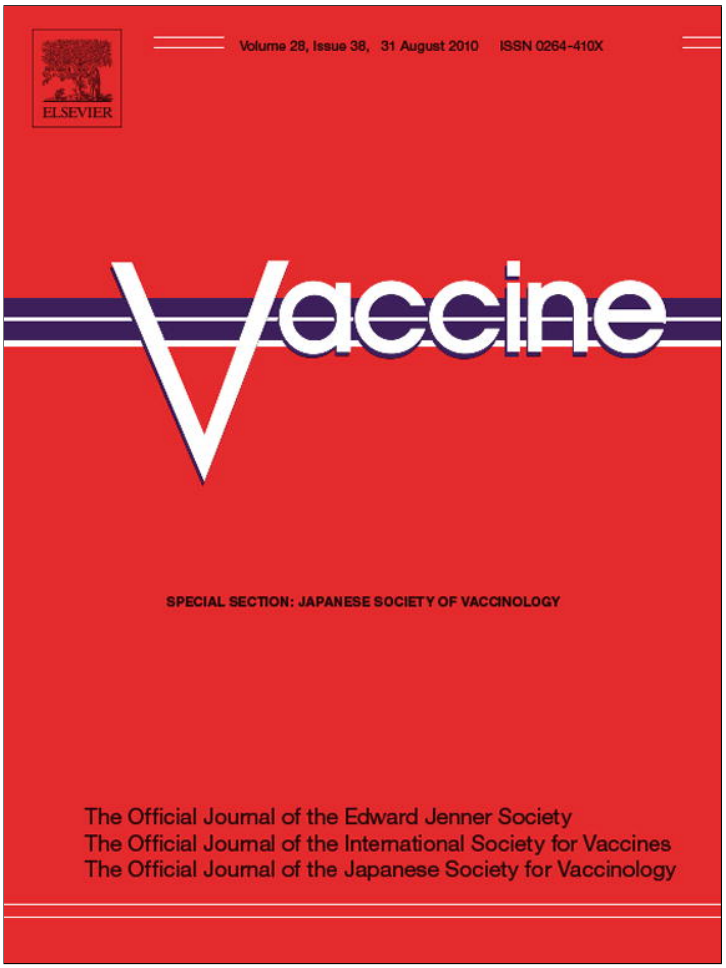

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Confidence in indirect assessment of foot-and-mouth disease vaccine potency and vaccine matching carried out by liquid phase ELISA and virus neutralization tests

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ABSTRACT

The necessity of avoiding the use of animals in vaccine potency testing has been widely recognized. The repeatability and reproducibility of the Expected Percentage of Protection (EPP) as a serological potency surrogate for A24 Cruzeiro foot-and-mouth disease virus (FMDV) strain was assessed, and compared with the results obtained with challenge in the Protection against Podal Generalization (PPG) test. To determine the EPPs, the serum titers obtained by liquid phase blocking competitive ELISA (lpELISA) and virus neutralization (VNT) in 10 potency trials using the same A24 Cruzeiro vaccine, were interpolated into previously validated logit transformation curves that correlate PPG with serology. Indirect serological assessment of vaccine matching between the serotype A FMDV strains A24 Cruzeiro and A/Argentina/01 was also carried out by lpELISA and VNT. The results obtained in this study strongly support the replacement of challenge tests for vaccine potency by indirect serological assays, at least for A24 Cruzeiro FMDV strain. While determination of EPPs by lpELISA titers showed an excellent repeatability, reproducibility and concordance with PPG for vaccine potency, assessments of cross-protection by VNT titers were more consistent with the PPG outcome.

1. Introduction

The gold standard test for foot-and-mouth disease (FMD) vaccine potency is the in vivo challenge procedure carried out in the target species to determine the efficacy of the vaccine. The manual of diagnostic tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health (OIE), describes two methods for assessing FMD vaccine potency in cattle, namely the 50% Protective Dose (PD_{50}) test and the South-American Protection against Podal Generalization (PPG) test [1]. However, the challenge tests have several drawbacks regarding standardization, cost, use of animals, facilities with high biosecurity levels, among others, and its replacement by reliable indirect tests, if possible by in vitro tests, is a priority task from the perspective of the 3R (refinement, reduction, and replacement) concept [2].

