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Avidity and subtyping of specific antibodies applied to the indirect assessment of heterologous protection against Foot-and-Mouth Disease Virus in cattle

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ABSTRACT

Serological assessment of the heterologous response among Foot-and-Mouth Disease Virus (FMDV) strains is mainly performed by virus neutralization test (VNT), liquid phase blocking ELISA and complement fixation assay. In this study two high-throughput ELISA techniques, avidity and IgG subtype ELISA, were developed and used to further characterize heterologous antibody responses in cattle during vaccination and challenge. Both assays were applied to a set of previously characterized sera from animals immunized with an inactivated A24 Cruzeiro/Brazil/55 (A24 Cruzeiro) strain monovalent FMDV vaccine and challenged with the heterologous A/Argentina/2001 (A/Arg/01) strain. Single dilution avidity ELISA assessment showed that animals that were protected against A/Arg/01 challenge had higher avidity antibodies to this heterologous strain than non-protected cattle. Animals with low or even undetectable anti-A/Arg/01 serum-neutralizing titers that passed the heterologous challenge presented higher IgG1/IgG2 ratio than non-protected animals. In this study, the three assessments (VNT and both ELISAs) discriminated between protected and not protected animals against a heterologous challenge. The combination of these techniques may be applied to complement current indirect serological vaccine-matching assessments. The measurement of these qualitative parameters may provide additional information to understand the mechanisms underlying FMD heterologous responses and the induction of cross-protection in cattle.

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1. Introduction

Foot-and-Mouth Disease Virus (FMDV) exists as seven different serotypes, with multiple variants within each serotype. New variants are arising continuously worldwide. Infection or vaccination with one strain, or even with other strains within the same serotype, does not necessarily protect against the others [1–4]. Serological tests are routinely used as part of the process for selecting the most appropriate vaccine strain to use against a given field isolate [5–8]. However, these methods that have been validated for homologous protection are not always accurate to estimate cross-protection [4,7], supporting the development of new techniques to measure different parameters of cross-reactive antibodies. Qualitative aspects of the antibody response, such as avidity or isotype profiles, may provide additional useful information.

Avidity of the specific antibodies has been explored in the serodiagnosis of several viral infections [9–12]. IgG avidity has been applied to differentiate current from past infections with Epstein–Barr virus [13,14], cytomegalovirus [15], and West Nile virus [16] and also to discriminate between acute and chronic infection caused by parasites, such as *Toxoplasma gondii* [17] and *Neospora caninum* [18,19]. For some viruses (*i.e.* bovine viral diarrhea virus and vesicular stomatitis virus) avidity measurement has been correlated with the virus neutralization test [20,21]. There are no studies, however, about the relationship between avidity of specific antibodies and cross-protection against a viral infection. Likewise, the avidity of antibody responses against FMDV and its relationship with protection has not been investigated, although the idea of its relevance in complementing quantitative assessments has been already proposed in early reports [22–25].

Regarding FMDV infection and vaccination, isotype profiles have been explored in a few publications as a source of information on the immune status of cattle [26,27]. The induction of high levels of serum FMDV-specific IgG1 subtype has been related to protection in vaccinated cattle, even at low levels of total IgG [27]. These studies have addressed the IgG-isotype response to FMDV infection and homologous vaccination, but never in cross-protection.

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We hypothesized that the avidity and isotype of the antibodies induced by vaccination with a strain may provide information on the protective potential of the vaccine against another strain. In this study, we used two non cross-protective FMDV strains [28]. We examined the avidity and immunoglobulin subtypes of antibody responses in a set of previously characterized sera from animals immunized with an inactivated A24 Cruzeiro/Brazil/55 (A24 Cruzeiro) strain monovalent FMDV vaccine and challenged with the heterologous A/Argentina/2001 (A/Arg/01) strain [7].

