Journal of Virological Methods 276 (2020) 113786

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

FMD vaccine matching: Inter laboratory study for improved understanding of r_1 values

Tom Willems[a,2], Annebel De Vleeschauwer[a,2], Mariano Perez-Filgueira[g], Yanmin Li[c], Anna Ludi[d], David Lefebvre[a], Ginette Wilsden[d], Bob Statham[d], Bernd Haas[e,1], Nora Mattion[f], Blanca Robiolo[f], Claudia Beascoechea Perez[b,1], Eduardo Maradei[b], Eliana Smitsaart[h], José La Torre[f,1], Kris De Clercq[a,*]

[a] Unit Exotic Viruses and Particular Diseases, SD Infectious Diseases in Animals, Sciensano (formerly CODA-CERVA), Groeselenberg 99, 1180 Brussel, Belgium
[b] Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), FMD Reference Laboratory, Talcahuano 1660, Martinez, Buenos Aires, Argentina
[c] The Chinese National/OIE Reference Laboratory for Foot and Mouth Disease, Lanzhou Veterinary Research Institute, The Chinese Academy of Agricultural Sciences (CAAS), Lanzhou, Gansu, PR China
[d] The Pirbright Institute, Ash Road, Pirbright, Surrey, GU24 0NF, United Kingdom
[e] Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Südufer 10, 17493 Greifswald-Insel Riems, Germany
[f] Centro de Virología Animal (CEVAN), Av Fleming 1653, Martinez, Buenos Aires, Argentina
[g] Instituto de Virología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA), N Repetto y De Los Reseros s/n, Hurlingham (1686), Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET), Buenos Aires, Argentina
[h] Biogénesis Bagó S.A., Garín, B1619 IEA, Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Foot-and-mouth disease virus
Liquid phase blocking ELISA (LPBE)
Virus neutralization test (VNT)
Vaccine matching
$_{r1}$-value

ABSTRACT

Foot-and-mouth disease virus (FMDV) is a highly variable RNA virus existing as seven different serotypes. The antigenic variability between and within serotypes can limit the cross-reactivity and therefore the *in vivo* cross-protection of vaccines. Selection of appropriate vaccine strains is crucial in the control of FMD. Determination of indirect relationships (r_1-value) between potential vaccine strains and field strains based on antibody responses against both are routinely used for vaccine matching purposes. Aiming at the investigation of the repeatability, reproducibility and comparability of r_1-value determination within and between laboratories and serological tests, a small scale vaccine matching ring test for FMDV serotype A was organized. Well-characterized serum pools from cattle vaccinated with a monovalent A24/Cruzeiro/Brazil/55 (A24) FMD vaccine with known *in vivo* protection status (homologous and heterologous) were distributed to four laboratories to determine r_1-values for the heterologous FMD strains A81/Argentina/87, A/Argentina/2000 and A/Argentina/2001 using the virus neutralization tests (VNT) and liquid phase blocking ELISA (LPBE). Within laboratories, the repeatability of r_1-value determination was high for both antibody assays. VNT resulted in reproducible and comparable r_1-values between laboratories, indicative of a lack of antigenic relatedness between the A24 strain and the heterologous strains tested in this work, thus corresponding to some of the *in vivo* findings with these strains. Using LPBE, similar trends in r_1-values were observed in all laboratories, but the overall reproducibility was lower than with VNT. Inconsistencies between laboratories may at least in part be attributed to differences in LPBE protocols as well as the in preexisting information generated in each laboratory (such as antibody titer-protection correlation curves). To gain more insight in the LPBE-derived r_1-values standard bovine control sera were included in the antibody assays performed in each laboratory and a standardization exercise was performed.

1. Introduction

Foot-and-mouth disease (FMD) remains one of the world's most important infectious diseases of livestock affecting multiple species of cloven-hoofed animals (Domingo et al., 2003). The FMD virus (FMDV) is an aphthovirus within the family of *Picornaviridae*. To date, seven

* Corresponding author at: Sciensano, Groeselenberg 99, 1180 Brussel, Belgium.
E-mail address: Kris.DeClercq@sciensano.be (K. De Clercq).
[1] In dedicated memory.
[2] These authors should be considered joint first author.

https://doi.org/10.1016/j.jviromet.2019.113786
Received 17 April 2019; Received in revised form 22 October 2019; Accepted 21 November 2019
Available online 22 November 2019

different serotypes have been identified (O, A, C, Asia 1, (Southern African territories) SAT1, SAT2 and SAT3) and new subtypes arise frequently (Belsham, 1993). Vaccination is key in the control of FMDV in both FMD-free and endemic regions (Lubroth et al., 2007). Elimination of FMDV by use of inactivated vaccines has been successful in Europe and South America (Lombard et al., 2007; Naranjo and Cosivi, 2013). The antigenic variability between and within serotypes however limits the cross-reactivity of immune responses elicited by one FMDV strain to another and therefore the probable cross-protection. Thus, assessing the antigenic and immunogenic relatedness of the vaccine strain and the field strain as well as matching the vaccine strain with the circulating field strains is crucial for optimization of vaccination programs. In addition, it is preferable that vaccines cover a broad antigenic spectrum to increase the level of cross-protection (Paton et al., 2005). *In vivo* vaccination-challenge experiments to assess the match between a particular combination of a vaccine and a field strain are considered the gold-standard for vaccine matching, but have limitations. *In vivo* trials need to be conducted in high-containment bio-security facilities, are labour-intensive and time-consuming resulting in high expenses. In addition *in vivo* trials cause suffering of animals and the outcome is prone to high variability that is seldomly taken into account (Goris et al., 2007). As a result the number of vaccine matching tests that can reliably and realistically be performed *in vivo*, within an acceptable timeframe in case of an epidemic, is limited. Until today, it is not possible to predict antigenic differences based on FMDV genomic sequence data alone (Paton et al., 2005; Ludi and Rodriguez, 2013). Several papers demonstrate that *in vivo* protection against FMD strains homologous to those of the vaccine correlates with antibody titers (Pay and Hingley, 1986; Van Maanen and Terpstra, 1989; Smitsaart et al., 1998; Ahl et al., 1983; Periolo et al., 1993; Barnett et al., 2003; Goris et al., 2008a; Maradei et al., 2008; Willems et al., 2012). This approach, however, proved more variable for correlations using heterologous strains and thus alternative serological assays, such as avidity assays and isotype ELISAs (Lavoria et al., 2012), have also been proposed to complement classical tests, *i.e.* virus neutralization tests (VNT) and liquid-phase blocking ELISA (LPBE). Despite the limited number of *in vivo* experimental cross protection studies correlated with serological results, *in vitro* serological methods have been used to estimate the antigenic and immunogenic differences between FMDV strains and the outcome of indirect *in vitro* assays is in general terms comparable to those observed *in vivo* (Brehm et al., 2008; Goris et al., 2008b; Maradei et al., 2011). As a result, in practice vaccine selection is mainly based on analyses of data from the field (investigations of outbreaks and collection of samples) and *in vitro* serological vaccine matching tests (Paton et al., 2005). These *in vitro* serological tests mainly comprise the comparison of the serum antibody titers from vaccinated animals [bovine vaccinal serum (BVS)] to the homologous vaccine strain and to heterologous field strains and the calculation of an indirect relationship (r_1-value) between the strains. However, many variables may affect the outcome of r_1 results and the debate on their interpretation has resulted in recommendations for determining FMD vaccine strain matching by serology. These recommendations include the use of VNT and LPBE, repeated testing, the addition of standardized BVS, the use of serum pools from at least five vaccinated animals collected 21–30 days after vaccination and the exclusion of sera with low antibody titers (Rweyemamu, 1984; Rweyemamu and Hingley, 1984; Paton et al., 2005; Mattion et al., 2009; OIE - World Organisation for Animal Health, 2019).