Goris et al. [3] reported the precision of the PPG test using six homologous (vaccine quality control) and four heterologous (vaccine matching) viral challenge trials. The results indicated that the homologous PPG test was more repeatable and reproducible than the PD_{50}, except for the full-dose group, which was similar to PPG. However, this study suggested that, in order to increase the test's statistical power, the number of animals should be increased, which is difficult to implement and unacceptable from the point of view of animal welfare. In this respect, indirect alternative tests for vaccine potency and vaccine matching purposes merit an important consideration, and work in this field is in progress in several laboratories.

According to the OIE Manual, indirect tests, such as measurement of virus neutralizing antibodies in cell culture, ELISA antibodies, or serum-protecting antibodies in suckling mice, may be used to assess the potency of a vaccine provided that a statistical evaluation has established a satisfactory correlation between

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Table 1
Intra-trial repeatability and inter-trial reproducibility of vaccine potency assessment by PPG or EPP for FMDV strain A24 Cruzeiro.

A24 Cruzeiro										
Trial[a]	PPG (%)	95%CI[b]	lpELISA				VNT			
			Mean titer	EPP[c] (%)	95% CI	CV%	Mean titer	EPP (%)	95% CI	CV%
1	100	[80.6–100.0]	2.50	89.9	[85.6–94.2]	8.90	1.76	93.0	[89.3–96.7]	7.50
2	93.8	[71.8–98.6]	2.66	91	[85.2–96.8]	11.98	1.81	91.9	[85.5–98.4]	13.25
3	ND	–	2.50	89.5	[86.1–92.9]	7.16	1.84	93.9	[89.6–98.2]	8.57
4	ND	–	2.27	83.7	[75.9–91.5]	17.56	1.74	92.7	[88.6–96.9]	8.35
5	ND	–	2.50	88.3	[83.5–93.1]	10.19	1.71	87.7	[81.3–94.1]	13.68
6	ND	–	2.67	91.8	[88.0–95.6]	7.74	1.57	84.0	[76.9–91.1]	15.87
7	93.8	[71.1–98.5]	2.15	80.4	[73.4–87.4]	16.31	1.38	75.4	[67.6–83.2]	19.37
8	81.3	[56.2–93.0]	2.21	83	[77.3–88.7]	12.80	1.53	83.6	[75.0–92.1]	19.20
9	87.5	[64.2–96.3]	2.38	87.3	[82.5–92.2]	10.41	1.41	73.5	[61.5–85.4]	30.47
10	75.0	[50.2–89.4]	2.49	88.9	[82.4–95.4]	13.74	1.49	82.1	[73.9–90.4]	18.85
Mean	88.5	[80.7–93.5]	2.43	87.4	[84.7–90.1]	4.32	1.63	85.8	[80.5–91.0]	8.58

ND: not determined.
[a] 16 animals per trial.
[b] From Goris et al. [3].
[c] Mean of 16 EPPs from individual animals.

the results obtained by the in vivo potency test in cattle and the alternative test, using the relevant vaccine serotype [1]. A thorough evaluation is provided by the Expected Percentage of Protection (EPP) method, which estimates the likelihood that cattle would be protected against a challenge of 10,000 infective doses after a single or boosted vaccination [4]. The clinical protection data is derived from previously performed experiments that have been carried out on a significant number of cattle immunized with the vaccine strain in question, and challenged with the homologous virus. Each animal is scored as protected or not protected, and tables of correlation based on logistic regression models are established between antibody titer and clinical protection. The