2. Materials and methods

2.1. Bovine serum samples

Serum samples from A24 Cruzeiro vaccinated animals (one single vaccine batch, 10 μg per dose) challenged with the heterologous A/Arg/01 FMDV strain correspond to a published study [7,29] and were kindly provided by the Argentine Animal Health Service (SENASA). We evaluated samples from four independent "Protection to Podal Generalization" (PPG) trials, challenged with A/Arg/01. Trials were conducted in Argentina according to the protocol established by SENASA in Act no. 351/2006 [7,30]. Sera used in the present study were collected at 30 days post vaccination (dpv). Sixty-four samples were assayed, 17 from protected and 47 from not-protected bovines. Protection levels against A/Arg/01 achieved in each of the four trials were 56.3, 25.0, 12.5 and 12.5% [7]. Published PPG data showed that cattle vaccinated with the A24 Cruzeiro vaccine were not protected against challenge with A/Arg/01 strain [7,29].

Three pooled-control samples were prepared to develop the avidity ELISA. The high positive control (HPC) contained sera from 5 cattle that had been immunized at least four times with a commercial tetravalent vaccine formulated with A24 Cruzeiro, A/Arg/01, O1/Campos/Brazil/58 (O1 Campos) and C3/Indaial/Brazil/71 (C3 Indaial) strains, with $\log_{10}$ titers higher than 3 by liquid phase blocking ELISA (LPB-ELISA) [31] against all the vaccine strains. The low positive control (LPC), consisted of sera from four single-vaccinated cattle (commercial vaccine) bled 21 dpv (mean log LPB-ELISA titers between 1.6 and 1.7, against all the vaccine strains) and the negative control (NC) contained four LPB-ELISA negative pooled sera from 10 naïve bovines from Patagonia (FMDV-free zone without vaccination).

In order to develop the avidity ELISA, we used a panel of 25 positive serum samples with various vaccination histories and 25 negative samples from Patagonia. To study avidity maturation, four bovines were immunized with a commercial tetravalent vaccine containing A24 Cruzeiro, A/Arg/01, C3 Indaial and O1 Campos strains. Blood samples were obtained prior to vaccination (day −1) and at several intervals (3, 7, 10, 14 and 21 dpv) between the first (0 dpv) and second dose (35 dpv), and followed up for 63 days, with samples also taken at 43 and 50 dpv. Antibody levels against the four vaccine strains were determined by LPB-ELISA [31].

2.2. Neutralization assay and indirect ELISA

Virus neutralization test (VNT) and LPB-ELISA assessments to measure specific antibodies against A/Arg/01 and A 24 Cruzeiro in the samples from challenged animals (n = 64) were performed before [7]. LPB-ELISA against the four vaccine strains was also conducted for the serum samples used to develop the avidity ELISA assay following the OIE standard protocol [32].

IgG subtype ELISA was performed as reported by Capozzo et al. [27] except that sheep-anti-bovine IgG1 and IgG2 HRP-conjugated

antibodies were used (1:1500; AbD Serotec, Raleigh, NC). Serum samples were run in two-fold serial dilutions starting at 1:20. Titers were expressed as the inverse dilution reaching the cut off value (0.2) calculated as mean OD + 2SD achieved by the FMDV-negative Patagonian bovine serum samples (n = 25). Results were expressed as the ratio between IgG1 and IgG2 titers. Total antibody ELISA was performed equally and revealed with anti-bovine conjugate (Jackson Laboratories, Bar Harbor, ME).