To gain further insight into the interpretation of r_1-values and to examine to what extent different laboratories can reproducibly determine r_1-values, a small scale vaccine matching ring test for r_1-values with FMDV serotype A was organized using well characterized serum pools from vaccinated animals with known *in vivo* protection status. The present study aimed to investigate (i) the repeatability of r_1-value determination within laboratories, (ii) the reproducibility of r_1-value determination between laboratories and (iii) the comparability of r_1-value determination based on VNT and LPBE.

2. Material and methods

2.1. Bovine sera and FMDV strains

Serum samples were obtained from a set of ten *in vivo* tests performed in Argentina following the "Protection against Podal Generalization" (PPG) protocol. Cattle were one time immunized intramuscularly with a water-in-oil single emulsion vaccine containing 10 µg of inactivated A24/Cruzeiro/Brazil/55 (A24) per dose. The overall PPG percentage (PPG%), taking into account results of all ten *in vivo* tests, when animals were challenged with the homologous A24 strain at 30 days post-vaccination (dpv) was 88.5 %. Sera collected 30 dpv were selected to create three serum pools containing medium (LPBE titer around 2.5) to high (LPBE titer of at least 3.0) antibody titers against the homologous A24 strain as determined by LPBE at the OIE FMD Regional Reference Laboratory located at the National Animal Health Service (SENASA) in Buenos Aires, Argentina and the Centro de Virología Animal (CEVAN, Buenos Aires, Argentina) (Mattion et al., 2009). Pool A consisted of five sera with high homologous antibody titers. Pool B consisted of five sera with a medium homologous antibody titers and pool C was made by mixing equal volumes of pool A and pool B (10 sera) (Table 1). The three serum pools were each further divided in three aliquots with different labels so in total nine samples (three replicates of pool A, B and C) were prepared for blind testing by the participating laboratories. Two standard control bovine sera (SCBS) derived from animals vaccinated at least three times with a tetravalent inactivated oil vaccine containing FMDV strains A24, A/Argentina/2001 (A2001), O1/Campos/Brazil/58 (O1C) and C3/Indaial/Brazil/71 (C3I) and containing high antibody titers against these virus strains were also included for standardization of the serological assays (Table 1).

The homologous A24 FMDV strain and the heterologous FMDV strains A81/Argentina/87 (A87), A/Argentina/2000 (A2000) and A2001 were provided by SENASA to participating laboratories for use in the serological assays. The heterologous virus strains were provided by SENASA in a blinded way. *In vivo* data indicated that the A24 vaccine did not induce cross-protection against the heterologous FMDV strains (PPG ≤ 37 %) (Alonso et al., 1987; Smitsaart et al., 2002; Goris et al., 2008b). An overview of the sera and viruses is given in Table 1.

Table 1
Overview of bovine sera and FMDV strains provided by SENASA to CEVAN, FLI, Pirbright and CODA-CERVA for blinded interlaboratory r_1-value determination.

Serum	Sample ID	Antibody titers by LPBE at SENASA / CEVAN against				FMDV strain[g]	Sample ID
		A24[a]	A2001[b]	O_1 C[c]	C3[d]		
Pool A	1-2-3	3.06	–	–	–	A24	A24
Pool B	4-5-6	2.51	–	–	–	A87[e]	2
Pool C	7-8-9	2.78	–	–	–	A2000[f]	3
Control	SBCS 1	4.06	4.03	4.34	4.5	A2001	1
	SBCS 2	4.22	4.11	4.27	3.93		

- : not available.
[a] A24/Cruzeiro/Brazil/55.
[b] A/Argentina/2001.
[c] O1/Campos/Brazil/58.
[d] C3/Indaial/Brazil/71.
[e] A81/Argentina/87.
[f] A/Argentina/2000.
[g] Live and binary ethylenimine-inactivated FMDV strains were provided by SENASA to perform VNT and LPBE, resp.

Table 2
Mean r_m-values and 95 % confidence intervals obtained in the different laboratories for A87, A2000 and A2001 using VNT on the three pools of sera.

