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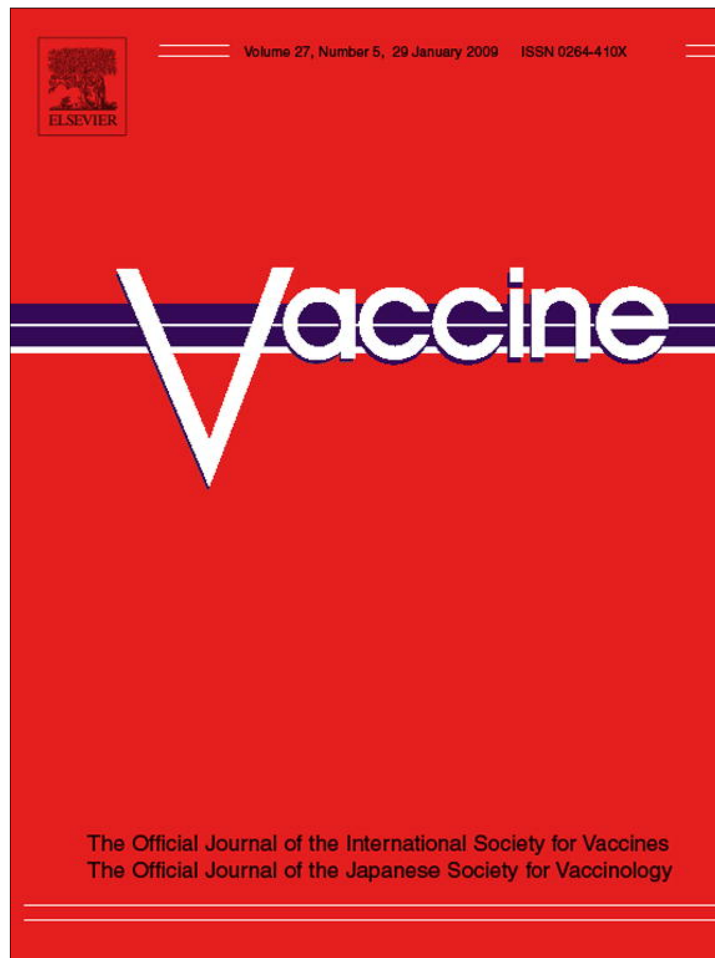

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Some guidelines for determining foot-and-mouth disease vaccine strain matching by serology

Nora Mattion [a,f,*,1], Nesya Goris [b,1], Tom Willems [b], Blanca Robiolo [a,f], Eduardo Maradei [c,f], Claudia Perez Beascoechea [c,f], Alejandro Perez [c,f], Eliana Smitsaart [e,f], Norberto Fondevila [d,f], Eduardo Palma [f], Kris De Clercq [b], José La Torre [a,f]

[a] *Centro de Virología Animal, Instituto de Ciencia y Tecnología Dr. César Milstein, CONICET, Saladillo 2468, (1440) Buenos Aires, Argentina*
[b] *Veterinary and Agrochemical Research Centre, Virology Department, Section of Epizootic Diseases, Groeselenberg 99, 1180 Brussels, Belgium*
[c] *Servicio Nacional de Sanidad Animal (SENASA), Fleming 1653, (1640) Martínez, Argentina*
[d] *Instituto Nacional de Tecnología Agropecuaria, Centro de Investigación de Ciencias Veterinarias y Agronómicas (INTA-CICVyA), CC77, (1708) Morón, Argentina*
[e] *Biogénesis Bagó S.A., Ruta Panamericana Km 38.2, (1619) Garín, Argentina*
[f] *Argentine FMD Interinstitutional Network for Research and Development in Foot-and-mouth Disease (RIIDFA), Rivadavia 1437, (1033) Buenos Aires, Argentina*

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ABSTRACT

The selection of matching strains for use in outbreaks of foot-and-mouth disease (FMD) virus can be assessed in vivo or by serological *r*-value determination. Sera from animals involved in vaccine potency and cross-protection trials performed using the “Protection against Podal Generalization” (PPG) test for two serotype A strains were collected and analyzed by the virus neutralization test (VNT) and liquid-phase ELISA (lpELISA) in three laboratories. The average VNT *r*-values for medium and high serum titer classes from the A_{24} Cruzeiro vaccinated animals were in line with the A/Arg/01 heterologous PPG outcome for all testing laboratories, suggesting that the vaccine strain A_{24} Cruzeiro is unlikely to protect against the field isolate A/Arg/01. The corresponding lpELISA *r*-values were slightly higher and indicate a closer relationship between both strains. Pooling of serum samples significantly reduced the inter-animal and inter-trial variation. The results suggest that a suitable reference serum for vaccine matching *r*-value experiments might be a pool or a medium to high VNT or lpELISA titer serum. Furthermore, the VNT seems to produce the most reproducible inter-laboratory results. More work is, however, needed in order to substantiate these claims.