2.3. Single dilution avidity ELISA (sd A-ELISA)

Ninety-six well flat bottom well plates (MICROLON®, Greiner Bio-One, Monroe, NC) were coated with 50 μl per well of a dilution that contained 15 ng/well of sucrose-gradient purified FMDV 146S particles [33] of one of the following serotypes: A/Arg/01, A24 Cruzeiro, O1 Campos or C3 Indaial in 50 mM carbonate/bicarbonate buffer pH 9.6, and incubated overnight at 4 °C. After five washes with PBS-T (PBS + Tween 0.05%), plates were blocked with dilution buffer [34] and after another PBS-T washing step, 50 μl of each serum sample was added in 8 two-fold serial dilutions starting at 1:10. The procedure was further optimized to perform a single dilution of the sample (1:50), which allowed to process 44 samples per plate (plus 3 controls and a blank) as samples were run in parallel with and without performing the urea washing step. Samples were incubated for 1 h at 37 °C. Plates were washed twice with PBS-T (300 μl/well), and subsequently washed with PBS–7 M urea (Promega, USA) for 15 min at room temperature and followed by two regular-PBS washing steps. FMDV-specific antibodies were detected with HRP-labeled anti-bovine conjugate (Jackson Laboratories) diluted 1:5000 and incubated for 1 h at 37 °C. The colorimetric reaction was revealed with chromogen/substrate mixture ABTS/H_2O_2 [ABTS: 2.2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] at room temperature, protected from light exposure. Color development was stopped after 20 min by the addition of 50 μl of 2% Sodium Floride. Absorbance was read at 405 nm (Multiskan® EX, Thermo Scientific, USA). Mean OD values of samples and controls were corrected subtracting mean blank OD values (cOD). The avidity index (AI) was calculated as the percentage of residual activity of the sera relative to the OD of the untreated (not washed with urea) sample: AI% = (cOD sample with urea/cOD sample without urea) × 100. The cut off value was fixed in OD = 0.1 (mean OD value of 25 negative samples + 2SD). Only untreated samples (PBS wash) with an OD value over 0.5 (corresponding to LPB-ELISA titer over 1.4) were considered to calculate the AI.

2.4. Data analysis

Statistical analyses were carried out using biostatistics, curve fitting and scientific graphing software (Statistix v8. Analytical Software, USA; GraphPad Prism v4.02, GraphPad Software, USA; MedCalc v11 Software, MedCalc, Belgium). Significance was established within the 95% confidence interval for all determinations.

Comparison between mean values of two groups (i.e. mean serology results against both viruses) was assessed by unpaired non-parametric t Student–Fisher test. Positive and negative control samples were assayed (with and without urea treatment) in 6 independent assays performed in quadruplicate. The OD values of 50 panel-samples in sd A-ELISA performed by the same and different operator/s were compared. Intra-assay, inter-assay and replicates' variations were calculated by ANOVA for a nested model of variance, applied to quantify the interaction between repeatability and intermediate precision. The variation coefficient was computed as the standard deviation/mean ratio (in percentage). Intra plate variations were calculated for each plate and averaged.

Table 1
Intermediate precision of the sdA- ELISA.

Treatment	Operator 1		Operator 2		Operator 3	
	No urea	Urea	No urea	Urea	No urea	Urea
1	0.710	0.140	0.752	0.134	0.738	0.147
2	0.784	0.151	0.747	0.129	0.728	0.130
3	0.756	0.129	0.794	0.109	0.768	0.136
4	0.742	0.129	0.718	0.145	0.721	0.134
5	0.767	0.123	0.733	0.135	0.769	0.134
6	0.765	0.115	0.711	0.130	0.732	0.117
Minimum	0.710	0.115	0.711	0.109	0.721	0.117
Maximum	0.784	0.151	0.794	0.358	0.769	0.147
Mean	0.754	0.131	0.742	0.324	0.743	0.133
Std. deviation	0.025	0.013	0.029	0.025	0.021	0.009
Std. error	0.010	0.005	0.012	0.010	0.008	0.003
Coefficient of variation	3.39%	9.70%	4.02%	7.85%	2.80%	7.31%

OD values (405 nm) obtained by 3 different operators assessing the low positive ELISA control sample in a 1:50 dilution with or without urea treatment as indicated, in 6 independent assays.

3. Results

3.1. Performance of the sd A-ELISA

Repeatability and intermediate precision of the assay were assessed using the low positive control, run by three different operators in six independent assays, using different batches of reagents. Results are shown in Table 1. Coefficients of variation for positive control samples were below 10% for all the operators. No significant differences were found between mean OD values determined by the three operators ($p > 0.05$). ANOVA analysis showed there were no differences in the OD values due to the operator, the urea treatment or their interaction ($p = 0.45$, 0.54 and 0.84 respectively).