Serum	Virus	Mean r_m-value [95%CI]			
		SENASA / CEVAN	FLI	PIRBRIGHT	CODA-CERVA
Pool A	A87	0.03 [0.01; 0.05]	0.11 [0.01;0.21]	0.12 [0.06; 0.17]	0.06 [0.03;0.08]
	A2000	0.09 [0.004;0.03]	0.11 [0.03;0.04]	0.10 [0.06; 0.14]	0.1 [0.06;0.15]
	A2001	0.007 [0.001;0.01]	0.17 [0.08;0.26]	0.06 [0.05;0.08]	0.07 [0.05;0.1]
Pool B	A87	0.01 [0.002;0.02]	0.13 [0.10;0.16]	0.11 [0.08; 0.13]	0.05 [0.02;0.08]
	A2000	0.01 [0.003; 0.02]	0.13 [0.13;0.14]	0.01 [0.01; 0.01]	0.06 [0.06;0.09]
	A2001	0.01 [0.002;0.02]	0.11 [0.09;0.13]	0.08 [0.02; 0.14]	0.06 [0.05;0.06]
Pool C	A87	0.01 [0.003;0.02]	0.16 [0.10;0.23]	0.06 [0.04; 0.09]	0.07 [0.05;0.09]
	A2000	0.02 [0.005;0.04]	0.20 [0.12;0.29]	0.07 [0.05; 0.09]	0.04 [0.02;0.07]
	A2001	0.01 [0.002;0.02]	0.13 [0.07;0.18]	0.05 [0.04; 0.07]	0.07 [0.05;0.08]
Overall	A87	0.07 [0.06;0.08]	0.14 [0.11; 0.16]	0.10 [0.07; 0.12]	0.06 [0.06;0.07]
	A2000	0.09 [0.08;0.10]	0.15 [0.11; 0.19]	0.06 [0.03; 0.09]	0.07 [0.05;0.09]
	A2001	0.06 [0.05;0.07]	0.14 [0.11; 0.16]	0.06 [0.05; 0.08]	0.06 [0.06;0.07]

2.2. Participating laboratories

Four laboratories participated in the ring trial: (i) Centro de Virología Animal (CEVAN, Buenos Aires, Argentina) (ii) the Friedrich-Loeffler-Institut (FLI, Insel-Riems, Germany), (iii) the Pirbright Institute (Pirbright, UK) and (iv) the Veterinary and Agrochemical Research Centre (CODA-CERVA, Brussels, Belgium). Each laboratory received the nine pooled serum samples, live FMDV strains to perform VNT and binary ethylenimine (BEI)-inactivated FMDV strains to perform LPBE. The sera and viruses were sent from SENASA to CEVAN and CODA-CERVA, and from the latter they were further distributed to the FLI and the Pirbright Institute. All nine pooled serum samples were tested in each laboratory against the four FMDV strains by VNT and this was repeated up to nine times, as well as by LPBE and this was repeated three times.

2.3. Serological assays

2.3.1. Virus neutralization assay

Virus neutralization test were performed according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE - World Organisation for Animal Health, 2019) with slight modifications. At Pirbright VNT were performed on IB-RS2 cells. At SENASA, FLI and at CODA-CERVA VNT were performed on BHK-21 cells.

2.3.2. Liquid phase blocking ELISA

The LPBE ring trial was performed in two stages. In the first stage all partner laboratories used their in-house LPBE which was optimized in terms of antigen concentration and detector monoclonal or guinea-pig antiserum dilution to obtain an optical density of approximate 1.5 in the antigen control for the A24 strain. This was done following OIE methods (OIE - World Organisation for Animal Health, 2019) to improve the detection of antibodies against this strain. In the second stage the laboratories adjusted in a similar way their LPBE protocol in order to improve determination of serum antibody titers for the strains A87, A2000 and A2001 in a blinded way.

2.4. The r_1-value determination and interpretation

VNT and LPBE antibody titers obtained in the participating laboratories were used to calculate individual r_1-values (r_i) for each serum pool at CODA-CERVA using the formula:

$$r_i = 10\text{exp (log10 serum titer against heterologous strain} - \text{log10 serum titer against homologous strain)} \quad (1)$$

Mean r_1-values (r_m) per pool were calculated using the formula:

$$r_m = 1/n \sum_{i=1}^{n} r_i \quad (2)$$

Overall mean r_1 values were calculated as in (2) with taking into account the data obtained for all three pools together.

OIE-recommended guidelines were used for the interpretation of the obtained r_1-values. When using VNT, r_1-values equal to or above 0.3 indicate a close antigenic relationship between the vaccine strain and the field isolate and that it is likely that the vaccine strain will confer cross-protection against the field strain whereas, r_1-values less than 0.3 indicate a lack of such cross-protection (Rweyemamu, 1984). When using LPBE, r_1-values between 0.4 and 1.0 are considered indicative of a close relationship between the vaccine and fields strain under investigation, *i.e.* it is considered likely that the vaccine strain will confer cross-protection against this fields strain; r_1-values between 0.2 and 0.39 indicate limited cross-reaction between the vaccine and field strain, *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 vaccinated preferably more than once; and r_1-values below 0.2 indicate a lack of cross-reactivity between the vaccine and field strain, *i.e.* it is considered unlikely that the vaccine strain will confer cross-protection against this fields strain (Ferris and Donaldson, 1992).

2.5. Statistical analyses

The r_i and r_m-values were compared by analysis of variance (1-way ANOVA). The r_m-values were compared pairwise using the Tukey test. A value of $P \leq 0.05$ was considered as the level of significance.

3. Results

In total, 303 individual r_i-values were obtained for A87, A2000 and A2001 using VNT and LPBE.

3.1. Data obtained with VNT

One hundred and ninety-five individual r_i-values were obtained by the four laboratories using the three serum pools: 62 for A87, 62 for A2000 and 71 for A2001. Mean r_m-values obtained with the three serum pools are shown in Table 2. In all laboratories, all individual r_i-values as well as the upper limit of the 95 % confidence intervals of the r_m were below 0.3, indicating a lack of antigenic relatedness between the virus strains under investigation and the A24 vaccine strain.

3.2. Data obtained with LPBE

In the first phase, when the LPBE set-up optimized for the homologous A24 strain was used, OD-values obtained in all laboratories with all three heterologous strains were below the guidance value set by the OIE (OD ~1.5) and results could not be validated (OIE - World Organisation for Animal Health, 2019). In the second phase, when the

Table 3
Mean r_m-values and 95 % confidence intervals obtained in the different laboratories for A87, A2000 and A2001 using the optimized LPBE on the three pools of sera.