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1. Introduction

Foot-and-mouth disease (FMD) is a very important and highly contagious veterinary disease causing serious concerns worldwide. The FMD virus (FMDV) is a picornavirus that affects cloven-hoofed animals, mainly the economically important bovine, porcine and ovine herds among others. Many countries are currently free from the disease, even though FMD remains endemic in large areas of the world (e.g. parts of the African and Asian continents). Some countries have mixed situations with FMD-free areas and areas where vaccination is practiced [1]. Regardless of its current FMD status, every country is in danger of suffering from a FMD outbreak at any moment in time as long as the disease has not been eradicated globally. Conventional, safe, inactivated vaccines are used for disease prevention, combat and control in numerous FMD outbreaks. Vaccine and antigen reserves have been composed mostly in countries without vaccination programs [2], but also in countries where vaccination is practiced (e.g. Argentina). Contingency planning is the main reason for establishing vaccine/antigen banks. However, FMDV is a highly variable RNA virus, and in general, there is little or no cross-protection between serotypes and even among different strains of the same serotype [3]. There are seven established serotypes of FMDV (A, O, C, Asia 1, SAT 1-2-3) and although worldwide most outbreaks are caused by viruses of serotypes O and A [1], any strain might emerge either by accidental or deliberate actions.

For these reasons, it is impossible for individual countries or consortia of countries to store every possible strain. Consequently, only several antigenic representative, cross-reactive (i.e. broad spectrum of reactivity) FMDV strains are kept over liquid nitrogen in antigen

* Corresponding author at: Centro de Virología Animal, Instituto de Ciencia y Tecnología Dr. César Milstein, CONICET, Saladillo 2468, (1440) Buenos Aires, Argentina. Tel.: +54 11 4686 6225; fax: +54 11 4686 6225.
E-mail addresses: nmattioncevan@centromilstein.org.ar, mattion@fibertel.com.ar (N. Mattion).
[1] These authors contributed equally and are considered to be joint first co-authors.

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reserves. In order to assess the suitability of the strains for a particular situation (e.g. outbreak scenario), a vaccine matching test should be readily performed. The direct comparative matching test is the in vivo cross-protection test which is on the one hand closely related to the FMD in-field situation, but on the other hand is costly, time-consuming, unreliable [4,5] and animal unfriendly. Consequently, by the time the result is known, the outbreak might have extended and caused irremediable damages.

Several indirect vaccine matching tests have been performed based on serological data in which an indirect relationship value (*r*-value) is considered for each reference bovine vaccinal sera (BVS) (reviewed by Ref. [3]). The *r*-value is calculated by determining the ratio of the reciprocal heterologous serum titer to the reciprocal serum titer against the homologous virus strain using one of three serological assays [complement fixation test (CF), ELISA and virus neutralization test (VNT)] [3].

There has been a lot of controversy surrounding indirect *r*-values as the protective immunity to FMDV infection is complex and involves not only humoral antibody responses but also factors derived from innate and cellular immunity [6]. The latter are not detected by VNT in cells or by antigen binding antibodies in ELISA. Moreover, a correlation between *r*-values and in vivo cross-protection has not always been observed. There are documented cases where cross-protection was found in spite of low *r*-values [7] and vice versa. Variation between batches of BVS has also led to inconsistent results [3,8]. There have been reports of *r*-values derived from sera of animals involved in a European Pharmacopoeia vaccine potency test (i.e. the 50% protective dose or PD_{50} trial) that differed depending on whether the sera were pooled ($n = 5$ and $r = 0.3$) or calculated based on the mean reciprocal serum titers of the full vaccine dose group sera only ($n = 5$ and $r = 0.12$) [7].

Furthermore, it is important to point out that the in vivo or serological assays and the reagents used in different experiments have not been harmonized worldwide, or even within a particular geographic region [3,5].

The objective of the present study was to use data and serum samples obtained from 10 "Protection against Podal Generalization" (PPG) FMD vaccine trials in which the animals were challenged with either of two antigenically different FMDV serotype A strains [5]. The serum samples taken post-vaccination were used to (i) establish the variability of *r*-value calculations, (ii) investigate the influence of vaccine potency/stability on *r*-value assessment and (iii) study the effect of pooling on *r*-value determination. For these purposes, *r*-values based on different sets of sera were analyzed in three different laboratories. The vaccine strain (FMDV A_{24} Cruzeiro) and a field virus isolated during the 2000–2001 Argentine outbreaks (FMDV A/Argentina/2001) [9,10] were used in the serological tests. The outcome of the study should help clarify selection criteria for reference BVS for future use in *r*-value assays worldwide.