The progression of the avidity maturation along one and two immunizations with a tetravalent vaccine was assessed as an indicator of the performance of the sd A-ELISA. After vaccination, following repeated exposures to the same antigen, a host will produce antibodies of successively greater affinities, which should be reflected by an increase in the antibody avidity index.

We evaluated total FMDV strain-specific antibodies and their avidity in sera from four calves vaccinated with a tetravalent vaccine containing A/Arg/01, A24 Cruzeiro, C3 Indaial and O1 Campos strains. Immunization induced a raise of total antibodies shortly after vaccination (3–4 dpv) that stayed at the same levels from 15 to 35 dpv for all four strains (Fig. 1). Total specific antibody titers increased about 0.5–1 log unit after the booster dose (35 dpv). Avidity maturation, however, followed a different kinetics. Mean avidity indexes remained below 30% after the first dose, indicating that primo-vaccination elicited large amounts of low avidity antibodies. As expected, AI increased after the booster, reaching values above 60–70% at 60 days after the fist vaccination and 25 days after the booster, for all the strains. Both total antibodies and avidity maturation were higher for O1 Campos than for the other serotypes after the first dose (Fig. 1). AI values from O1 Campos vaccinated animals were significantly higher than the others at 35 dpv ($p < 0.05$).

3.2. Avidity and IgG subtypes of FMDV antibodies to the homologous and heterologous virus

Avidity and subtyping ELISAs were applied to a set of previously characterized sera from animals immunized with an inactivated A24 Cruzeiro strain monovalent FMDV vaccine and challenged with the heterologous A/Arg/01 strain. Serum samples from 4 PPG trials were tested against A24/Cruzeiro and A/Arg/01. Mean neutralizing antibodies and IgG1/IgG2 ratio were significantly higher for anti-A24/Cruzeiro antibodies than those against A/Arg/01 ($p < 0.001$). Notably, mean avidity values of cross-reactive antibodies were

significantly higher ($p = 0.0013$) than those against the homologous strain (Table 2).

Among the 64 serum samples, protected and not-protected animals were not equally represented, as 47 samples belonged to animals that did not pass the challenge test. Therefore, the assays were analyzed for the protected and non-protected animals respectively. A/Arg/01 VNT titers, avidity indexes (Fig. 2A) and IgG1/IgG2 ratios (Fig. 2B) were significantly lower ($p < 0.05$) in those bovines that did not surpass the PPG test. VNT, isotype and avidity ELISAs, differentiated between protected and unprotected bovines.

3.3. Avidity, subtypes and neutralizing activity of cross-reactive antibodies

Mean values show that A/Arg/01 neutralizing antibodies in A24 Cruzeiro vaccinated bovines were present in low amounts for both protected (mean titer 1.21 ± 0.39, $n = 17$) and non-protected animals (mean titer 0.87 ± 0.04, $n = 47$). However, levels of total cross-reactive antibodies detected by LPB-ELISA were similar between both groups (2.23 ± 0.56 and 1.91 ± 0.38 for protected and non-protected animals, respectively), showing that a significant fraction of the antibodies reactive to A/Arg/01 were non-neutralizing antibodies.

Analyzing individual data, we observed that differences in avidity and IgG1/IgG2 ratio were particularly relevant at low neutralizing titers. Therefore, data were organized based on the performance of each animal in the PPG test to heterologous virus; comparing VNT titers with IgG1/IgG2 ratio and Avidity Index (Fig. 3A and B, respectively).

Fig. 3A shows that even though all non-protected animals had undetectable or low VNT titers (<1.2), there were many protected animals (10 out 17) with low titers as well. Remarkably, all these protected animals with low or negative VNT titers had IgG1/IgG2 ratios over 10. When higher VNT titers were achieved, the isotype ratio was not associated to the protection status. Then, if both tests were considered simultaneously, the indirect prediction could be improved by classifying as protected those animals that have either high VNT or low VNT but high IgG1/IgG2 ratio.