Serum	Virus	r_m-value [95%CI]			
		SENASA / CEVAN	FLI	PIRBRIGHT	CODA-CERVA
Pool A	A87	0.06 [0.04;0.08]	**0.58 [0.57;0.60]**	0.19 [0.05;0.32]	0.27 [0.21;0.32]
	A2000	0.18 [0.11;0.25]	**0.77 [0.64;0.91]**	0.26 [0.18;0.34]	**1 [1;1]**
	A2001	0.03.[0.016;0.38]	**0.43** [0.36;**0.50**]	0.20 [0.15;0.25]	0.003 [0.003;0.003]
Pool B	A87	0.21 [0.20;0.23]	**0.70 [0.66;0.74]**	0.14 [0.12;0.17]	0.28 [0.23;0.33]
	A2000	0.36 [0.33;0.38]	**0.99 [0.86;1.12]**	0.14 [0.12;0.17]	**0.79 [0.68;0.90]**
	A2001	0.08 [0.07;0.08]	**0.61 [0.58;0.63]**	0.12 [0.08;0.17]	0.006 [0.005;0.007]
Pool C	A87	0.15 [0.13;0.17]	**0.55 [0.46;0.63]**	0.31 [0;**0.65**]	0.30 [0.26;0.33]
	A2000	0.36 [0.32;0.39]	**0.67 [0.56;0.79]**	**0.41** [0;**0.99**]	**1 [0.90;1.15]**
	A2001	0.07 [0.05;0.09]	**0.42** [0.32;**0.53**]	0.32 [0;**0.66**]	0.004 [0.004;0.004]
Overall	A87	0.14 [0.07;0.21]	**0.61 [0.54;0.68]**	0.21 [0.14;0.28]	0.28 [0.21;0.35]
	A2000	0.30 [0.17;**0.41**]	**0.81 [0.69;0.94]**	0.27 [0.15;0.40]	**0.94 [0.82;1.06]**
	A2001	0.057 [0:0.13]	**0.49 [0.42;0.56]**	0.21 [0.14;0.28]	0.04 [0;0.07]

r_m-values > 0.4 are marked in bold.

optimized LPBE set-up for the heterologous virus strains was used, 108 r_i-values were obtained: 9 for A87, 9 for A2000 and 9 for A2001 in each laboratory. Results are summarized in Table 3.

In all four laboratories, a pattern of increasing r_i-values from A2001 over A87 to A2000 was observed (Fig. 1). In general, the r_i-values obtained within the different laboratories were highly repeatable per sample and per pool. All r_i-values obtained at CEVAN were below 0.4 of which 18 were below 0.2, whereas at FLI all r_i-values were above to 0.2 with 25 values being higher than 0.4. With LPBE at Pirbright, 24 r_i-values were below 0.4 of which 19 were below 0.2 and at CODA-CERVA 18 r_i-values were below 0.4 of which 9 were below 0.2. At Pirbright, r_i-values for sample 9 (pool C) were consistently above 0.4 for all three heterologous strains A87, A2000 and A2001, resulting in a lower repeatability for pool C compared to pool A and B. At CODA-CERVA r_i-values for A2000 were consistently above 0.4 for all three serum pools.

In all four laboratories r_i-values for pool A were generally lower than those of pool B with values of pool C in between, but these differences between pools were not significant. The overall mean r_1-values of all 9 samples (pool A + B + C) for A87 were comparable between CEVAN, Pirbright and CODA-CERVA. The overall mean r_1-values of all 9 samples for A2000 were comparable between FLI and CODA-CERVA on the one hand and CEVAN and Pirbright on the other hand. The overall mean r_1-values of all 9 samples for A2001 were comparable between CEVAN and CODA-CERVA, but those of Pirbright and FLI were significantly different from the three other laboratories. Within laboratories, correlations between r_1-values obtained by VNT and LPBE were generally low (Pearson's correlation coefficient below $|0.40|$) except for A87 and A2001 in CEVAN with correlation coefficients of 0.51 and 0.90, resp.

Taking into account the known lack of *in vivo* cross-protection induced by the A24 vaccine against the heterologous A87, A2000 and A2001 strains (Alonso et al., 1987; Smitsaart et al., 2002; Goris et al., 2008b) on the one hand and the observed differences in r_1-values determined in the participating laboratories on the other, it was examined whether LPBE outcomes could be standardized to increase the reproducibility of r_1-value determination between the laboratories. Standardized bovine control sera (SBCS 1 and 2) were included in LPBEs performed in each laboratory. In each laboratory and with both SBCS, the highest LPBE antibody titers were detected against A24 and the lowest against A2001. This resulted in r_1-values for A24 well above 1 when A2001 was considered as homologous strain, whereas low r_1-values were obtained for A2001 when A24 was considered as

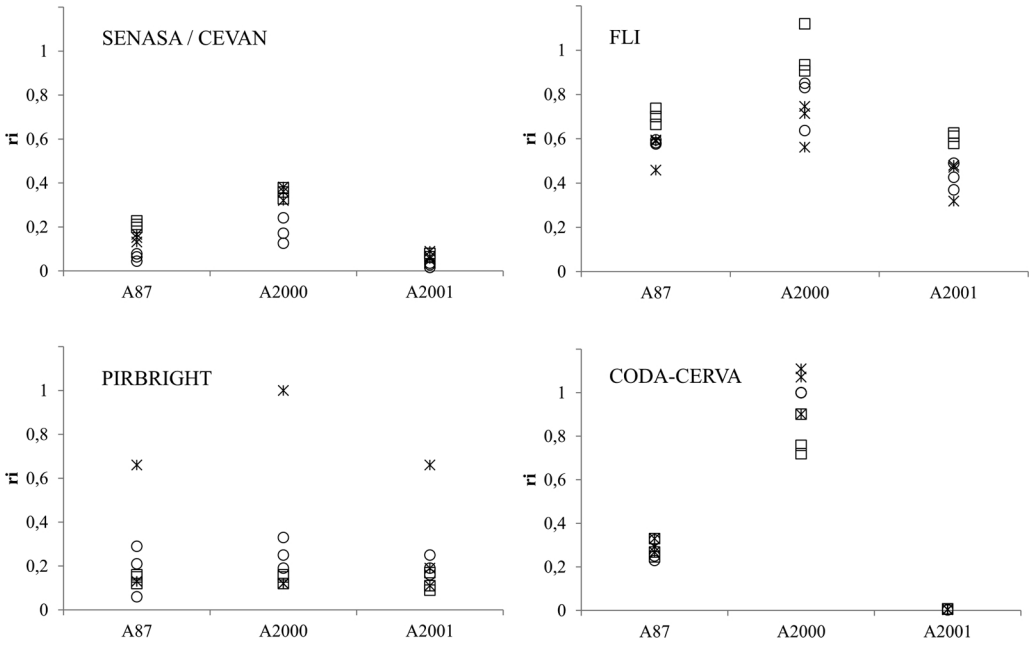

Fig. 1. Distribution of individual ri values obtained by the LPBE optimized in different laboratories for detecting A87, A2000 or A2001 strains using 21 dpv sera from cattle immunized with a monovalent FMDV A24 vaccine. ○: serum 1, 2 and 3 (pool A); Ж: serum 4, 5 and 6 (pool B); Ж: serum 7, 8 and 9 (pool C).

