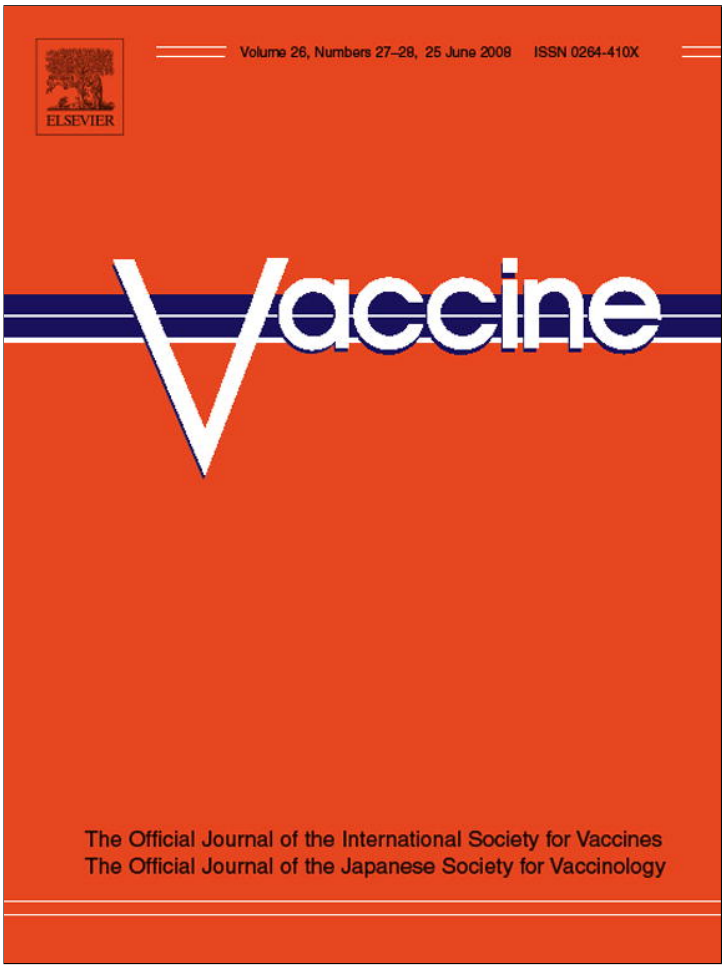

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Foot-and-mouth disease vaccine potency testing in cattle using homologous and heterologous challenge strains: Precision of the "Protection against Podal Generalisation" test

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ABSTRACT

The level of protection conferred by foot-and-mouth disease (FMD) vaccines in primovaccinated animals primarily depends on the potency of the vaccine and the relatedness of the vaccine strain and circulating field isolate. The "Gold Standard" FMD vaccine potency test is the in vivo test performed in the target species. The objective of the study was to determine the precision of the in vivo "Protection against Podal Generalisation" (PPG) FMD vaccine potency test in cattle using homologous (vaccine quality control) and heterologous (vaccine matching) viral challenge. The overall level of protection induced by the A_{24} Cruzeiro/Brazil/55 vaccine used in six homologous PPG tests was 88.5%. Vaccine accordance (VACC) and vaccine concordance (VCON) were estimated to be 75.9% and 73.7%, respectively. In four heterologous challenge PPG tests, the overall level of cross-protection induced by the A_{24} Cruzeiro/Brazil/55 vaccine against A Argentina/2001 challenge was 26.6%, with VACC and VCON values of 65.7% and 59.2%, respectively. Results indicate that the homologous PPG test is more reliable than the European Pharmacopoeia potency test, but that a larger number of animals should be used in order to increase the test's statistical power. In this regard, indirect alternative tests for vaccine potency and vaccine matching merit consideration.