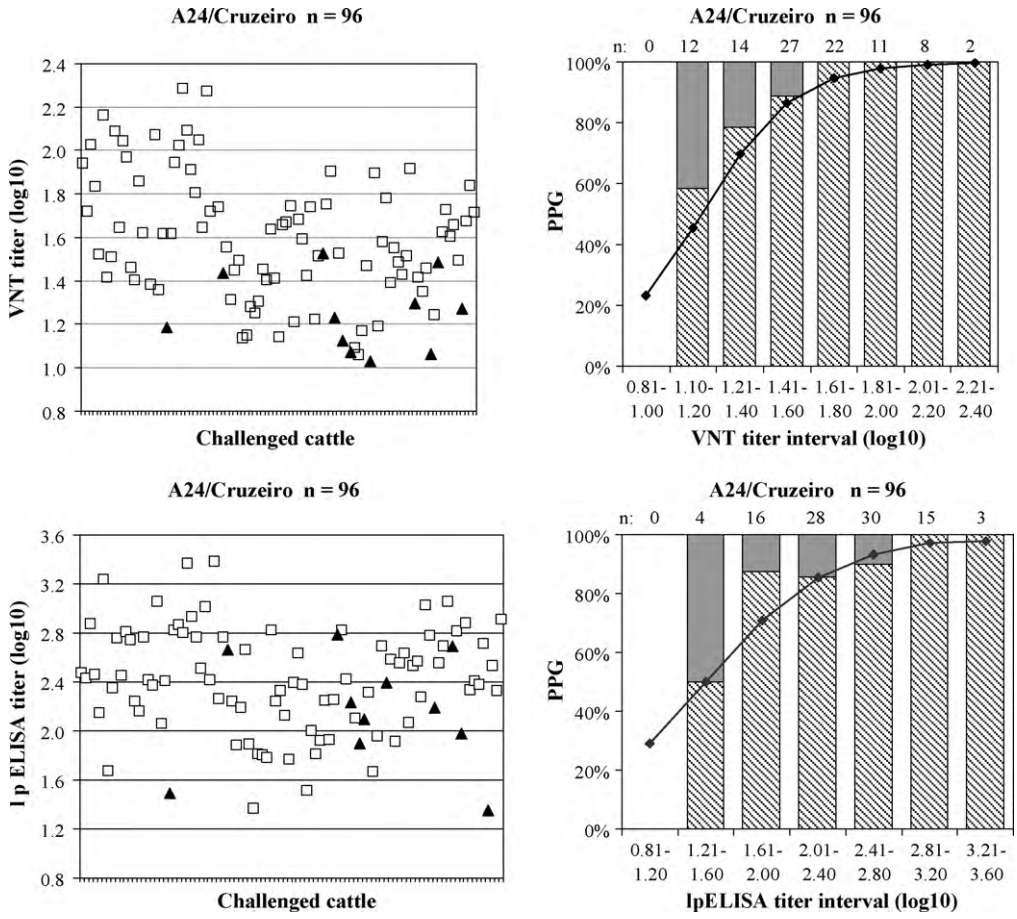

Fig. 1. Potency trials. Distribution of VNT and lpELISA titers for A24 Cruzeiro FMDV strain in six challenge trials. Left panels: dispersion of individual VNT and lpELISA titer values of protected cattle (open squares) or non-protected cattle (filled triangles) in PPG trials. Right panels: histograms showing the data of percentage of protection (PPG) for each VNT or lpELISA titer interval for FMDV strain A24 Cruzeiro. The VNT PANAFTOSA's logit transformation curve for A24 Cruzeiro is shown superimposed in the top right panel. The SENASA-CEVAN's logit transformation curve for A24 Cruzeiro is shown superimposed in the lower right panel. The number of animal sera included in each titer interval is shown at the top of each bar. EPP, Expected Percentage of Protection and *n*, number of animal sera tested.

Table 2
Comparison of cross-protection assessment between FMDV strains A24 Cruzeiro and A/Arg/01 by PPG or EPP.

A/Arg/01										
Trial[a]	PPG (%)	95%CI[b]	lpELISA					VNT		
			Mean titer	EPP[c] (%)	95%CI	EPP[c] (%)	95%CI	Mean titer	EPP[c] (%)	95%CI
1	ND	–	1.73	41.7	[31.7–51.7]	66.2	[59.1–73.3]	0.98	32.5	[21.3–43.6]
2	ND	–	2.01	59.6	[47.9–71.4]	76.8	[70.0–83.7]	0.98	38.6	[27.6–49.5]
3	56.3	[33.2–76.6]	2.01	57.9	[46.7–69.1]	76.4	[70.3–82.6]	1.17	50.6	[34.9–66.2]
4	25.0	[10.5–50.0]	1.74	41.9	[30.8–53.0]	66.0	[58.3–73.8]	0.97	29.1	[18.5–39.7]
5	12.5	[3.8–36.4]	2.15	61.9	[48.0–75.8]	78.3	[70.5–86.0]	0.85	19.8	[19.8–19.8]
6	12.5	[3.9–36.6]	2.09	63.3	[50.7–75.9]	78.9	[72.1–85.7]	0.85	19.8	[19.8–19.8]
7	ND	–	1.83	47.3	[33.6–60.9]	69.1	[60.5–77.6]	0.89	23.6	[15.5–31.8]
8	ND	–	1.83	47.7	[35.6–59.7]	69.8	[62.4–77.2]	1.18	56.7	[48.6–64.8]
9	ND	–	2.09	66.2	[54.5–77.9]	79.9	[72.5 –87.2]	0.91	25	[21.7–28.4]
10	ND	–	2.22	69.5	[55.8–83.2]	81.7	[73.6–89.7]	0.85	19.8	[19.8–19.8]
Mean	26.6	[17.4–38.5]	1.97	55.7	[48.4–63.0]	74.3	[70.1–78.5]	0.96	31.6	[22.1–41.0]
				SENASA's curve for A/Arg/01		SENASA's curve for A24		PANAFTOSA's curve for A24		