Table 4
Individual r_i-values for A24, A87, A2000 and A2001 obtained by the optimized LPBE in the different laboratories, with either A24 or A2001 as homologous strains and using standard bovine control sera (SBCS) from cattle immunized with a tetravalent formulation (A24/O1C/C3I/A2001).

Serum[1]	Virus	r_i-value considering A24 as homologous strain/considering A2001 as homologous strain			
		SENASA / CEVAN	FLI	PIRBRIGHT	CODA-CERVA
SBCS 1	A24	1/10.68	1/1.96	1/3.74	1/99.61
	A87	0.37/3.94	0.69/1.36	0.27/1.00	0.14/14.25
	A2000	1.49/15.95	0.83/1.63	0.27/1.00	0.45/45.71
	A2001	0.09/1	0.51/1	0.26/1	0.01/1
SBCS 2	A24	1/5.36	1/1.55	1/5.09	1/32.74
	A87	0.79/4.22	0.81/1.26	0.20/1.00	0.45/14.67
	A2000	2.02/10.81	0.77/1.19	0.20/1.00	1.30/42.71
	A2001	0.19/1	0.64/1	0.20/1	0.03/1
Overall	A24	1/7.57	1/1.75	1/4.37	1/57.11
	A87	0.54/4.07	0.75/1.31	0.23/1.01	0.25/14.46
	A2000	0.86/6.52	0.80/1.39	0.23/1	0.77/43.87
	A2001	0.13/1	0.57/1	0.23/1	0.02/1

homologous strain (Table 4). As with the serum pools, with the SBCSs a pattern of increasing r_i-values from A2001 over A87 to A2000 was observed in all laboratories when A24 was considered as homologous strain. The r_1-values for A87, A2000 and A2001 with the SBCSs obtained at FLI, Pirbright and CODA-CERVA were comparable to those obtained with the serum pools. Conversely, r_1-values for A87 and A2000 in the SBCSs at CEVAN were higher than 0.4 with A24 as homologous strain, unlike with the serum pools.

Using the SBCSs, a baseline for each of the four virus strains was calculated as the geometric mean of homologous LPBE antibody titers obtained by all laboratories. Next, factors were calculated for each laboratory taking into account the difference between the obtained antibody titer in the SBCSs and the baseline. Using these factors, LPBE antibody titers against all viruses obtained at FLI as well as those against A24 and A2001 obtained at Pirbright were adjusted downwards. For CEVAN and CODA-CERVA antibody titers against all viruses as well as those against A87 and A2000 from Pirbright were adjusted upwards. Fig. 2 shows the resulting r_i corrected ($r_{i,c}$) values. All $r_{i,c}$-values obtained at CEVAN and FLI were lower than their corresponding r_i-values, while at Pirbright and CODA-CERVA only those $r_{i,c}$-values obtained for A2001 and A2000 were lower than their corresponding r_i-values (Table 5). In total, the number of individual r_1-values above 0.4 for A87, A2000 and A2001 strains, decreased from 37 for r_i to 28 for $r_{i,c}$. The observed change was due to FLI scoring $r_{i,c}$ below 0.4 for A87 and A2001 strains. In contrast, the adjustment did not alter the r_1-value classification for Pirbright and CODA-CERVA for serum sample 9 to A87 and A2000 or for pool A, B and C to A2000. Within laboratories, correlations between r_1 obtained by VNT and LPBE remained low (Pearson correlation coefficient < 0.40) except for A87 in CEVAN with a correlation coefficient of 0.51.

4. Discussion

In order to allow optimal and rapid selection of the most suitable FMD vaccine strain at all times, reliable and reproducible estimation of the antigenic relatedness between FMDV vaccine strains and FMDV field strains is required. *In vitro* serological tests that compare the reactivity of BVS against the homologous vaccine strain and heterologous field strains have distinct advantages compared to *in vivo* challenge tests. Therefore calculation of r_1-values based on antibody titers is most frequently used in practice to support the choice of an appropriate vaccine strain. In the present study, well characterized serum pools from vaccinated animals with known *in vivo* protection status were used to examine the repeatability of r_1-value determination within laboratories. The present data show that using the VNT, all laboratories determined consistent r_1-values within their laboratory for A87, A2000 and A2001 in the three serum pools from cattle vaccinated with A24. Similar observations were made for the LPBE, with only one laboratory obtaining a deviating r_1-value for one sample of serum pool C with the deviation in itself being consistent. Our findings are similar to those of Mattion et al. (2009) who reported the reducing effect of pooling of serum samples on the inter-animal and inter-trial variation of calculated r_1-values, especially when sera from *in vivo* protected animals were used (Mattion et al., 2009).

The second aim was to examine the reproducibility of r_1-value determination between laboratories. Using the VNT, all laboratories

