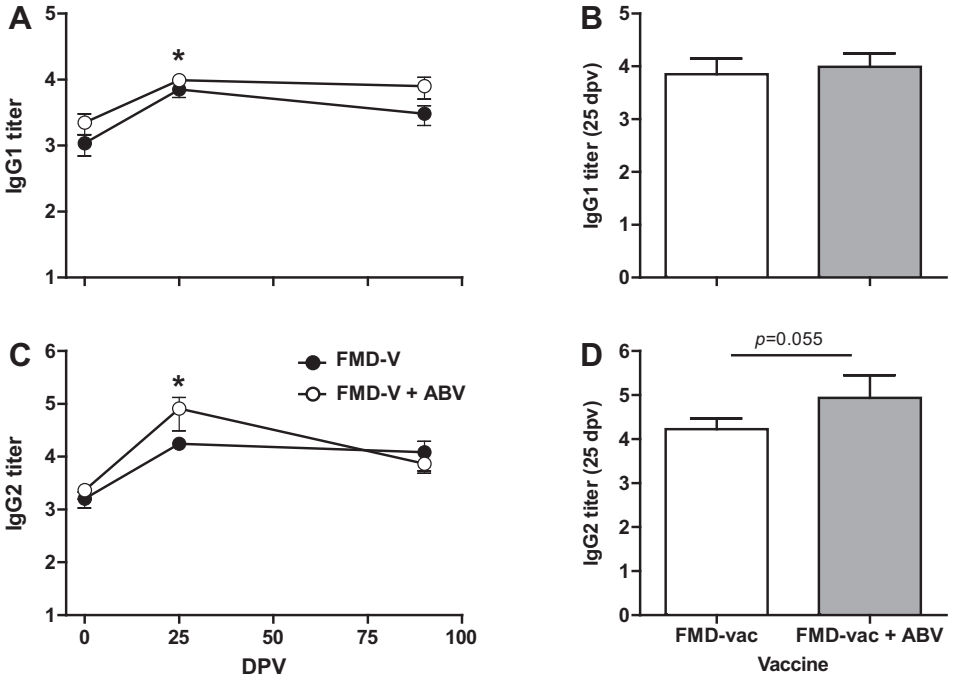

Fig. 3. Kinetics of anti FMDV IgG 1 (A) and IgG2 (C) serum titers (Mean ± SEM) against O1/Campos strain in FMD-vac (black circles) and FMD-vac + ABV (white circles) vaccinated animals. *Differences were significant from 0 dpv in both groups ($p < 0.01$; Mann Whitney test). Bars depict IgG1 (B) and IgG2 (D) mean serum titers (±SD) measured at 25 dpv comparing the two groups of vaccinated animals. Significant differences between FMD-vac and FMD-vac + ABV were determined by Mann Whitney test (p value <0.05 is shown).

similar kinetics curve, also comparable to AI% responses measured before for booster vaccination. This is another argument to support the lack of interference of the simultaneous vaccination with FMD-vac and ABV.

We observed a significant increase in total antibody levels at 25 dpv, for the O1/Campos strain, when the vaccines were co-administered compared to the FMD-vac applied alone. We also showed that this increase may be explained by the presence of higher IgG2 titers. There is no information in the literature about the cytokine milieu provoked by anthrax Sterne-strain vaccine in cattle, which may explain this isotype-profiling. However, there are reports supporting the induction of pro-inflammatory cytokines during murine anthrax [29] and by anthrax lethal toxin in human endothelium [30], meaning that this bacterium can promote a Th1-biased pro-inflammatory environment. Is it possible that the cytokine environment generated by the anthrax vaccine promoted an expansion of Th1 clones which in term helped to induce the proliferation of IgG2-producing B cells.

Altogether, the data provided in this study demonstrate that the Sterne-strain live anthrax vaccine can be administered simultaneously with an oil-based tetravalent FMD vaccine in adult cattle, producing no impact in the humoral responses against FMDV booster responses. It will be also interesting to evaluate the impact of primary simultaneous immunization of both vaccines and the immune responses against anthrax. This is the first study on the FMD responses elicited in cattle simultaneously immunized with FMD and anthrax vaccines. The information provided here can be particularly useful for endemic regions with compulsory FMD vaccine programs.

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