Avidity values over 25% were observed in 16 out of 17 protected animals, even though some of them had low VNT titers (9 out of 17). However, avidity measurement proved less useful for samples from non-protected animals with low or negative VNT titers. Ten out of the 47 samples, showing VNT titers between 0.85 and 0.92, yielded very low OD values in the avidity ELISA and were not acceptable for processing according to the assay's parameters, and 13 of the remaining samples showed AI > 25% and thus were indistinguishable from the protected ones (Fig. 3B).
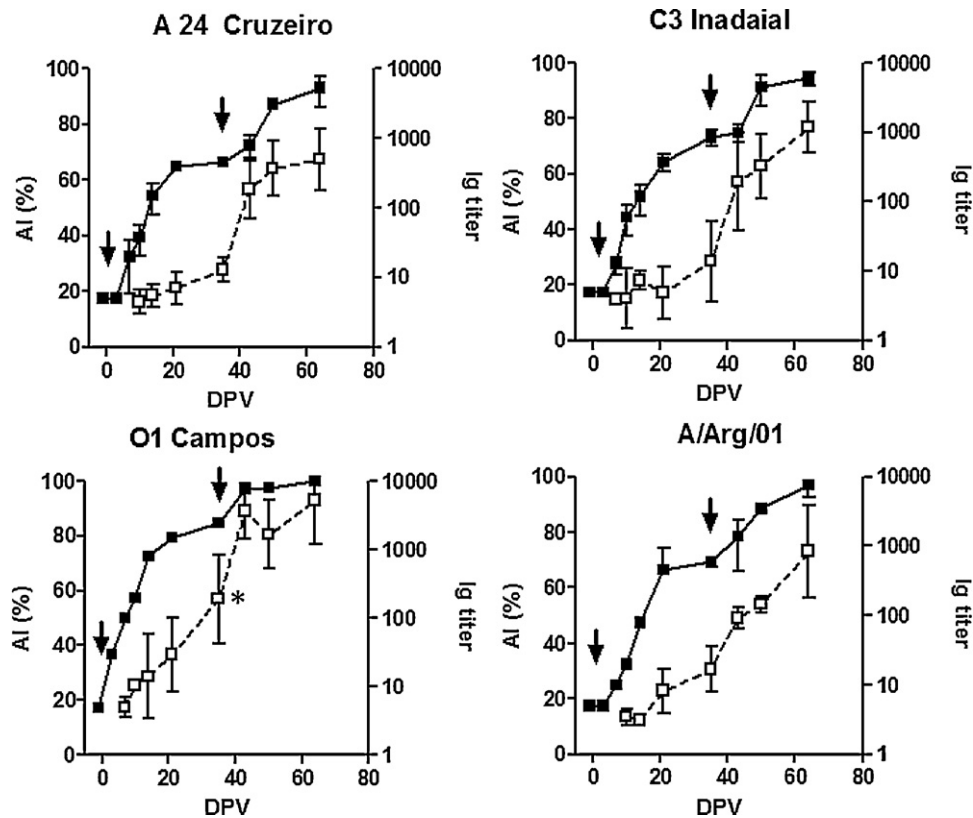
Fig. 1. Assessment of avidity maturation. Four calves were vaccinated twice (indicated with arrows) with a tetravalent vaccine containing A/Arg/01, A24 Cruzeiro, C3 Indaial and O1 Campos strains; and bled at several time points pre- and post each vaccination. Total immunoglobulin levels measured in LPB-ELISA (black squares, right "Y" axes) and avidity indexes (open squares, left "Y" axes) of antibodies against the four FMDV-strains are depicted. AI against O1 Campos were significantly higher than AI measurements against other strains at the same time point ($p < 0.05$).

Table 2
Comparative results of serological assessments.

|	Ab. against A24 Cruzeiro	Ab. against A/Arg/01	p values between groups
VNT (log 10)	1.88 ± 0.51	0.99 ± 0.29	<0.001
Avidity index (%)	17.51 ± 8.94	34.66 ± 16.26[a]	0.0013
IgG1/IgG2 ratio	11.22 ± 12.73	6.04 ± 8.34	<0.001

Comparative serology results against A24 Cruzeiro and A/Arg/01 performed on 64 serum samples from cattle vaccinated with the monovalent A24 Cruzeiro FMDV vaccine. Results for each assay were compared using Student's t-test (p values).
[a] 54 samples were evaluated (OD > 0.5, without urea treatment).