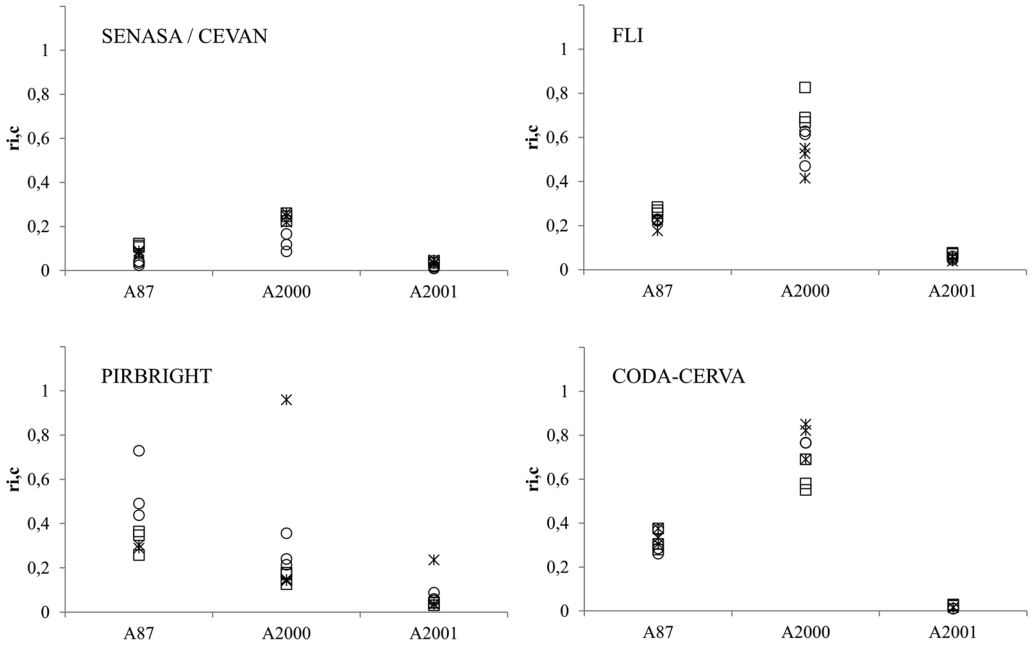

Fig. 2. Distribution of individual ri,c values obtained in different laboratories by LPBE antibody titres scaled to the baseline for A87, A2000 and A2001 strains using 21 dpv sera from cattle immunized with a monovalent FMDV A24 vaccine. ○: serum 1, 2 and 3 (pool A); Ж: serum 4, 5 and 6 (pool B); Ж: serum 7, 8 and 9 (pool C).

Table 5
Mean $r_{m\text{-}c}$ values obtained in the different laboratories for A87, A2000 and A2001 using the optimized LPBE on the three pools of sera.

Serum	Virus	$r_{m,c}$-value [95%CI]			
		SENASA / CEVAN	FLI	PIRBRIGHT	CODA-CERVA
Pool A	A87	**0.04 [0.03;0.07]**	**0.29 [0.29;0.30]**	0.35 [0.24;0.46]	0.39 [0.30;0.46]
	A2000	**0.12 [0.08;0.17]**	**0.57 [0.47;0.66]**	0.54 [0.37;0.72]	**0.74 [0.74;0.74]**
	A2001	**0.02 [0.01;0.03]**	**0.07 [0.06;0.08]**	**0.09 [0.06;0.12]**	0.01 [0.01;0.01]
Pool B	A87	**0.14 [0.13;0.16]**	**0.35 [0.33;0.37]**	0.20 [0.16;0.25]	0.40 [0.34;0.48]
	A2000	**0.24 [0.22;0.26]**	**0.72 [0.63;0.81]**	0.32 [0.26;0.7]	**0.59 [0.50;0.67]**
	A2001	**0.05 [0.05;0.06]**	**0.10 [0.09;0.11]**	**0.05 [0.04;0.06]**	0.03 [0.03;0.04]
Pool C	A87	**0.10 [0.09;0.12]**	**0.27 [0.23;0.32]**	0.54 [0;1]	0.43 [0.38;0.48]
	A2000	**0.24 [0.22;0.27]**	**0.49 [0.41;0.58]**	0.84 [0;1]	**0.76 [0.67;0.86]**
	A2001	**0.05 [0.04;0.07]**	**0.07 [0.05;0.09]**	**0.14 [0;0.31]**	0.02 [0.02;0.02]
Overall	A87	**0.098 [0.068;0.12]**	**0.31 [0.28;0.33]**	0.36 [0.14;0.58]	0.41 [0.37;0.44]
	A2000	**0.020 [0.12;0.28]**	**0.59 [0.51;0.67]**	0.57 [0.22;0.92]	**0.70 [0.59;0.81]**
	A2001	**0.041 [0.030;0.054]**	**0.081 [0.070;0.10]**	**0.091 [0.036;0.15]**	0.024 [0.018;0.030]

A decrease of $r_{m,c}$-value compared to the r_m-value (Table 2) is marked in bold.

obtained consistent r_1-values below 0.3 indicating a lack of relatedness between the homologous A24 strain and the heterologous strains (Rweyemamu, 1984). In contrast, r_1-values obtained by LPBE antibody titers presented a lower reproducibility in all samples to all three heterologous viruses ranging from all below 0.4 for one laboratory to all above 0.4 for another laboratory. These data are similar to and substantiate previous reports on the VNT producing more reproducible inter-laboratory results for r_1-value calculation than LPBE (Mattion et al., 2009). The observed differences between laboratories with the LPBE are most likely due to different LPBE protocols that were applied in the different laboratories, whereas VNT were conducted following more standardized methodologies. It is well-known that antibody titers obtained in different laboratories cannot be compared directly (Barnett et al., 2003; Brehm et al., 2008; Goris et al., 2008a). Although r_1-values represent the ratio of antibody titers against different virus strains obtained within the same laboratory, the differences in LPBE antibody titers obtained for different FMD virus strains seem to still prevent immediate comparability of r_1-values between laboratories.

The r_1-values that corresponded best with the VNT and the *in vivo* findings were obtained by use of a LPBE at CEVAN based on trapper and detector monoclonal antibodies (Mabs) developed against the A24 strain. This assay managed to capture all examined virus strains with an optimal optical density and without large differences in concentrations of the reagents. Therefore it represented the blocking reactions with the different FMDV strains to a similar degree resulting in r_1-values that corresponded well with *in vivo* and VNT findings. LPBE set up with polyclonal trapper and/or detector reagents developed against the A24 strain, on the other hand, seem to capture the heterologous virus strains less efficiently, thus producing higher LPBE antibody titers and resulting in overestimation of r_1-values. This seemed the case for all three heterologous strains at FLI and for A2000 at CODA-CERVA. The order of magnitude LPBE-derived r_1-values obtained for A2001 at CODA-CERVA are similar to previous findings (Mattion et al., 2009). Interestingly, Mattion et al. (2004) reported the reactivity with a set of Mabs raised against A24, A87 and an A/Argentina/79 strain, to be lower for the A2000 strain than for the A2001 and A87 strains (Mattion et al., 2004). These antigenic characteristics of the A2000 strain may have contributed to the lower capture efficiency of the A2000 strain in the LPBE at CODA-CERVA. It should be noted that all LPBE were performed in a blinded way with the participating laboratories not being aware of the nature of the serum and the nature of viruses at the time of testing. This situation would not necessarily reflect a practical situation in which laboratories would use their knowledge, experience and skills to optimize test approaches and interpretations. The present findings however emphasize once more the importance of using optimized and standardized reagents and methods and the need for consistent testing conditions to improve the comparability of serological test outcomes between laboratories.