ND: not determined.
[a] 16 animals per trial.
[b] From Goris et al. [3,7].
[c] Mean of 16 EPPs from individual animals.

EPP is determined from the serological titer obtained for each individual serum, by reference to these predetermined tables of correlation between serological titers and clinical protection. The mean EPP is then calculated from the EPP obtained for each individual serum [1,3,5,6]. In Argentina, the validation of the lpELISA and the construction of correlation curves of lpELISA titers with PPG, have been reported for the four FMDV strains present in the vaccines (A24 Cruzeiro, A/Argentina/01, O1 Campos and C3 Indaial). The concordance between the in vivo and the serological outcomes was established considering 40 bovine PPG trials carried out with batches of commercial vaccines manufactured during the years 2001–2008 [6]. In a recent study, 10 PD_{50} replicates potency trials of a FMDV strain O1 Manisa vaccine were used to propose an indirect FMD vaccine potency test that directly

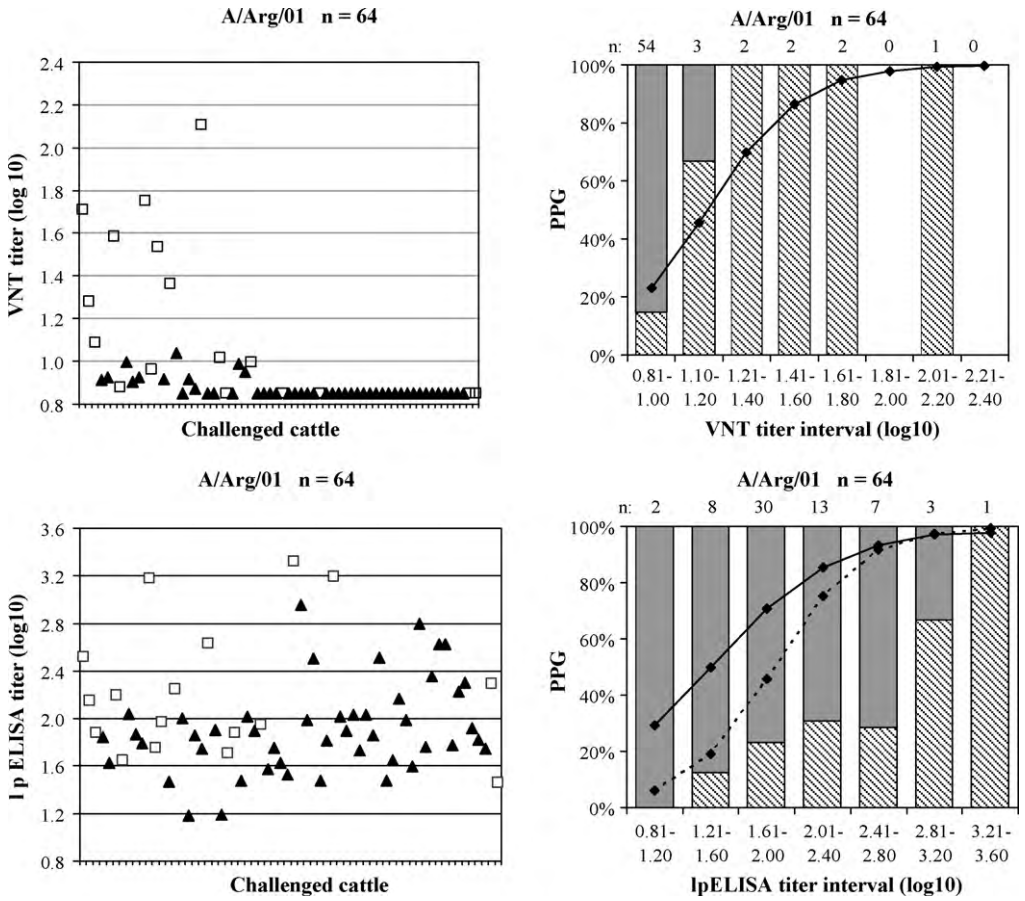

Fig. 2. Cross protection trials. Distribution of VNT and lpELISA titers for A/Arg/01 FMDV strain in four challenge trials. Left panels: dispersion of individual VNT and lpELISA titer values of protected cattle (open squares) and non-protected cattle (filled triangles) in PPG trials. Right panels: histogram showing the data of PPG for each VNT or lpELISA titer interval for FMDV strain A/Arg/01. The lpELISA SENASA-CEVAN's logit transformation curves for A24 Cruzeiro (full line) and A/Arg/01 (dotted line) are shown superimposed in the lower right panel. The number of animal sera in each titer interval is shown at the top of each bar. EPP, Expected Percentage of Protection and *n*, number of animal sera tested.

Table 3
Concordance between viral challenge and indirect serological tests.

		PPG		Total
		Approved (A24/Cruz)	Rejected (A/Arg/01)	
(A)				
EPP (lpELISA)	Approved	6	0	6
	Rejected	0	4	4
	Total	6	4	10
(B)				
EPP (VNT)	Approved	5	0	5
	Rejected	1	4	5
	Total	6	4	10

results in an estimate of the true PD_{50} content of the vaccine batch [7].

Appropriate vaccine strain selection is an important element in the control of FMD, and it is necessary for the application of vaccination programmes in FMD-affected regions, as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions. The most direct method to measure cross-protection between different strains is to vaccinate animals of the relevant target species and then to challenge them by exposure to the virus isolate against which protection is required. However, this approach is slow, expensive, and variable [3]. Moreover, the use of animals challenged with live virus for such studies should be avoided, where possible, by the use of indirect (*r* values) [8] or alternative in vitro analytical methods.