2. Materials and methods

2.1. Vaccine trials and virus strains

Serum samples were derived from 10 individual in vivo PPG experiments using cattle primo-vaccinated with a monovalent vaccine batch formulation of purified A_{24} Cruzeiro/Brazil/55 (A_{24} Cruzeiro) antigen in a water-in-oil emulsion [5]. The vaccine had an antigenic mass of 10 μg and an average potency of 88.5% PPG [95% confidence interval (CI): 80.7–93.5] in six homologous A_{24} Cruzeiro challenge PPG potency trials and 26.6% PPG [95%CI: 17.4–38.5] in four heterologous A/Argentina/2001 (A/Arg/01) challenge PPG cross-protection trials as reported previously [5]. All PPG trials were conducted in Argentina according to the Argentine Animal Health Service (SENASA) Act No. 351/2006 [11], except that the animals were challenged at 30-day post-vaccination (dpv) instead of at 90 dpv. The PPG percentage was calculated according to the following formula [11]:

$$\%\text{PPG} = \frac{\text{number of protected animals}}{\text{number of vaccinated animals}} \times 100 \quad (1)$$

According to SENASA Act No. 351/2006 [11], animals are considered to be protected against live viral challenge if no foot lesions are observed at 7-day post-challenge. In the presence of such lesions, animals are classified as unprotected.

All PPG trials were performed within an 11-month period (January–November 2006), during which a slight decrease in vaccine potency/stability was noted after September 2006 [5]. First, *r*-values were calculated for all individual serum samples taken at 30 dpv. Then, given the decline in vaccine potency/stability as of trial 7 and to minimize external sources of variability not related to the actual serological *r*-value test, sera from the first six PPG trials (carried out between January and September 2006) were first analyzed separately for establishing the variability of *r*-value determination. Subsequently, to study the influence of vaccine potency/stability on the *r*-value outcome, *r*-values based on data from the first four (January–February 2006) and last four PPG trials (October–November 2006) were used for comparison.

The following FMDV serotype A virus strains provided by SENASA were used for serological *r*-value determination in VNT and liquid-phase ELISA (lpELISA): A_{24} Cruzeiro [origin: Pan American Center for Foot-and-mouth Disease (PANAFTOSA)] and A/Arg/01. Additionally, FMDV strains O1/Campos/Brazil/58 (O1 Campos) and C3/Indaial/Brazil/71 (C3 Indaial) were also used in lpELISA to study the variability of *r*-value determination for more distantly related strains.

2.2. Participating laboratories and serological tests

All animals were bled at 0, 14 and 30 dpv, and at 7 dpc. A collection of BVS was constructed by fractionating the collected serum samples into different size aliquots (a total of 120 ml per animal of 30 dpv sera was collected) and stored at −80 °C at different locations.

The 30 dpv serum collection was used in the present study and tested at three different laboratories for *r*-value determination: the Veterinary and Agrochemical Research Centre (VAR, Brussels, Belgium), Centro de Virología Animal (CEVAN, Buenos Aires, Argentina) and SENASA (Martínez, Argentina). The VAR used the FMDV A_{24} Cruzeiro and A/Arg/01 VNT and lpELISA as previously described [12]. CEVAN made use of the FMDV A_{24} Cruzeiro and A/Arg/01 lpELISA [13,14], whereas SENASA applied the FMDV A_{24} Cruzeiro and A/Arg/01 VNT [15].

The base 10 logarithmic ($\log_{10}$) VNT and lpELISA serum titers ($\log_{10} t$) against the vaccine strain were further classified in low, medium and high (Table 1). The limits of these classes were assigned based on experience, and a SENASA VNT $\log_{10} t$ of 1.60 and

Table 1
Classification of base 10 logarithmic serum titers in the respective homologous A_{24} Cruzeiro serological assays.

	VNT			lpELISA		
	Low	Medium	High	Low	Medium	High
CEVAN		NA		$t < 2.20$	$2.20 \le t \le 2.80$	$t > 2.80$
SENASA	$t < 1.60$	$1.6 \le t \le 2.00$	$t > 2.00$		NA	
VAR	$t < 1.81$	$1.81 \le t \le 2.26$	$t > 2.26$	$t < 2.11$	$2.11 \le t \le 2.41$	$t > 2.41$

NA = not applicable; *t* = homologous serum titer; VNT = virus neutralization test; lpELISA = liquid-phase ELISA.

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a CEVAN lpELISA $\log_{10}$ of 2.20 are around the values that may be considered as protective antibody levels for bovines [unpublished data]. The VAR VNT and VAR lpELISA $\log_{10} t$ of 1.81 and 2.11, respectively, correspond to approximately 70% probability of protection levels based on logistic regression models [unpublished data].

2.3. r-Value determination and effect of vaccine potency/stability on r-values

In total, 160 individual r-values (r_i) were determined using VNT and lpELISA titers according to the following formula [15]:

$$r_i = \frac{\text{reciprocal titer of reference serum against field virus}}{\text{reciprocal titer of reference serum against vaccine virus}} \quad (2)$$

The interpretation of the results was based on Ferris and Donaldson [16] for lpELISA and on Rweyemamu [17] for VNT. Following these guidelines, r-values based on lpELISA between 0.4 and 1.0 or VNT r-values superior to 0.3 indicate a close relationship between the vaccine strain and the field isolate. In other words, a potent vaccine containing the vaccine strain is likely to confer protection against the field isolate. lpELISA r-values between 0.2 and 0.39 signify that the vaccine strain might be suitable for use if no closer match can be found provided that a potent vaccine is used and animals are immunized preferably more than once, whereas a lpELISA r-value inferior to 0.2 or a VNT r-value smaller than 0.3 points toward a vaccine strain that is unlikely to protect against challenge with the field isolate [15–17].