The third aim was to examine the comparability of r_1-value calculations based on VNT and LPBE assays. Using the VNT, all laboratories obtained r_1-values below the established threshold being indicative of a lack of antigenic relatedness (Rweyemamu, 1984), and corresponding to what has been reported from *in vivo* studies (Alonso et al., 1987, Smitsaart et al., 2002, Goris et al., 2008a, 2008b). Findings with LPBE on the other hand were more complex and correlations between r_1-values obtained by VNT and LPBE were low. Although non-neutralizing antibodies as well as cell-mediated immune mechanisms may also contribute to protection (Paton et al., 2005), *in vitro* neutralizing antibodies, which are not necessarily distinguished by LPBE, may be most relevant to *in vivo* protection. Moreover, tests like LPBE do not assess some qualitative aspects such as the affinity of the antibodies, which may affect *in vitro* virus neutralization, as early described (Blank et al., 1972), also correlating to protection in cattle (Steward et al., 1991). It would be too hasty to conclude from this inter laboratory study that r_1-values obtained by LPBE are not suitable for the assessment of possible *in vivo* protection as Maradei et al. (2008) showed that their LPBE can reliably be used to evaluate the potency of FMD vaccines.

In an attempt to improve the comparability of VNT and LPBE-based r_1-values and the reproducibility of LPBE-based r_1-values between laboratories, a standardization exercise was conducted using two standardized polyvalent SBCS. Using a rough and uniform approach of scaling LPBE antibody titers of all laboratories to a baseline showed that the overall number of r_1-values indicating limited cross-reaction between A24 and the heterologous strains tested in these assays had increased. LPBE r_1-values improved for the initially most deviating r_1-values. This general improvement suggests the usefulness of using SBCSs to standardize serological tests relating antibody responses between laboratories. It must however be admitted that the currently used polyvalent SBCS originating from animals repeatedly vaccinated with a tetravalent vaccine may not be most ideal for this purpose. The use of a vaccine containing more than one FMDV subtype as well as the use of booster vaccinations is known to stimulate a strong immunity resulting in a broadened spectrum of antibodies (Paton et al., 2005). The use of well-characterized monovalent sera to each of the virus strains under examination may be more appropriate in this context. Further, antibody titers related to protection are often different for different strains. Despite both A24 and A2001 being contained in the vaccine administered to the animals from which the SBCS originated, all laboratories detected the highest LPBE antibody titers against A24 and the lowest against A2001. The use of r_1-value determination with fixed values for every virus strain in every laboratory to predict probable cross-protection may therefore not necessarily be quite appropriate. For improved assessment of the cross-protective potential in each laboratory, it may be worthwhile investigating settings of r_1-value limits depending on virus

strains used.

Although the present findings suggest that a solution is not found in a single approach and that further research is needed, the present study provides valuable insight and suggests a path to harmonized r_1-value determination in the participating laboratories. Shortly after this inter laboratory study Lavoria et al. (2012) showed that a combined use of their newly developed single dilution avidity ELISA with subtyping of IgG and VNT discriminated better between protected and unprotected animals than VNT or LPBE alone. It would be interesting to include these tests in future inter laboratory studies.

Declaration of Competing Interest

None.

Acknowledgements

This research received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 226556 (FMD-DISCONVAC), from the Belgian Federal Public Service of Health, Food Chain Safety and Environment (grant RT-05/06-ALTANDI-2), from DEFRA project SE1129 and from CODA-CERVA (grants ALTANDI-1 and ALTANDI-3). The authors thank Anja Landmesser, Sofie Diez, Ina Musch, Coralie Willeman and Rim Dhambri for their excellent technical assistance. Angele Breithaupt's contribution to the DISCONVAC project is gratefully acknowledged. We are grateful to Dr. Michael Eschbaumer (Friedrich-Loeffler-Institut, Germany) for critically reading the manuscript. TW and ADV contributed equally.

References

Ahl, R., Lorenz, R.J., Wittman, G., 1983. A trial to estimate the potency of FMD vaccines by means of serum antibody assays with sera from groups of vaccinated cattle. Report of the Session of Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease. FAO, Rome, Lelystad, Netherlands, 20-22 September; 1983, pp. 56–61.

Alonso, A., Casas Olascoaga, R., Graciela Fernandez, R., Vianna Filho, Y.L., Sondhal, M.S., Gomes, I., Astudillo, V.M., Auge de Mello, P., Bahnemann, H.G., 1987. Epidemiologic, antigenic, immunogenic and biochemical characterization of aptho-virus A-81 Argentina/87. Bol. Centr. Panam. Fiebre Aftosa 53, 37–42.

Barnett, P.V., Statham, R.J., Vosloo, W., Haydon, D.T., 2003. Foot-and-mouth disease vaccine potency testing: determination and statistical validation of a model using a serological approach. Vaccine 21 (23), 3240–3248.

Belsham, G.J., 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. Prog. Biophys. Molec. Biol. 60, 241–260.

Blank, S.E., Leslie, G.A., Clem, L.W., 1972. Antibody affinity and valence in viral neutralization. J. Immunol. 108 (3), 665–673.

Brehm, K.E., Kumar, N., Thulke, H.H., Haas, B., 2008. High potency vaccines induce protection against heterologous challenge with foot-and-mouth disease virus. Vaccine 26 (13), 1681–1687.

Ferris, N.P., Donaldson, A.I., 1992. The World Reference Laboratory for Foot and Mouth Disease: a review of thirty-three years of activity (1958–1991). Rev. Sci. Technol. 11 (3), 657–684.

Domingo, E., Escarmís, C., Baranowski, E., Ruiz-Jarabo, C.M., Carrillo, E., Núñez, J.I., Sobrino, F., 2003. Evolution of foot-and-mouth disease virus. Virus Res. 91 (1), 47–63.

Goris, N., Maradei, E., D'Aloia, R., Fondevila, N., Mattion, N., Perez, A., Smitsaart, E., Nauwynck, H.J., La Torre, J., Palma, E., De Clercq, K., 2008b. Foot-and-mouth disease vaccine potency testing in cattle using homologous and heterologous challenge strains: precision of the "Protection against Podal Generalisation" test. Vaccine. 26 (27–28), 3432–3437.

Goris, N., Merkelbach-Peters, P., Diev, V.I., Verloo, D., Zakharov, V.M., Kraft, H.P., et al., 2007. European Pharmacopoeia foot-and-mouth disease vaccine potency testing in cattle: between test variability and its consequences. Vaccine 25, 3373–3379.