1. Introduction

Foot-and-mouth disease (FMD) is caused by a member of the genus *Aphthovirus* within the *Picornaviridae* family [1]. The FMD virus (FMDV) affects multiple species, has an extremely high mutation rate (7 different serotypes and multiple subtypes have been identified) [2] and is very disruptive to normal life and economic activity [3]. Moreover, FMDV is globally ranked by veterinary authorities as the first and foremost priority [4]. Given the severe socio-economic consequences related to FMD outbreaks and incursions, different control strategies are implemented worldwide to contain and/or eradicate the disease (reviewed by ref. [5]). In regions of the world where FMD is endemic (e.g. India, China, and certain African countries) vaccination is at the forefront of disease control tools. Other countries and zones are officially recognised by the World Organisation for Animal Health (OIE) as free from FMD with vaccination (e.g. Argentina and Uruguay) [6], and the majority of FMD-free countries/zones without vaccination store strategic FMDV inactivated antigens over liquid nitrogen for rapid formulation into vaccines in case of an emergency (i.e. antigen banks) [7]. The latter is also considered in countries/zones where vaccination is practiced by storing antigens from strains different from those included in the current vaccine.

Regardless of the setting, the efficacy of any vaccination programme will largely depend on the quality (purity, safety and potency) and suitability (vaccine matching by selecting appropriate FMDV strains for inclusion into vaccines) of the chosen vaccine. Whereas guidelines exist to ensure the production of high

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quality FMD vaccines (e.g. European Pharmacopoeia Monograph 04/2005:0063 (Ph.Eur.) [8], Act No. 351/2006 of the Argentine Animal Health Service (SENASA) [9]), neither reagents nor methods for vaccine matching and FMDV strain selection are fully harmonised and only limited in vivo cross-protection data is available (reviewed by ref. [10]).

The most important vaccine quality parameter in conferring protection against FMDV infection in primovaccinated animals is the potency of the vaccine, which is usually determined by experimentally infecting vaccinated cattle. The OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals describes two methods for assessing FMD vaccine potency in cattle, namely the Ph.Eur. 50% protective dose (PD_{50}) test and the South-American "Protection against Podal Generalisation" (PPG) test [11]. Whereas three groups of five animals are vaccinated 21 days prior to live FMDV challenge with, respectively, the full, a fourth or a 16th of the vaccine dose in the PD_{50} test, the PPG test uses 16 cattle, all of which are vaccinated with the full vaccine dose before viral challenge. In both in vivo tests, two unvaccinated control animals are also included. Recently, Goris et al. [12], using a 9.99 PD_{50} monovalent FMDV O_1 Manisa vaccine, showed that the Ph.Eur. FMD vaccine potency test suffers from low in vivo repeatability and reproducibility making it impossible to distinguish between a potency of 3, 6 or even 10 PD_{50} based on the outcome of a single potency trial.

One of the objectives of the present study is, thus, to determine in vivo measures for intra-potency test repeatability and inter-potency test reproducibility of the South-American PPG FMD vaccine potency test by using a monovalent FMDV A_{24} Cruzeiro/Brazil/55 vaccine and the homologous FMDV A_{24} Cruzeiro/Brazil/55 challenge virus in six replicate PPG tests. The study further aims at providing data on in vivo cross-protection and on the precision/reliability of such in vivo data. Therefore, naïve cattle vaccinated with the same FMDV A_{24} Cruzeiro/Brazil/55 vaccine as used in the homologous replicate trials, were challenged using an intratypic heterologous FMDV A Argentina/2001 strain. The heterologous PPG set-up was performed four times under standard operating conditions.

2. Materials and methods

2.1. Vaccine and challenge strains

The vaccine batch was an experimental single water-in-oil emulsion of partially purified and polyethylene glycol concentrated, inactivated FMDV strain A_{24} Cruzeiro/Brazil/55 [origin of the strain: Pan American Centre for Foot-and-Mouth Disease (PANAFTOSA); provided by SENASA]. The monovalent emulsion consisted of a 60% Montanide ISA 50® (Seppic, France) oil phase and a 40% aqueous phase in which the virus and saponin (3 mg/vaccine dose) was suspended. The full cattle vaccine dose contained 10 μg of purified 140S antigen. The vaccine was produced according to Good Manufacturing Practice (GMP) by Biogénesis Bagó S.A. (Buenos Aires, Argentina). The in-process and final vaccine batch was sterility and safety tested and complied with all requirements of the OIE Manual [11] and Argentine regulations [9]. The vaccine batch was divided in 93 identical 100 ml polypropylene bottles, stored at 4–8 °C and used in subsequent vaccine potency trials.