For each PPG trial (T), an r-value (r_T) was calculated from the reciprocal mean homologous and reciprocal mean heterologous serum titers of all 16 vaccinated animals according to the following formula:

$$r_T = \frac{\text{reciprocal mean serum titer per trial against A/Arg/01}}{\text{reciprocal mean serum titer per trial against A}_{24}\text{ Cruzeiro}} \quad (3)$$

The mean r_T ($\overline{r_T}$) was subsequently calculated for the group of PPG trials considered (i.e. the first six PPG trials, the first four PPG trials and the last four PPG trials):

$$\overline{r_T} = \frac{1}{n}\sum_{i=1}^{n} r_{T,i} \quad \text{with } n = \text{number of trials under consideration} \quad (4)$$

When the $\log_{10}$ serum titers were classified into groups of low, medium and high, an r-value for each $\log_{10} t$ class (C) was calculated per trial ($r_{T,C}$) similarly to formula (3) and the mean per C for all trials under consideration was also determined ($\overline{r_{T,C}}$) in line with formula (4). Furthermore, an r-value of a group (G) of trials was alternatively calculated for each $\log_{10} t$ class (C) using the reciprocal mean serum titers of all the animals belonging to the group of PPG trials and to the $\log_{10} t$ class considered ($r_{G,C}$).

The standard deviations (S.D.) and 95%CI were calculated for each mean r-value.

2.4. Serum pooling and r-value determination

In order to evaluate the effect of serum sample pooling on r-value determination, different serum pools were composed based on 30 dpv sera and tested in the VAR VNT. Pools were made of the serum samples of either all the vaccinated animals per PPG trial, or all the protected animals per PPG trial, or all the unprotected animals per PPG trial, or 10 randomly selected animals, or 10 randomly selected protected animals, or, finally, 10 randomly selected unprotected animals. The random sampling of the pools was done computer aided using the Random Samples and Permutations function of the freely distributed R programme software (www.r-project.org). In total, 58 pools containing between 2 and 16 pooled sera were analyzed.

For comparative purposes, a set of r-values were determined. First of all, the r-value of the pool (r_{pool}) was calculated according to formula (2) and the mean r_{pool} ($\overline{r_{pool}}$), S.D. and 95%CI were determined for all similar pools (e.g. all pools based on all protected animals per PPG trial or all pools based on 10 randomly selected animals). Subsequently, per pool, the average r-value ($\overline{r_i}$) based on the individual r_i-values (2) of each serum sample in the pool was calculated as follows:

$$\overline{r_i} = \frac{1}{n}\sum_{i-1}^{n} r_i \quad \text{with } n = \text{number of sera/pool} \quad (5)$$

The mean $\overline{r_i}$, S.D. and 95%CI were calculated for all similar pools.

A third r-value (r_t) was estimated based on the reciprocal mean serum titers (t) for all serum samples that constitute the pool:

$$r_t = \frac{\text{reciprocal mean serum titer per pool against A/Arg/01}}{\text{reciprocal mean serum titer per pool against A}_{24}\text{ Cruzeiro}} \quad (6)$$

The mean r_t ($\overline{r_t}$), S.D. and 95%CI were also determined for all similar pools.

3. Results

3.1. Serum collection

The 10 PPG trials allowed for the collection of an important well-characterized set of sera from vaccinated animals with known in vivo protection status at 30 dpv. In total, 640 serum samples were collected corresponding to 160 serum samples collected at each sampling stage (0, 14 and 30 dpv and 7 dpc). The 160 sera collected at 30 dpv were analyzed in four different assays for assessing the (i) variability of r-value determination, (ii) the influence of vaccine potency/stability on r-values and (iii) the effect of serum pooling on r-value assessment.

3.2. r-Values for non-pooled serum samples calculated from VNT and lpELISA titers

In total, four times 160 individual r-values (r_i) for A/Arg/01 were obtained based on 30 dpv SENASA VNT serum titers, VAR VNT serum titers, CEVAN lpELISA serum titers and VAR lpELISA serum titers. For the SENASA VNT, r_i ranged from 0.016 to 1.000, whereas a range from 0.004 to 1.000 was noted for VAR VNT. A similar range from 0.059 to 1.000 and 0.006 to 1.000 was found for CEVAN lpELISA and VAR lpELISA r_i-values, respectively.

Overall, 107 out of the 160 SENASA VNT r_i-values were found to be below 0.3, whereas 124 VAR VNT r_i-values also indicated a lack of relatedness between both FMDV serotype A strains. For the CEVAN lpELISA r_i-values, 26 were below the 0.2 threshold, 66 were situated between 0.2 and 0.39, and 68 were greater than 0.4. A total of 106, 29 and 25 were recorded for VAR lpELISA r_i-values.