In the present study, the repeatability and reproducibility of the EPP determined by two ex vivo serological tests, lpELISA and virus neutralization (VNT) was carried out using the sera from animals involved in the 10 PPG trials described by Goris et al. [3]. The suitability of serological measurements of cross protection for vaccine matching purposes was also assessed.

2. Materials and methods

2.1. Vaccine and animal trials

PPG trials were carried out as reported previously [3,9]. Briefly, 16 animals were vaccinated with 2 ml of a monovalent vaccine batch formulation of purified A24 Cruzeiro/Brazil/55 (A24 Cruzeiro) antigen in a water-in-oil emulsion and challenged at 30 days post-vaccination (dpv) with homologous (A24 Cruzeiro) or heterologous [A/Argentina/2001 (A/Arg/01)] FMDV strains. From the number of vaccinated protected animals (i.e. absence of FMDV-induced lesions at the feet), the PPG percentage was determined. Ten independent PPG trials were conducted in Argentina according to the protocol established by the Argentine Animal Health Service (SENASA) in Act no. 351/2006 [10], except that the animals were challenged at 30 days post-vaccination (dpv) instead of at 90 dpv. All PPG trials were carried out using the same vaccine within an 11-month period (January–November 2006), during which a slight decrease in vaccine potency/stability was noted after September 2006 [3]. The sera collected at 30 dpv were used in the present study. The 95% confidence intervals (95%CI) were calculated using Soft Graph Pad PRISM 4.

The serotype A FMDV strains, A24 Cruzeiro [origin: Pan American Center for Foot-and-Mouth Disease (PANAFTOSA)] and A/Arg/01 used in VNT, were provided by SENASA.

2.2. Serological tests

lpELISA was carried out and validated as described previously [6]. VNT was conducted following the protocol of the OIE Manual [1], using BHK-21 c13 cell monolayers. The virus titers and the titer of positive working control sera were charted, monitored and compared to their predetermined values [6].

It is important to emphasize that in potency tests, all the reagents (antigen and sera) were homologous, but when determining cross-protection, the sera used were raised against the vaccine strain A24 Cruzeiro, whilst the antigen used was the heterologous A/Arg/01.