The challenge FMDV strain A_{24} Cruzeiro/Brazil/55 (abbreviated as A_{24} Cruzeiro) was a baby hamster kidney (BHK-21) cell adapted strain obtained from PANAFTOSA and passaged once in bovine tongue epithelium before use. The FMDV strain A Argentina/2001 (abbreviated as A Arg 2001) was a field isolate obtained from the FMD outbreak in 2001 in Trenque Lauquen (Buenos Aires, Argentina) that was passaged twice in bovine tongue epithelium. Both tongue epithelia were homogenised in Eagle's medium and subsequently fractionated in individual vials conserved over liquid nitrogen until the day of experimental challenge. Both challenge virus strains were titrated in 4-day-old suckling mice to determine the 50% lethal dose ($SMLD_{50}$), a titration method considered to be equivalent to in vivo titration in cattle tongue epithelium to determine the 50% bovine infectious dose [9].

2.2. Animals

The cattle used originated from Patagonia (Argentina), a region officially recognised by the OIE as free from FMD without vaccination [6]. All were steers, between 24 and 30 months of age and weighing 280–350 kg. The animals selected for this study were healthy, had a good nutritional status and were free of parasites. Prior to the study, all cattle were bled and the absence of anti-FMDV antibodies was checked using the liquid-phase blocking ELISA (lpELISA) [13,14], the 3ABC ELISA [15] with enzyme-linked immunoelectrotransfer blot (EITB) [16], and the solid phase competition ELISA (SPCE) [17].

2.3. PPG potency tests and data recording

In total, six identical FMDV A_{24} Cruzeiro PPG vaccine potency tests were performed using homologous challenge with FMDV A_{24} Cruzeiro, and four identical FMDV A_{24} Cruzeiro PPG vaccine potency tests were performed using heterologous challenge with FMDV A Arg 2001. All potency tests were conducted according to SENASA Act No. 351/2006 [9], except that animals were challenged at 30 days post-vaccination (dpv) instead of 90 dpv. Challenge at 30 dpv is, however, considered in the OIE Manual [11].

Briefly, 16 individually, ear-tag marked cattle were vaccinated intramuscularly in the upper part of the neck with a full cattle vaccine dose (2 ml) and two unvaccinated control animals were included in each potency trial. Thirty dpv all vaccinated animals and both control cattle were challenged by inoculating 10^4 $SMLD_{50}$ of challenge virus intradermally into four different sites on the upper surface of the tongue (0.25 ml per site). Seven days post-challenge (dpc) all animals were clinically checked for FMDV-induced lesions at the site of challenge and on the feet [9]. From the number of vaccinated protected animals (i.e. absence of FMDV-induced lesions at the feet), the PPG percentage was determined according to the following formula [9]:

$$\%\text{PPG} = \frac{s}{n} \times 100 \quad (1)$$

in which s is the number of vaccinated-protected animals and n is the total number of vaccinated animals. Uncertainty (i.e. 95% confidence intervals (CI)) around s/n was estimated by simulation from the Bayesian posterior distribution of s/n as described by Goris et al. [12].

The trial was considered valid if both non-vaccinated control animals showed FMDV-induced foot lesions. According to SENASA Act No. 351/2006 [9], a vaccine batch is approved for licensing if at least 12 out of the 16 vaccinated animals are found to be protected (i.e. 75.0% PPG). A vaccine batch is retested if 10–11 vaccinated cattle are protected against challenge (i.e. 62.5–68.8% PPG), and a vaccine batch is rejected if 9 or less vaccinates show absence of lesions on the feet (i.e. inferior to 62.0% PPG).

All six homologous PPG vaccine potency tests were performed within a timeframe of 11 months; whereas the four heterologous PPG tests were concluded within 7 months following vaccine production. Serum samples were collected prior to vaccination (0 dpv) and at 30 dpv.