The 10 SENASA VNT r_T-values ranged from 0.19 to 0.53, whereas values of 0.048–0.405 were recorded for VAR VNT r_T-values, with mean $\overline{r_T}$-values and S.D. of 0.28 ± 0.17 [95%CI: 0.17–0.39] and 0.188 ± 0.127 [95%CI: 0.098–0.278], respectively. A similar range of lpELISA r_T-values of 0.19–0.60 and 0.101–0.337 was observed for CEVAN and VAR, respectively, with mean $\overline{r_T}$-values and S.D. of 0.40 ± 0.17 [95%CI: 0.29–0.51] and 0.197 ± 0.062 [95%CI: 0.153–0.241], respectively.

The data derived from the SENASA VNT $\log_{10} t$ class smaller than 1.60 resulted in some $r_{T,C}$-values for A/Arg/01 above 0.3 as shown by the $\overline{r_{T,C}}$-value and 95%CI of the first six PPG trials (Table 2). The same was noted for the VAR VNT $\log_{10} t$ class smaller than 1.81. The

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Table 2
Virus neutralization r-values for FMDV strain A/Arg/01 using FMDV A_{24} Cruzeiro vaccination (trials 1–6).

	SENASA VNT			VAR VNT		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	*0.317*[a]	0.145	0.117	*0.339*	0.196	0.091
S.D.	0.079	0.038	0.083	*0.352*	0.083	0.058
95%CI	*0.253–0.380*	0.115–0.175	0.050–0.183	*0.017–0.661*	0.120–0.272	0.038–0.144
$r_{G,C}$	0.300	0.145	0.092	*0.454*	0.208	0.081

S.D. = standard deviation; 95%CI = 95% confidence interval.
[a] r-Values superior to 0.3 are depicted in italics.

Table 3
Liquid-phase ELISA r-values for FMDV strain A/Arg/01 using FMDV A_{24} Cruzeiro vaccination (trials 1–6).

	CEVAN lpELISA			VAR lpELISA		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	*0.436*[a]	0.273	0.245	0.190	0.266	0.225
S.D.	0.124	0.076	0.131	0.056	0.105	0.032
95%CI	*0.337–0.534*	0.212–0.334	0.140–0.350	0.139–0.241	0.170–0.362	0.196–0.254
$r_{G,C}$	*0.426*	0.261	0.215	0.200	0.243	0.217

S.D. = standard deviation; 95%CI = 95% confidence interval.
[a] r-Values superior to 0.4 are depicted in italics.

data derived from the CEVAN lpELISA $\log_{10} t$ class smaller than 2.20 resulted in a $\overline{r_{T,C}}$-value above 0.4 as well, whereas this phenomenon was not observed for the VAR lpELISA serum titers smaller than 2.11 (Table 3). For SENASA VNT $\log_{10} t$, VAR VNT $\log_{10} t$, CEVAN lpELISA $\log_{10} t$ and VAR lpELISA $\log_{10} t$ classes of at least 1.60, 1.81, 2.20 and 2.11, respectively, serological values of relatedness were below 0.3 or 0.4, as shown by the medium or high $\log_{10} t$ columns in Tables 2 and 3, which is in line with the obtained in vivo PPG cross-protection results.

A similar trend was observed for the data derived from O1 Campos and C3 Indaial lpELISA titers where $\overline{r_{T,C}}$-values above 0.2 were found for the low $\log_{10} t$ classes (Table 4). For higher $\log_{10} t$ classes, $\overline{r_{T,C}}$-values and upper limits of 95%CI were always below the 0.2 threshold for distantly related strains.

The bottom lines of Tables 2 and 3 show $r_{G,C}$-values calculated on the basis of the mean of the A_{24} Cruzeiro and A/Arg/01 VNT or lpELISA serum titers of all animals involved in trials 1–6 per $\log_{10} t$ class. The same calculation carried out using the mean of serum titers for all animals involved in the 10 trials (data not shown) also led to high r-values when using low titer sera. For the more distant FMDV strains O1 Campos and C3 Indaial, all $r_{G,C}$-values based on lpELISA titers were found to be below 0.2 regardless of the number of trials considered (i.e. trials 1–6 or trials 1–10) (Table 4).

3.3. Influence of vaccine potency/stability on r-value determination

Given the fact that vaccine potency decreased from 100% PPG to 75% PPG during the 11-month period in which the 10 PPG trials were performed, further studies were performed to analyze the influence of vaccine potency/stability on r-value determination. The calculation of r-values was thus based on reciprocal VNT and lpELISA serum titers from the first four (i.e. trials 1–4, January–February 2006) and the last four trials (i.e. trials 7–10, October–November 2006) (Tables 5 and 6).