2.3. Expected Percentage of Protection

The EPP was determined from the serological titers obtained for each individual serum, referenced to predetermined tables of correlation between serological titers and clinical protection against virus challenge, based on logistic regression models, as reported previously [6,11]. The tables of correlation used in this study were: (i) SENASA-CEVAN's correlation curves for lpELISA titers for A24 Cruzeiro and A/Arg/01 strains, and (ii) the A24 Cruzeiro PANAFTOSA's curve for VNT titers. In each trial, the mean EPP (average of the 16 individual EPPs) and the 95% CI were calculated. An EPP < 75% is an indication that the vaccine will give a low protection

Table 4
Number of animals with an EPP ≥ 75% compared to the individuals protected in PPG, in six replicates of protection or four replicates of cross protection trials.

FMDV[a]	Animal status	PPG[b] (*n*)	lpELISA A24[c]	lpELISA A/Arg/01[d]	VNT[e]
Potency trials					
A24 Cruzeiro	Protected	85	84	–	74
	Unprotected	11	12	–	22
	% Protected	88.5	87.5		77.1
Cross protection trials					
A/Arg/01	Protected	17	33	17	6
	Unprotected	47	31	47	58
	% Protected	26.6	51.6	26.6	9.4

Protected: EPP ≥ 75%; unprotected: EPP < 75%.
[a] Challenge strain.
[b] No. of animals protected from challenge.
[c] SENASA-CEVAN's curve for A24 Cruzeiro.
[d] SENASA-CEVAN's curve for A/Arg/01.
[e] PANAFTOSA's curve for A24 Cruzeiro.

against homologous challenge [6]. For the calculation of the variability within each trial (intra-trial variability) and among the trials (inter-trial variability), the standard deviation (SD) and coefficient of variation (CV %) were calculated.

It is worth mentioning that in cross protection studies, A/Arg/01 was considered alternatively as an emerging strain or as a vaccine strain. This is because this strain, which represents the predominant isolate from the year 2001 outbreaks, was subsequently incorporated into the Argentine vaccine used in regular vaccination campaigns. In the first case, the A24 Cruzeiro logit regression curve was used for the determination of cross protection by EPP, while in the second case, the homologous A/Arg/01 logit regression curve [6] was used for comparison purposes.

3. Results

3.1. Vaccine potency assessment by EPP for FMDV strain A24 Cruzeiro

The A24 Cruzeiro monovalent vaccine exceeded a potency of 75%, determined by EPP using lpELISA titers in 10 replicate trials (Table 1), and by PPG after homologous challenge, in six of those trials [3]. The vaccine also reached an EPP >75% in 9 out of 10 replicate trials, when the potency was calculated with VNT titers and the PANAFTOSA's logit regression curve. In one trial (trial 9), the vaccine was rejected by VNT EPP (EPP = 73.5%), while it was approved by PPG and lpELISA.

The mean EPP of the 10 trials, calculated from lpELISA titers (87.4%) or VNT (85.8%), was very close to the mean outcome of the 6 challenge trials (PPG = 88.5%). The 95% CI of the mean lpELISA EPP or VNT EPP of the 10 trials was well above the approval limit of 75%. However, in some individual trials, the lower limit of the 95% CI was slightly below the approval value (trial 7 for lpELISA EPP, and trials 7, 9 and 10 for VNT EPP). The lower limit of the 95% CI for the PPG test was below 75% in five out of six trials (Table 1).

VNT and lpELISA titers were subsequently grouped in fixed intervals (Fig. 1, right panels). The protection levels corresponding to each group of titers (shown by the hatched bars), matched closely with the VNT and lpELISA logit regression curves for A24 Cruzeiro.

At the individual animal level, it was found that when challenged with virus homologous to the vaccine strain (A24 Cruzeiro), all animals with VNT titers above 1.53 were protected (Fig. 1, left top panel).

It is worth mentioning, that in a previous work [6] a statistically suitable correlation was established by logit regression for strains A/Arg/01, O1/Campos and C3/Indaial FMDV FMDV strains, but not for A24 Cruzeiro. In this case, the regression could not be obtained with the data available at that times ($p > 0.05$) in which only commercial vaccines were used. Afterwards, more PPG tests were carried out with A24 Cruzeiro experimental vaccines, including the six described in Goris et al. [3], and the curve is presently under validation.