Table 1
Summary of six identical, replicate FMDV A_{24} Cruzeiro vaccine potency tests using homologous FMDV A_{24} Cruzeiro challenge

Trial	Date of vaccination	Date of challenge	Vaccinated animals		PPG (%) [95% confidence interval]
			Protected	Total	
1	18 January 2006	17 February 2006	16	16	100.0 [80.6–100.0]
2	18 January 2006	17 February 2006	15	16	93.8 [71.8–98.6]
3	10 October 2006	9 November 2006	15	16	93.8 [71.1–98.5]
4	10 October 2006	9 November 2006	13	16	81.3 [56.2–93.0]
5	7 November 2006	7 December 2006	14	16	87.5 [64.2–96.3]
6	7 November 2006	7 December 2006	12	16	75.0 [50.2–89.4]
Overall	–	–	85	96	88.5 [80.7–93.5]

2.4. Vaccine accordance and vaccine concordance analysis

The terms vaccine accordance (VACC) and vaccine concordance (VCON) were used for FMDV vaccine potency testing by Goris et al. [12] to assess vaccine intra-potency test repeatability (i.e. the percentage chance of finding the same result for two similarly vaccinated animals with regard to their protection status within the same potency test under standard operating conditions) and vaccine inter-potency test reproducibility (i.e. the percentage chance of finding the same result for two similarly vaccinated animals in different potency tests using the same vaccine batch under standard operating conditions), respectively, and were estimated according to the following formulae [12]:

$$\mathrm{VACC} = \frac{1}{n}\sum_{i=1}^{n} p_{\mathrm{o},i}^2 + p_{1,i}^2 \quad (2)$$

$$\mathrm{VCON} = P_{\mathrm{o},i}^2 + P_{1,i}^2 \quad (3)$$

in which n is the number of individual vaccine potency tests performed, $P_{\mathrm{o}} = (1/n)\sum_{i=1}^{n} p_{\mathrm{o},i}$, $P_1 = (1/n)\sum_{i=1}^{n} p_{1,i}$, $p_{\mathrm{o},i}$ and $p_{1,i}$ are defined for each vaccine potency test i as the proportion of unprotected and protected animals, respectively.

Confidence intervals around VACC and VCON were obtained by Bayesian inference as described above. Following their definition, VACC and VCON range from 50.0 to 100.0%. Hence, the worst possible result for both parameters is 50.0%. Consequently, the 50.0% value will never be included in the CI around both parameters of vaccine potency test precision.

3. Results

3.1. Animal trials

Based on the results of the lpELISA, the 3ABC ELISA with EITB and the SPCE on serum samples taken prior to vaccination, no anti-FMDV antibodies were found in any of the animals used in the study. Moreover, none of the control animals had detectable anti-FMDV antibody levels at 30 dpv in any of the assays used. No disease or injury contracted during the course of the animal trials led to the exclusion of cattle from the study.

Furthermore, inspection of the site of challenge and the feet of all non-vaccinated control cattle at 7 dpc revealed primary FMDV-induced lesions on the tongue epithelium of all control animals and secondary lesions on 76 out of 80 feet clearly indicating signs of generalised FMD. Hence, all 160 vaccinated and 20 control animals were included in subsequent analyses.

3.2. Precision of the homologous PPG vaccine potency test

Table 1 depicts the results obtained for six identical, replicate A_{24} Cruzeiro PPG vaccine potency tests using homologous FMDV strain A_{24} Cruzeiro challenge. The number of protected animals per PPG potency test ranged from 12 to all animals protected with a corresponding %PPG ranging 75.0 to 100.0%. Overall, 88.5% PPG was observed when all 96 vaccinated animals were taken into account. The 95% CI around the overall %PPG ranged from 80.7 to 93.5%. Although a slight decreasing trend in mean %PPG was noted in time, the 95% CI of each individual vaccine potency test were largely overlapping indicating no significant differences in %PPG for all six replicate potency trails (Fig. 1). This justified the pooling of all data to calculate VACC and VCON, which were estimated to be 75.9% [95% CI: 64.9–86.2] and 73.7% [95% CI: 62.1–84.3], respectively (Table 2).