For r-values based on reciprocal VNT titers, the results obtained for trials 1–4 were not significantly different from the results obtained for trials 1–6 as seen by the highly overlapping 95%CI for both participating laboratories. Slightly, although not-significantly, higher r-values based on SENASA VNT were obtained when using data from trials 7 to 10 for the low and medium $\log_{10} t$ class; whereas slightly lower r-values were found in all serum titer classes for the VAR VNT (Tables 2 and 5). Interestingly, for the last four trials, no SENASA VNT $\log_{10} t$ greater than 1.60 was obtained (Table 5).

The r-values based on reciprocal lpELISA titers based on the first four trials are also more in line with the in vivo heterologous PPG results (%PPG < 75%), when the sera from the low $\log_{10} t$ class are discarded (Table 6). Moreover, r-values based on lpELISA titers seemed to be more influenced by vaccine potency/stability as shown by the high CEVAN lpELISA $\overline{r_{T,C}}$-values for all serum titer classes (Table 6). Interestingly, no VAR lpELISA $\log_{10} t$ greater than 1.81 was obtained for the last four trials (Table 6).

3.4. Influence of pooling on r-value determination

Table 7 depicts the influence of pooling on r-value determination. Irrespective of the type of samples and the number of samples pooled, the 58 individual VAR VNT r_{pool}-values were found to be

Table 4
CEVAN liquid-phase ELISA r-values for FMDV strains O1 Campos and C3 Indaial using FMDV A_{24} Cruzeiro vaccination (trials 1–6).

	O1 Campos			C3 Indaial		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	*0.227*[a]	0.087	0.050	0.188	0.075	0.056
S.D.	0.087	0.019	0.027	0.079	0.028	0.043
95%CI	0.157–*0.296*	0.072–0.102	0.028–0.071	0.125–*0.250*	0.052–0.097	0.022–0.090
$r_{G,C}$	0.171	0.078	0.044	0.143	0.065	0.044

S.D. = standard deviation; 95%CI = 95% confidence interval.
[a] r-Values superior to 0.2 are depicted in italics.

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Table 5
Virus neutralization *r*-values for FMDV strain A/Arg/01 determined for the first four and the last four trials using FMDV A_{24} Cruzeiro vaccination.

	(a) SENASA VNT					
	n = 4 (trials 1–4)			n = 4 (trials 7–10)		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	*0.329*[a]	0.160	0.151	*0.427*	0.200	na
S.D.	0.099	0.038	0.082	0.161	0.086	na
95%CI	0.230–*0.426*	0.122–0.198	0.071–0.232	0.269–*0.585*	0.115–0.284	na

	(b) VAR VNT					
	n = 4 (trials 1–4)			n = 4 (trials 7–10)		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	*0.383*	0.204	0.104	0.279	0.194	0.045
S.D.	0.488	0.100	0.064	0.028	0.144	0.027
95%CI	−0.164 to *0.930*	0.092–*0.316*	0.032–0.176	0.248–*0.310*	0.033–*0.355*	0.015–0.075

na = not available; S.D. = standard deviation; 95%CI = 95% confidence interval.
[a] *r*-Values superior to 0.3 are depicted in italics.

Table 6
Liquid-phase ELISA *r*-values for FMDV strain A/Arg/01 determined for the first four and the last four trials using FMDV A_{24} Cruzeiro vaccination.

	(a) CEVAN lpELISA					
	n = 4 (trials 1–4)			n = 4 (trials 7–10)		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	*0.439*[a]	0.240	0.192	*0.524*	*0.457*	*0.496*
S.D.	0.140	0.059	0.065	0.038	0.078	0.093
95%CI	0.301–*0.577*	0.183–0.298	0.128–0.255	*0.486–0.562*	0.381–*0.533*	*0.405–0.588*

	(b) VAR lpELISA					
	n = 4 (trials 1–4)			n = 4 (trials 7–10)		
	Low	Medium	High	Low	Medium	High
$\overline{r_{T,C}}$	0.198	0.271	0.233	0.178	0.087	na
S.D.	0.067	0.103	0.021	0.057	0.069	na
95%CI	0.123–0.273	0.156–0.386	0.209–0.257	0.114–0.242	0.010–0.164	na

na = not available; S.D. = standard deviation; 95%CI = 95% confidence interval.
[a] *r*-Values superior to 0.4 are depicted in italics.

below the anticipated 0.3 threshold (range: 0.006–0.250). Consequently, the $\overline{r_{pool}}$-values and upper limits of the 95%CI were also below 0.3, which correlates with the in vivo cross-protection data. In contrast, the $\overline{r_i}$-values and mean $\overline{r_i}$-values of the pools were significantly higher as shown by the non-overlapping 95%CI for the mean $\overline{r_i}$- and $\overline{r_{pool}}$-values. Moreover, two out of six upper limits of the mean $\overline{r_i}$-values 95%CI were superior to 0.3 (Table 7). Interestingly, they correlated to serum pools from animals that were found to be in vivo unprotected against live virus challenge. Similarly higher *r*-values were also obtained based on the reciprocal

Table 7
The effect of pooling on *r*-value determination for FMDV strains A/Arg/01 and A_{24} Cruzeiro using the VAR virus neutralization test and FMDV A_{24} Cruzeiro vaccination.