3.2. Intra-trial repeatability and inter-trial reproducibility of vaccine potency assessment by EPP

The vaccine used was the same in the 10 trials, and although it showed a slight decrease in potency over time [3], it always protected over 75% of the animals by PPG. Much variation was found in the individual animal titers determined by both serological methods (Fig. 1) and consequently the EPPs showed a considerable dispersion. The CV% for intra-indirect potency repeatability (variations of the 16 EPPs calculated from the titer of individual animals within each trial) were ≤20% in the 10 trials for lpELISA and in 9 out of 10 trials of VNT, except trial 9 (CV% = 30.47) in which the vaccine was rejected by VNT EPP (Table 1). The CV% for inter-indirect potency test reproducibility (variations of the 10 EPP replicates) was 4.32% for lpELISA EPP and 8.58% for VNT EPPs.

3.3. Cross protection trials

As it was reported previously, animals vaccinated with the A24 Cruzeiro vaccine and challenged with A/Arg/01 strain in four cross protection trials (trials 3–6), were not protected by PPG [3]. In the current study, cross protection was evaluated indirectly in the 10 trials by EPP using lpELISA or VNT titers and relating them to the SENASA-CEVAN's or the PANAFTOSA logit regression curve for A24 Cruzeiro, respectively. In this case, A/Arg/01 was considered as a hypothetical emerging strain. The rationale of this procedure was based on the suggestion of the OIE Manual to determine vaccine matching through measuring the reactivity of post-vaccination sera raised against relevant vaccine strains (in this case A24 Cruzeiro), with the emerging strain (in this case A/Arg/01). The probability of protection was then assessed using the correlation curves that associate antibody titers (VNT or lpELISA) with protection from viral challenge (logit regression curves for A24 Cruzeiro).

In addition, we used those antibody titers to determine the EPPs using the homologous lpELISA logit regression curve for A/Arg/01, as it was available from previous work [6]. It has to be pointed out that this is not the case for most emerging FMDV strains, where the homologous logit regression curve would not be available. Moreover, this was not done for VNT because such curve for A/Arg/01 is unavailable from SENASA or PANAFTOSA.

The animals were not cross protected by the A24 Cruzeiro vaccine in any trial, when measured through VNT EPP and the PANAFTOSA A24 Cruzeiro curve (Table 2). The mean EPP of 10 trials obtained by VNT (31.6%) was close to the mean PPG value of the four challenge trials (26.6%).

In the case of lpELISA EPP, the results varied according to the regression curve used to relate the titers (Table 2). When the reference vaccine strain (A24 Cruzeiro) curve was used, the mean EPP (74.3%) was very close to the approval value. However, using the homologous curve for the challenge strain (A/Arg/01), the mean EPP (55.7%) was more consistent with the PPG outcome.

The upper limit of the 95% CI for lpELISA EPPs was above the approval value in 8 of the trials. In contrast the upper limit of the 95% CI for VNT EPPs was always below 75%, while for PPG exceeded 75% in one out of four A/Arg/01 trials (trial 3, Table 2).

At the individual animal level, the 47 animals unprotected after heterologous challenge with A/Arg/01 had VNT titers below 1.04, and 5 animals with titers below the detection limit (VNT < 0.85) were protected (Fig. 2). There were no unprotected animals with high VNT titers, although this number was quite small.

The distribution of A/Arg/01 VNT titers is shown in the histogram of Fig. 2 (top right panel), where the number of animals included in each titer interval is displayed at the top of the bars. Most VNT titers (84%) were found at the test detection limit (≤0.85), which corresponded to an EPP = 19.8% (Fig. 2, left panel). The histogram of lpELISA titers grouped in fixed intervals showed a different distribution, with protection values distant from both type A logit regression curves (Fig. 2, lower right panel; full line A24 Cruzeiro; dotted line, A/Arg/01).

3.4. Concordance of EPP and PPG

The concordance of the outcome of the three methods in six homologous and four heterologous trials was described in double entry tables (Table 3, panels A and B). EPPs determined by serology were highly concordant with challenge results, except for one

trial, where the A24 Cruzeiro vaccine was rejected by VNT EPP, but approved by PPG and lpELISA. The EPPs used in these tables were obtained from the correlation curve corresponding to the challenge strain.