3.3. Precision of the heterologous PPG vaccine potency test

Table 3 depicts the results obtained for four identical, replicate A_{24} Cruzeiro PPG vaccine potency tests using heterologous FMDV strain A Arg 2001 challenge. The number of protected animals per PPG potency test ranged from 2 to 9 animals protected with a corresponding %PPG ranging from 12.5 to 56.3%. Overall, 26.6% PPG was observed when all 64 vaccinated animals were taken into account. The 95% CI around the overall %PPG ranged from 17.4 to 38.5%. As for the homologous PPG vaccine potency tests, the 95% CI for each individual PPG test were markedly wider due to the smaller number of animals used.

One of the two heterologous vaccine potency trials performed between 14 February 2006 and 16 March 2006 had a significantly higher percentage of animals protected against challenge (i.e. 9 out of 16 vaccinated cattle were protected against generalised FMD) than the %PPG observed for the remaining vaccine potency tests. Therefore, VACC and VCON were calculated based on all four heterologous PPG vaccine potency tests and based on the results of the last three heterologous PPG vaccine potency tests (Table 2). When excluding the first heterologous PPG vaccine potency test, slightly

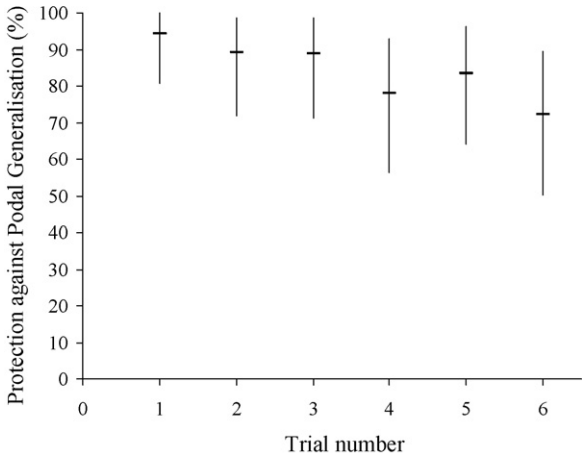

Fig. 1. The Bayesian mean percentage PPG and 95% confidence intervals for six identical FMDV A_{24} Cruzeiro vaccine potency trials with FMDV A_{24} Cruzeiro challenge.

Table 2
Vaccine accordance and vaccine concordance for FMDV A_{24} Cruzeiro PPG vaccine potency testing using homologous FMDV A_{24} Cruzeiro and heterologous A Arg 2001 challenge

	FMDV vaccine strain/FMDV challenge strain		
	A_{24} Cruzeiro/A_{24} Cruzeiro ($n=6$)	A_{24} Cruzeiro/A Arg 2001 ($n=4$)	A_{24} Cruzeiro/A Arg 2001 ($n=3$)
Vaccine accordance (%)	75.9 [64.9–86.2][a]	65.7 [50.7–80.3]	69.8 [52.5–86.0]
Vaccine concordance (%)	73.7 [62.1–84.3]	59.2 [50.0–74.0]	68.1 [50.3–84.4]

[a] 95% confidence interval was calculated using Bayesian simulation from posterior [12].

Table 3
Summary of four identical, replicate FMDV A_{24} Cruzeiro vaccine potency tests using heterologous FMDV A Arg 2001 challenge

Trial	Date of vaccination	Date of challenge	Vaccinated animals		PPG (%) [95% confidence interval]
			Protected	Total	
1	14 February 2006	16 March 2006	9	16	56.3 [33.2–76.6]
2	14 February 2006	16 March 2006	4	16	25.0 [10.5–50.0]
3	19 September 2006	19 October 2006	2	16	12.5 [3.8–36.4]
4	19 September 2006	19 October 2006	2	16	12.5 [3.9–36.6]
Overall	–	–	17	64	26.6 [17.4–38.5]

higher estimates of VACC and VCON were obtained although these differences were not significant at the 95% confidence level.