4. Discussion

Virus neutralization is an accurate protection-related test commonly used to determine $r1$ values as a measure of vaccine matching [6]. However, previous reports have shown that $r1$ values may not always agree with *in vivo* challenge experiments [4,8].

Moreover, animals that do not have *in vitro*-measurable neutralizing antibodies and are protected from challenge can also be found, suggesting that humoral responses entailing non-neutralizing antibodies, and probably cellular-mediated immunity, may also be protective. We hypothesized that antibody avidity and IgG subtyping may provide additional evidence in investigating if cross
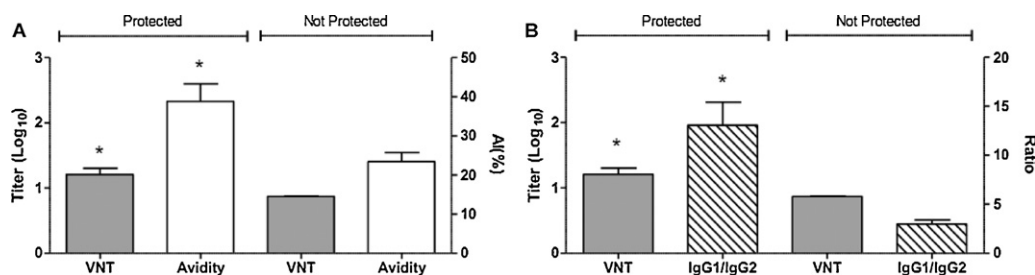
Fig. 2. Serology and cross-protection. Serum samples classified according to the PPG tests results as indicated, were tested by virus neutralization assay (VNT, gray bars) and avidity index (avidity, white bars) (AI) (Panel A) or IgG1 to IgG2 ratio (IgG1/IgG2, striped bars) (B) against A/Arg/01. Mean values and standard error of the mean (SEM) are depicted. Serum samples included in this study: $n = 47$ from protected cattle and $n = 17$ from non-protected cattle. Ten samples were excluded from the AI assessment since they did not comply with the assay's validation parameters. *Significantly higher to unprotected animals ($p < 0.05$).
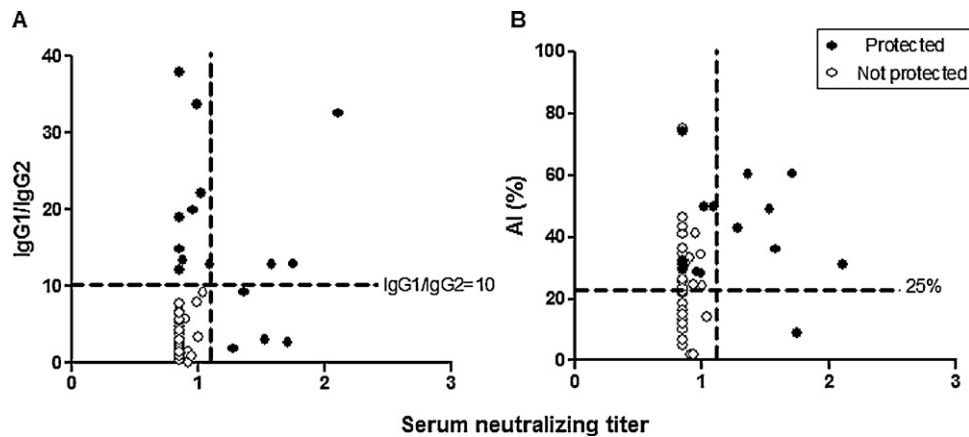
Fig. 3. Combined assessment of avidity, isotopes and neutralization titers of antibodies against A/Arg/01. (A) Anti A/Arg/01 IgG1/IgG2 ratio ($n = 64$) and (B) avidity index ($n = 54$) for each A24 Cruzeiro vaccinated animal are depicted relative to their virus neutralizing titer (30 dpv). Protected or unprotected animals (results from PPG tests) are depicted in black or white circles, respectively. Data correspond to four independent trials. (A) IgG1/IgG2 = 10 is indicated with a horizontal dotted line, and VNT titer 1.2, with a vertical dotted line. (B) The horizontal dotted line indicate AI = 25%; and VNT titers = 1.2 are indicated with a vertical dotted line.

reacting antibodies induced by vaccination are likely to be associated with protective serological responses.