Pool identification	Number of pools	Number of sera/pool	$\overline{r_{pool}}$ [95%CI]	Mean $\overline{r_i}$ [95%CI]	$\overline{r_t}$ [95%CI]
All animals per PPG trial	10	16	0.048 ± 0.034[a] [0.024–0.072]	0.207 ± 0.098[a] [0.138–0.277]	0.186 ± 0.125[a] [0.097–0.275][b]
Protected animals per PPG trial	10	2–16	0.045 ± 0.029 [0.025–0.066]	0.190 ± 0.122 [0.104–0.277]	0.174 ± 0.122 [0.088–0.260]
Unprotected animals per PPG trial	7	2–14	0.055 ± 0.042 [0.019–0.091]	0.229 ± 0.142 [0.109–*0.349*]	0.210 ± 0.122 [0.107–*0.313*]
Randomly selected animals	15	10	0.105 ± 0.067 [0.066–0.144]	0.202 ± 0.092 [0.148–0.255]	0.142 ± 0.068 [0.103–0.181]
Randomly selected protected animals	10	10	0.057 ± 0.031 [0.035–0.079]	0.170 ± 0.058 [0.129–0.211]	0.103 ± 0.042 [0.073–0.133]
Randomly selected unprotected animals	6	10	0.107 ± 0.045 [0.066–0.148]	0.280 ± 0.076 [0.210–*0.350*]	0.213 ± 0.069 [0.150–0.276]

95%CI = 95% confidence interval.
[a] Results are expressed as mean ± standard deviation.
[b] *r*-Values superior to 0.3 are depicted in italics.

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mean serum titers (r_t and $\overline{r_t}$). In this case, the upper limit of the 95%CI was superior to 0.3 for one of the six $\overline{r_t}$-values, which again corresponded to serum pools of groups of animals that were found to be unprotected in vivo.

4. Discussion

For vaccine matching purposes, FMDV strain selection is based on indirect serological methods (r-values), on sequence data [3] or alternatively on the calculation of the relatedness between the field isolate and available vaccine strains using in vivo challenge tests [5,7]. Recently, we performed 10 PPG trials using a single FMDV A_{24} Cruzeiro vaccine batch with homologous A_{24} Cruzeiro or intratypic heterologous A/Arg/01 challenge [5]. Based on the cross-protection PPG results, A_{24} Cruzeiro and A/Arg/01 cannot be regarded as closely related FMDV strains, since a single vaccination with the former did not induce a sufficient level of cross-protection against the later (%PPG < 30%). The same conclusion was drawn based on sequence and deduced amino acid data [10] and on the results of several other PPG trials performed in Argentina between 2001 and 2008 (Ref. [14] and unpublished results). Furthermore, the mean Expected Protection Percentage (EPP) [15] calculated for the 10 PPG trials as a function of the lpELISA titers using SENASA/CEVAN logit regression curves for A_{24} Cruzeiro and A/Arg/01 [Robiolo et al., unpublished data] was 86.2% [95%CI: 83.2–89.2] and 56.1% [95%CI: 51.1–61.1], respectively. The mean EPP calculated with the VNT titers using PANAFTOSA logit regression curves for A_{24} Cruzeiro [18] was 85.8% [95%CI: 80.8–90.8], while no logit regression curves are available for A/Arg/01. Field observations confirm these findings as routine vaccination against A_{24} Cruzeiro did not protect Argentine livestock against the FMDV A/Arg/01 outbreak [9,10]. Therefore, we hypothesized that the in vivo cross-protection test is the "Gold Standard" procedure for assessing the relatedness between different FMDV strains. Consequently, serological r-values calculated from VNT serum titers must be below 0.3, and inferior to 0.2 when using lpELISA serum titers.

At first, r-values were calculated using sera from all A_{24} Cruzeiro vaccinated animals (r_i), irrespective of their in vivo protection status, and from all 10 PPG trials (r_T) performed within 11 months. Upon analysis of the complete dataset based on four times 160 individual r_i-values and even four times 10 r_T-values, a high inter-animal and inter-trial variation was observed which severely hampered and complicated the interpretation of the relatedness of both FMDV serotype A strains. For instance, serological (VNT and lpELISA) r-values varied from indicating that both strains were sufficiently antigenically similar for the A_{24} Cruzeiro vaccine to induce protection against the A/Arg/01 field isolate, to indicating that the vaccine strain is unlikely to protect against A/Arg/01 challenge. However, when the animals were classified into classes of low, medium or high responders based on the antibody levels induced by vaccination and serum samples from the first six trials were considered, medium and high titer sera seemed to be more suitable for the purpose of calculating the antigenic relatedness between both FMDV strains ($\overline{r_{T,C}}$-values in Tables 2 and 3), even when more distant related strains belonging to different FMDV serotypes are used (e.g. O1 Campos and C3 Indaial) (Table 4). In other words, low titer sera seem to be poor estimators of the relatedness between FMDV strains and should preferably not be used for vaccine matching purposes. Instead, medium to high titer sera result in r-value estimates that more closely correlate to in vivo cross-protection data [5,14], field and sequence observations [9,10]. It is nonetheless worth mentioning that $\log_{10}$ serum titer values of 2.20 for CEVAN lpELISA and 1.60 for SENASA VNT are only indicative, and not meant as fixed limits as shown by the differences in chosen limits for the VAR VNT and lpELISA. The limits of the titer classes should be set based on experience and can for instance be based on serum titers that correspond to antibody pass-levels for protection which can differ among laboratories [12].