4. Discussion

Historically, FMD vaccine potency testing is performed in vivo and to date the OIE prescribes two such direct methods in cattle, the PD_{50} test and the PPG test. Given the current stringent demands of accreditation guidelines such as ISO/IEC 17025:2005 [18], vaccine potency test procedures, much like any other test system, should produce reliable and reproducible results with a certain degree of statistical confidence (usually 95%). This is of particular importance since pursuant to Ph.Eur. and OIE requirements alternative indirect potency methods based on serology, for instance (e.g. refs. [14,19]) are only accepted if deemed suitable and validated [8,11]. Suitable tests are subsequently defined as those tests for which the correlation with the in vivo test has been demonstrated [8]. '*In the establishment of any correlation it is important to remember that one may be attempting to correlate a relative precise alternative test with a statistically questionable but prescribed test. Our natural prejudice, particularly if the alternative test is an in vitro procedure and the prescribed test is an in vivo procedure is to give the benefit of the doubt to the latter*' [20]. It is, therefore, absolutely vital to understand the performance characteristics of the prescribed in vivo "Gold Standard" FMD vaccine potency tests.

The Ph.Eur. PD_{50} test has been in use for over 50 years, but up until recently a measure of its in vivo between-test variability was lacking. In 2007, however, Goris et al. [12] demonstrated that the Ph.Eur. vaccine potency test suffers from low in vivo repeatability and reproducibility due to the limited number of animals used in each vaccine dose group. Based on computer simulation, it was postulated that the South-American PPG test in which all animals are vaccinated with a full cattle dose of vaccine would be a more reliable method for measuring vaccine potency if at least 15 animals were used [21]. This hypothesis was put to the test by Vianna Filho et al. [22] who compared the Ph.Eur. PD_{50} test to the PPG test. Their study concluded that the PPG test is the preferred direct method for FMD vaccine potency testing in cattle since it produced consistent results in 64 out of 65 replicate tests (20 replicate tests using batch 005/88 of a FMDV O_1 Campos-Br/58 vaccine; 17 replicate tests using batch 003/88 of a FMDV O_1 Campos-Br/58 vaccine; 13 replicate tests using batch 002/87 of a FMDV A-79 Venceslau-Br/76; 8 replicate tests using batch 001/87 of a FMDV O_1 Campos-Br/58 vaccine; 7 replicate tests using batch 004/87 of a FMDV O_1 Campos-Br/58 vaccine), in which consistent was interpreted as giving a %PPG equal to or greater than 75.0%. The authors, however, did not quantify this level of consistency and information on the 140S content of the vaccines, one of the major determinants in conferring protective immunity [23,24], was lacking. The present study was undertaken to address these gaps and also to provide valuable in vivo data on the reliability of cross-protection studies, which are due to financial considerations more frequently conducted through in vitro serological methods based on *r*-values (i.e. a measure of the antigenic relatedness between a FMD vaccine strain and a heterologous field strain) [25–27] and sequencing [28].

The data presented here indicate that the %PPG of six valid, replicate PPG tests using homologous live FMDV A_{24} Cruzeiro challenge ranged from 75.0 to 100.0%. In other words, even if a slight decrease in the potency of the experimental vaccine was noted in time (Fig. 1), the vaccine batch would still fulfil potency requirements for official batch release (i.e. licensing authorisation) at any given moment during the course of the study (%PPG $\geq$ 75.0). The overall level of protection induced by the A_{24} Cruzeiro vaccine was 88.5% [95% CI: 80.7–93.5], which confirms the statement made by Sutmöller [21] that approximately 99.0% of all vaccines batches that induce a level of protection of 90% in the population, which corresponds to a vaccine of approximately 6 PD_{50} [22], will be approved with a pass mark of 12/16 protected cattle. This means that based on the 75.0% acceptance criterion, a distinction between good ($\geq$90.0% protection at population level) and poorer vaccines should be possible. However, when calculating the 95% confidence limits around the %PPG using Bayesian simulation from posterior (Table 1 and Fig. 1), it should be noted that due to the limited number of animals ($n=16$) used in vaccine potency testing, only 1 out of the six trials (i.e. trial 1) provides sufficient confidence (i.e. lower limit of the 95% CI equal or superior to 75.0% PPG) to allow licensing of the product. This in turn means that all 16 vaccinated animals have to be protected to enable acceptance of the vaccine with 95% confidence. The moment one unprotected-vaccinated animal is present in any PPG trial, the lower 95% confidence limit drops below the pass mark of 75.0% and was found to be 71.1–71.8%. Increasing the number of animals to 19 would be one way of solving the lack of statistical power. However, in practice usually more vaccinated-unprotected animals are observed during FMD vaccine potency testing. Realistically, further increasing the number of animals to 25 to allow for 2 unprotected vaccinated animals (data not shown) is not an option as this would make vaccine potency testing unfeasible and unethical from an animal welfare point of view. Nevertheless for