The lpELISA EPPs were highly reliable for determination of vaccine potency, while they were not so useful in cross protection trials, when the heterologous strain A/Arg/01 was treated as an emerging strain. Considering the difference found in the results using different curves for determination of vaccine matching by lpELISA titers, the bovines were further categorized as "protected" or "unprotected" according to the individual serum titer needed to reach 75% protection in each correlation curve (Table 4). The number and the percent protected animals were consistent with the previous observation that PPG and VNT seemed more reliable than lpELISA for vaccine matching assessment, provided an lpELISA correlation curve for the emerging strain is unavailable. Interestingly, when using the homologous A/Arg/01 curve, the lpELISA results matched exactly the PPG results (26.6%). On the other hand, lpELISA results for vaccine potency purposes (Fig. 2, top panel) showed a better match with PPG than VNT.

4. Discussion

There is a strong consensus worldwide on the necessity to replace experiments using live viral challenge. Considering the economical importance of FMD, the confidence in indirect (ex vivo) tests is crucial and a correlation with the in vivo test has to be thoroughly statistically demonstrated. In Argentina, the PPG test has been partially replaced by EPP determined by lpELISA titers in sera of vaccinated bovines [6], although viral challenge is still used in case of vaccines under licensing process, for new strains to be included in the vaccines, or for vaccine matching purposes.

In this study, we determined the repeatability and reproducibility of the EPP in 10 replicates of PPG trials with the same A24 Cruzeiro vaccine. Although potency trials are intrinsically very variable and the influence of many factors must be considered, the 10 in vitro trials behaved similarly and the inter-trial variation was within acceptable limits of reproducibility, especially for homologous EPPs determined by lpELISA titers (CV 4.32%, Table 1). This variation was lower than the variation obtained in PPG (CV 10.4%) for the same A24 Cruzeiro vaccine.

On average, the mean EPP values obtained from VNT and lpELISA titers for the A24 Cruzeiro vaccine were highly comparable between each other and with PPG. One of the conclusions arising from this study is that the EPP determined by lpELISA titers is an indirect method statistically reliable to be used as a surrogate of protection for vaccine potency purposes, with an excellent concordance with the PPG outcome. Based on EPP results, the VNT falsely rejected the vaccine batch in one out of 10 occasions (trial 9, Table 1). As the CV% variation obtained in this particular VNT EPP determination was high (CV 30.47%), it should be necessary to introduce in the future more appropriate validation criteria for the VNT, similarly to what has been done for the lpELISA test in use [6]. Based on the limited data available, the results indicated that the VNT correlation curve obtained in this study for A24 Cruzeiro strain had a good fitting with the one developed by the Cuenca del Plata project [11] (Fig. 1).

When a new FMDV strain emerges in the field, there is no statistical correlation curve readily available for that isolate. It has been suggested that, besides the challenge method, a post vaccinal serum panel derived from existent vaccines strains may be used for indirect assessment of the degree of matching by *r* values or by EPP. In view of the results obtained in this study, the use of an indirect assay more related with protection, such as VNT, may reflect better the outcome of PPG trials for vaccine matching. It has been reported previously that the same is true for *r* values determination [8]. Although non-neutralizing antibodies may also be protective, in vitro neutralization may be more relevant to in vivo protection than other measures of virus–antibody interaction [12].

In cross protection trials, it is worth mentioning, that the results obtained using the lpELISA logit regression homologous curve for A/Arg/01 were consistent with those of PPG in heterologous challenge tests. However, in most outbreaks, a curve for the new emerging strain would not be available. In this regard, the results obtained with the lpELISA curve of A24 Cruzeiro are misleading; although consistent with the fact that protection against A/Arg/01 requires higher lpELISA titers than for A24 Cruzeiro [6]. The lpELISA EPPs (homologous A/Arg/01 curve) indicated in all cases a lack of cross protection, although the data appeared to overestimate the degree of protection observed in vivo, and there were four trials in which it was not possible to state with 95% confidence that less than 75% of the animals would be protected. On the other hand, the analysis of VNT EPPs for A/Arg/01 was limited by the fact that a validated correlation homologous curve of VNT titers with PPG is unavailable for this strain.

The possibility of including and monitoring standard reagents and internal control sera increases the level of confidence in the results from serological tests and in the overall potency test system, a procedure which is not feasible for the in vivo challenge tests. It is known that indirect methods need to be standardized in each country or laboratory for the particular conditions (reagents, vaccines, serological techniques), and that serum titers obtained by different groups with different test systems cannot be directly compared [13]. Nevertheless, they are less variable, more easily standardized and in addition save the lives of many animals, contributing to the 3R rule.

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