Goris, N., Willems, T., Diev, V.I., Merkelbach-Peters, P., Vanbinst, T., Van der Stede, Y., Kraft, H.-P., Zakharov, V.M., Borisov, V.V., Nauwynck, H.J., Haas, B., De Clercq, K., 2008a. Indirect foot-and-mouth disease vaccine potency testing based on a serological alternative. Vaccine 26, 3870–3879.

Lavoria, M.Á., Di-Giacomo, S., Bucafusco, D., Franco-Mahecha, O.L., Pérez-Filgueira, D.M., Capozzo, A.V., 2012. Avidity and subtyping of specific antibodies applied to the indirect assessment of heterologous protection against Foot-and-Mouth Disease Virus in cattle. Vaccine 30 (48), 6845–6850.

Lombard, M., Pastoret, P.P., Moulin, A.M., 2007. A brief history of vaccines and vaccination. Rev. Sci. Technol. 26 (1), 29–48.

Lubroth, J., Rweyemamu, M.M., Viljoen, G., Diallo, A., Dungu, B., Amanfu, W., 2007. Veterinary vaccines and their use in developing countries. Rev. Sci. Tech. Off. Int. Epiz. 26, 179–201.

Ludi, A., Rodriguez, L., 2013. Novel approaches to foot-and-mouth disease vaccine development. Dev. Biol. (Basel) 135, 107–116.

Maradei, E., La Torre, J., Robiolo, B., Esteves, J., Seki, C., Pedemonte, A., Iglesias, M., D'Aloia, R., Mattion, N., 2008. Updating of the correlation between lpELISA titers and protection from virus challenge for the assessment of the potency of polyvalent aphtovirus vaccines in Argentina. Vaccine 26 (December (51)), 6577–6586. https://doi.org/10.1016/j.vaccine.2008.09.033. 2008.

Maradei, E., Perez Beascoechea, C., Malirat, V., Salgado, G., Seki, C., Pedemonte, A., Bonastre, P., D'Aloia, R., La Torre, J.L., Mattion, N., et al., 2011. Characterization of foot-and-mouth disease virus from outbreaks in Ecuador during 2009–2010 and cross-protection studies with the vaccine strain in use in the region. Vaccine 29, 8230–8240.

Mattion, N., König, G., Seki, C., Smitsaart, E., Maradei, E., Robiolo, B., Duffy, S., León, E., Piccone, M., Sadir, A., Bottini, R., Cosentino, B., Falczuk, A., Maresca, R., Periolo, O., Bellinzoni, R., Espinoza, A., Torre, J.L., Palma, E.L., 2004. Reintroduction of foot-and-mouth disease in Argentina: characterisation of the isolates and development of tools for the control and eradication of the disease. Vaccine 22 (31-32), 4149–4162.

Mattion, N., Goris, N., Willems, T., Robiolo, B., Maradei, E., Beascoechea, C.P., Perez, A., Smitsaart, E., Fondevila, N., Palma, E., De Clercq, K., La Torre, J., 2009. Some guidelines for determining foot-and-mouth disease vaccine strain matching by serology. Vaccine 27, 741–747.

Naranjo, J., Cosivi, O., 2013. Elimination of foot-and-mouth disease in South America: lessons and challenges. Philos. Trans. R. Soc. Lond. B Biol. Sci. 368 (1623) 20120381.

OIE - World Organisation for Animal Health, 2019. Foot-and-mouth disease (infection with foot and mouth disease virus) [chapter 3.1.8]. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019. OIE, Paris, France. available at http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/3.01.08_FMD.pdf (Accessed 4 July 2019).

Paton, D.J., Valarcher, J.-F., Bergmann, I., Matlho, O.G., Zakharov, V.M., Palma, E.L., Thomson, G.R., 2005. Selection of foot and mouth disease vaccine strains – a review. Rev. sci. tech. Off. int. Epiz. 24 (3), 981–993.

Pay, T.W., Hingley, P.J., 1986. The use of serum neutralizing antibody assay for the determination of the potency of foot and mouth disease (FMD) vaccines in cattle. Dev. Biol. Stand. 1986 (64), 153–161.

Periolo, O.H., Seki, C., Grigera, P.R., Robiolo, B., Fernández, G., Maradei, E., D'Aloia, R., La Torre, J.L., 1993. Large-scale use of liquid-phase blocking sandwich ELISA for the evaluation of protective immunity against aphthovirus in cattle vaccinated with oil-adjuvanted vaccines in Argentina. Vaccine 11 (7), 754–760.

Rweyemamu, M.M., 1984. Antigenic variation in foot-and-mouth disease: studies based on the virus neutralization reaction. J. Biol. Stand. 12 (3), 323–337.

Rweyemamu, M.M., Hingley, P.J., 1984. Foot and mouth disease virus strain differentiation: analysis of the serological data. J. Biol. Standard. 12 (2), 225–229.

Smitsaart, E., Mattion, N., Mazzuca, G., Robiolo, B., Maradei, E., Filippi, J., Sadir, A., Falczuk, A., La Torre, J., Pedemonte, A., D´aloia, R., Periolo, O., Cadenazzi, G., Palma, E., Bellinzoni, R., 2002. Foot-and-mouth disease in Argentina: development of vaccines for emergency, control and eradication of the disease. Session of the Research Group of the Standing Technical Committee of the European Commmission for the Control of FMD, Izmir, Turkey, 17-20 September 2002 Appendix 40 336-349.

Smitsaart, E.N., Zanelli, M., Rivera, I., Fondevila, N., Compaired, D., Maradei, E., Bianchi, T., O'Donnell, V., Schudel, A.A., 1998. Assessment using ELISA of the herd immunity levels induced in cattle by foot-and-mouth disease oil vaccines. Prev. Vet. Med. 33 (1–4), 283–296.

Steward, M.W., Stanley, C.M., Dimarchi, R., Mulcahy, G., Doel, T.R., 1991. High-affinity antibody induced by immunization with a synthetic peptide is associated with protection of cattle against foot-and-mouth disease. Immunology 72 (1), 99–103.

Van Maanen, C., Terpstra, C., 1989. Comparison of a liquid-phase blocking sandwich ELISA and a serum neutralization test to evaluate immunity in potency tests of foot-and-mouth disease vaccines. J. Immunol. Methods 124 (1), 111–119.

Willems, T., Lefebvre, D., Goris, N., Diev, V.I., Kremenchugskaya, S.R., Paul, G., Haas, B., De Clercq, K., 2012. Characteristics of serology-based vaccine potence models for foot-and-mouth disease virus. Vaccine 30, 5849–5855.