Table 4
Vaccine accordance and vaccine concordance for replicate PPG vaccine potency tests and overall percentage PPG for FMDV serotypes O and A performed by Vianna Filho et al. [22]

Vaccine batch	FMDV strain	PPG (%)	VACC (%) [95% confidence interval]	VCON (%) [95% confidence interval]
001/87	O1 Campos-Br/58	92.2	79.8 [70.5–88.2]	78.3 [68.6–86.9]
002/87	A-79 Venceslau-Br/76	97.6	87.1 [81.0–92.4]	85.6 [79.1–91.1]
004/87	O1 Campos-Br/58	84.6	70.0 [59.3–80.4]	67.6 [56.5–78.3]
003/88	O1 Campos-Br/58	90.8	78.7 [72.4–84.5]	76.3 [69.8–82.5]
005/88	O1 Campos-Br/58	93.8	82.0 [76.7–87.0]	80.3 [74.6–85.6]

those vaccines that, under the current SENASA guideline [9], have to undergo retesting (10–11 protected animals out of 16 vaccinated cattle), confidence in the outcome of the PPG test could be increased by combining the re-test results with the initial test results as confidence limits could then be calculated on the basis of 32 animals.

The between-test variability (i.e. VACC and VCON) of the test procedure should also be taken into account when interpreting the results of vaccine potency tests. The homologous set-up, which is the best approach to assess the precision of the PPG test, results in measures of VACC and VCON of 75.9% [95% CI: 64.9–86.2] and 73.7% [95% CI: 62.1–84.3], respectively, indicating that the between-test variability of PPG vaccine potency test is lower than for the Ph.Eur. PD_{50} vaccine potency test (i.e. 67.6% [95% CI: 63.2–72.1] and 58.8% [95% CI: 54.8–63.1], respectively [12]), with the difference in VCON between both potency test methods being significant at the 95% confidence level. In other words, the percentage chance of finding the same result with regard to their protection status for two similarly vaccinated animals in different PPG tests is significantly higher than for PD_{50} FMD vaccine potency tests. The lower reliability of the Ph.Eur. test is likely due to the inclusion of a group of animals receiving a 16th of the vaccine dose (i.e. 0.125 ml of vaccine), which is at the limit of what can be administered to animals with an acceptable level of reproducibility. The difference in VACC and VCON between both vaccine potency tests is in fact not significant when only animals receiving a full vaccine dose are considered for the PD_{50} test [12]. Furthermore, regardless of the potency test procedure, when animals are vaccinated with the full vaccine dose, there is about as much chance of obtaining two protected or two unprotected animals within one trial as between trials, meaning that all other variation observed is due to sources other than the vaccine itself. These results are in line with the outcome of previously performed replicate PPG potency tests for FMDV serotypes O and A, for which the estimates of VACC and VCON are calculated here according to the above-described method based on the results published by Vianna Filho et al. [22] and are given in Table 4. Based on the combined results of Table 4 and the present study, a positive linear correlation between %PPG and VACC ($y = 1.29x - 38.5$; $R^2 = 0.9961$) and VCON ($y = 1.37x - 47.6$; $R^2 = 0.9985$) is observed at least for the range of PPG values used (Fig. 2). Not surprisingly, this means that the precision of the PPG test depends on the overall potency of the vaccine batch, with more potent vaccines resulting in more consistent results.