The study further showed that r-value estimations become less precise when using data from the last four PPG trials (i.e. trials 7–10, Tables 5 and 6). This observation was more evident for lpELISA than for VNT r-values, and might be due to a slight decrease in vaccine potency/stability [5]. These observations, however, are preliminary and additional experiments with low potency or even degenerated vaccines are needed to substantiate such claims.

Previous work suggested that even in vivo PPG cross-protection studies might be of questionable reliability when dealing with primo-vaccinated animals and with FMDV strains of low antigenic relatedness [5]. It might be possible that the potency of the A_{24} Cruzeiro vaccine (i.e. 88.5% PPG) used was not sufficiently high for the purpose of producing appropriate BVS for r-value determination. A high potency vaccine of 100% PPG might be preferred since they generally lead to a more elevated antibody response or serum titer post-vaccination [19] and an increased likelihood of cross-protection [7].

The VNT $r_{G,C}$-values for medium and high titer classes were also below the 0.3 threshold value and, thus, in line with the heterologous challenge PPG outcome for both testing laboratories, suggesting that the vaccine strain A_{24} Cruzeiro is unlikely to protect against the field isolate A/Arg/01. The corresponding lpELISA r-values are slightly higher and indicate a closer relationship between both strains (i.e. the vaccine strain might be suitable for use if no closer match can be found provided that a potent vaccine is used and animals are immunized preferably more than once). This seems to indicate that the VNT test, rather than the lpELISA, is the preferred test for vaccine matching purposes. In the case of the more distant FMDV strains O1 Campos and C3 Indaial, however, lpELISA r-values also clearly indicate a complete lack of cross-protection regardless of the trials considered, except for the O1 Campos low titer class (Table 4). Unfortunately, VNT titers were unavailable for these strains.

Pooling of serum samples significantly reduced the inter-animal and inter-trial variation, irrespective of the number of serum samples in the pool (ranging from 2 to 16) and the type of serum samples pooled (ranging from sera from unprotected animals to randomly selected animals). Moreover, a consistently low r_{pool}-value was observed indicating that the A_{24} Cruzeiro vaccine would be unlikely to confer protection against challenge with the A/Arg/01 field strain. In contrast, the upper limit of 95%CI based on alternative r-value calculations for these pools (i.e. $\overline{r_t}$ and mean $\overline{r_i}$) were found to be above 0.3 when sera from unprotected animals were used. This is not surprising, as generally unprotected animals display lower serum titers [12] and this study demonstrates that low titer sera are less suitable for r-value determination. It should be noted that even though, this study suggests that the number of serum samples constituting a pool has little or no influence on the r_{pool}-value, it is advisable to pool at least five different serum samples to limit the influence of outliers on the r-value outcome as suggested by Brehm et al. [7] and prescribed by the World Organisation for Animal Health [15].

During the course of this study a set of well-characterized sera against the FMDV A_{24} Cruzeiro strain suitable for use in vaccine matching assays was collected. The SENASA and VAR VNT, CEVAN and VAR lpELISA, the 3ABC-ELISA [20] and the PrioCHECK® FMDV NS status is known for all 0, 14 and 30 dpv and 7 dpc sera. Moreover, the in vivo protection result in a valid PPG test was determined as well. This set of sera is available upon request for further analysis in different laboratories and potential future reference A_{24} Cruzeiro BVS for r-value determination may result from a selection of the complete serum set.

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In conclusion, although highly potent vaccines (100% PPG) may be of particular importance in case of a FMD outbreak in a FMD-free zone, vaccine matching is also very important for regular vaccination programs with normal strength vaccines. Therefore, for animals vaccinated with a normal strength vaccine as used in the present study, *r*-values calculated from low titer sera are poor estimators of the antigenic relationship between strains. According to our findings a suitable reference BVS for vaccine matching experiments might be a medium to high VNT or lpELISA titer serum or a pool of at least five sera, but more work using additional serotype A strains will be needed in order to substantiate these claims especially for those strains for which a closer antigenic relationship is expected. The most reliable *r*-values are determined using the VNT data. Similar studies should also be conducted using other antigenically variable FMDV serotypes (e.g. FMDV SAT2 strains). Moreover, the effect of using more than one field virus isolate from the same FMDV outbreak on the matching result should also be elicited.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2008.11.026.

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