In this study, a single-dilution ELISA was developed to determine avidity indexes of FMDV-specific immunoglobulins induced in cattle after vaccination. We verified the performance of the test by showing the progression of the avidity maturation of antibodies against four South American strains in cattle, along a vaccination-revaccination schedule. Results were similar for all strains, although AI values from O1 Campos vaccinated animals were significantly higher than the others at 35 days after a single vaccination. Even though the actual amount of antigens in this vaccine was not disclosed, this may be due to a higher payload of this particular strain in the multivalent formulation, since other authors demonstrated the relationship between vaccine-payload and cross-protection [7,8,33]. Additionally, Ig avidity measurement may be a useful diagnostic tool to differentiate between recently primo- and multi-vaccinated cattle which may be of interest for vaccine-based control programs. This application of avidity testing that has been utilized for other diseases [16,18,35], needs to be explored and further validated for FMD.

We observed that avidity indexes of cross-reactive antibodies were higher than those of antibodies binding the homologous virus (Table 2). As it shown in Fig. 1, avidity of FMDV-specific homologous antibodies is low in primo-vaccinated animals at 30 dpv, even when total antibody titers are high. Consequently, it is probable that low avidity antibodies may compete with high avidity antibodies (in low number at this time) for the antigen bound to the plate and may be washed away before the urea incubation. This situation is not observed when using the heterologous antigen since cross-reactive antibodies are low in number and thus steric impairments attributable to the large number of antibodies binding the antigen are minimized. Experiments designed-for-purpose are needed to confirm this idea.

Isotyping the heterologous anti-FMDV response in vaccinated cattle gave complementary information to the VNT assessment. Animals that passed the PPG test had higher levels of FMDV-IgG1 serum titers than non-protected animals. We also observed that this differential IgG1/IgG2 ratio was associated with protection in those animals with low VNT titers (<1.2). These data are in agreement with our previous report [27], in which we found that serum IgG1 but not IgG2 levels were related to protective immune responses in vaccinated animals with low anti-FMDV antibody titers.

Almost 59% of the A24 Cruzeiro vaccinated animals that passed the A/Arg/01 challenge, exhibited low neutralizing antibodies against this heterologous strain (VNT titers ≤1.2) and all of them had IgG1/IgG2 ratios >10. Moreover, all animals with low VNT titers and IgG1/IgG2 ratio <10 were not protected. These data suggest that there are antibody mediated cross-protective mechanisms besides neutralization that become evident when neutralizing antibodies titers are low or undetectable. Also, these results would suggest that IgG isotype profile elicited after vaccination may influence the outcome of a heterologous infection. Early reports have highlighted that qualitative and functional aspects of the specific antibodies may impact on different virus clearance mechanisms and consequently, on the protection against FMDV [22–24]. Currently, the *in vitro* functionality of bovine IgG1 and IgG2 is not completely discerned [36] to explain differential clearance mechanisms associated to the IgG subclass. The fact that IgG1 is the main antibody found in mucosal surfaces as well as in colostrum and milk [36,37] may indicate a differential role of this isotype in antibody-mediated effector mechanisms.

The data presented here suggest that avidity ELISA and isotype ELISA may be applied as tools to evaluate cross-protection at 30 dpv. The competence of these tests in evaluating protective responses at earlier time points, which can be useful for countries that perform emergency vaccination, needs to be further explored. In this study, VNT as well as avidity and subtype ELISAs discriminated between protected and not protected animals against a heterologous challenge. We also found that the combination of these techniques may be applied to improve the ability of indirect testing in reflecting the outcome of PPG trials for vaccine matching. Since the biological function of antibodies is regulated by specificity, isotype and avidity, all these parameters should be addressed when studying protection.

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