The second objective of the study was to provide data on in vivo cross-protection and on the reliability of using heterologous PPG vaccine challenge tests as indicators of vaccine strain selection and thus cross-protection in the field. The results obtained indicate that vaccination with a fairly potent FMDV A_{24} vaccine (i.e. 88.5% PPG or approximately 6 PD_{50} [22]) does not provide sufficient protection against experimental infection with FMDV A Arg 2001 since four replicate PPG test resulted in percentage of protection levels ranging from 12.5 to 56.3% with an overall potency of 26.6% PPG [95% CI: 17.4–38.5] far below the acceptable level of 75.0%. These findings support observations in the field during the 2001 FMDV type A outbreak in Argentina [29] and subsequent molecular epidemiological results indicating RNA sequence and critical deduced amino acid changes between FMDV A_{24} Cruzeiro and FMDV A Arg 2001 [30], which led to the inclusion of FMDV A Arg 2001 in the current vaccine formulation. Hence, PPG vaccine potency testing using heterologous challenge strains are good predictors of cross-protection, but require time and money.

However, one heterologous trial stands out (i.e. trial 1) for which it cannot be stated with 95% confidence that less than 75.0% of the animals would be protected against live virus challenge. A clear explanation for this more elevated level of protection, apart from a possible decrease in vaccine potency over time, cannot be provided at present, especially not since trial 2 was performed at exactly the same time and resulted in 25.0% PPG [95% CI: 10.5–50.0]. It may well be due to the lack of precision of PPG testing using vaccine strains and challenge isolates for which the level of cross-protection is expected to be low (based on either field observations, sequence date or *r*-values) as poorer estimates of precision (VACC and VCON) of the PPG potency test are found than for homologous PPG trials. Moreover, the lower level of the 95% CI around the VACC and VCON estimates (based on three or three replicate PPG tests) approach 50.0%, meaning that it is just as likely that two similarly vaccinated animals are both protected, both unprotected or differ in their protection status. It may be more reliable to perform a series serological indirect vaccine matching tests and base the vaccine strain selection on the in vitro expected percentage protected [25] or on a significantly high number of *r*-value tests. For instance, Mattion et al. [29] reported, based on the virus neutralisation test, an *r*-value of 0.15 against A Arg 2001 for animals vaccinated with a FMDV A_{24} Cruzeiro vaccine which is consistent with the low level of cross-protection observed in vivo.

In conclusion, the in vivo "Gold Standard" test for official vaccine potency quality control in cattle, either the Ph.Eur. PD_{50} or South-American PPG test, has proven its value in the past and should

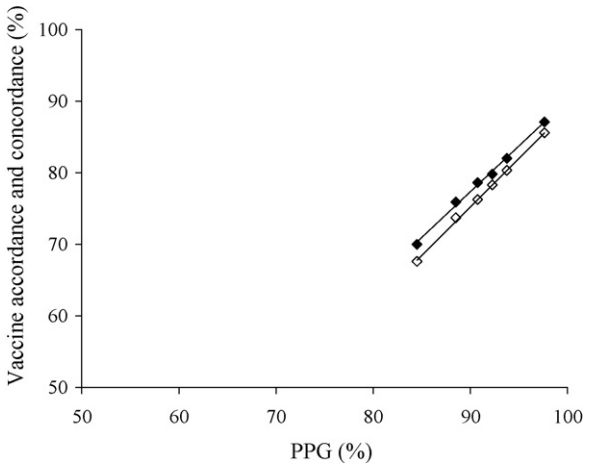

Fig. 2. Correlation between precision [vaccine accordance (filled diamonds) and vaccine concordance (open diamonds)] and the percentage PPG for FMDV serotypes O and A.

not be dismissed *per se*, although its limitations should be better recognised. Alternative indirect potency methods are usually more precise (repeatable and reproducible) and easier to standardise. With regard to vaccine matching, heterologous PPG tests are good indicators of the protection observed in the field, but are expensive, and when low levels of protection are expected, their reliability is questionable. From the perspective of the 3R (Refinement, Reduction, Replacement) [31] concept, the results of this study and of previous publications [12,22] thus favour further research into and acceptance of indirect alternatives to in vivo potency testing and in vivo vaccine matching.

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