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Characterization of a type O foot-and-mouth disease virus re-emerging in the year 2011 in free areas of the Southern Cone of South America and cross-protection studies with the vaccine strain in use in the region

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ABSTRACT

Molecular, antigenic and vaccine matching studies, including protective response in vivo, were conducted with a foot-and-mouth disease type O virus isolated during the outbreak in September 2011 in San Pedro, Paraguay, country internationally recognized as free with vaccination in 1997. The phylogenetic tree derived from complete VP_1 sequences as well as monoclonal antibody profiling indicated that this isolate was related to viruses responsible for previous emergencies in free areas of the Southern Cone of South America occurring sporadically between the years 2000 and 2006. Marked differences with the vaccine strain O_1/Campos, including the loss of reactivity with neutralizing MAbs, were recognized. Levels of protective antibodies induced by the vaccine containing the O_1/Campos strain against the San Pedro virus and the virus responsible for the previous emergency in 2006 in the Southern Cone assessed by in vitro vaccine matching studies pointed to an insufficient protective response 30 days after vaccination (DPV), which was properly attained at 79 DPV or after revaccination. In agreement with the in vitro assessment, the in vivo challenge in the Protection against Podal Generalization test in cattle indicated appropriate protection for the San Pedro strain at 79 DPV or after revaccination. The complementary conclusions that can be derived from vaccine matching tests designed differently to fit the various objectives intended: prophylaxis, emergency vaccination or incorporation of new field strains into antigen banks, is evaluated. This is the first report of the antigenic and immunogenic characterization of the variants responsible for emergencies in the Southern Cone of South America and the putative impact of the changes on the cross protection conferred by the vaccine strain.

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious vesicular disease of cattle and other cloven-hoofed animals. Although mortality due to the disease is very low and mostly restricted to young animals, drastic

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decrease in productivity and working capacity of the animals causes great losses to the livestock industry. The disease has an important socio-economic impact in countries where it is endemic (Perry et al., 1999), provokes huge economic consequences when outbreaks occur in disease free regions (Correa Melo et al., 2002) and is considered one of the most important constrains to international trade of livestock and animal products.

FMD virus (FMDV) is a member of the *Picornaviridae* family, genus *Aphthovirus* (Pereira, 1981). It possesses a single-stranded positive RNA molecule of about 8200 nucleotides, within an icosahedral capsid made of 60 copies each of four proteins VP_1, VP_2, VP_3, and VP_4. There are seven immunologically distinct serotypes, O, A, C, Asia 1, South African Territories (SAT) 1, SAT 2 and SAT 3, in circulation worldwide and intratypic variants (subtypes) arise continuously (Brooksby, 1982). Infection or vaccination with one serotype of FMDV does not cross-protect against other serotypes and may also fail to protect fully against other subtypes of the same serotype (Brooksby, 1982; Cartwright et al., 1982; Mattion et al., 2004). Serotypes O, A and C have been recorded in South America.

Vaccination is widely applied to control, eradicate and prevent FMD (Garland, 1999; Bergmann et al., 2005). Moreover, a considerable transformation is ongoing regarding the acceptance of the benefits of vaccination as an alternative to stamping out policies, particularly after recurrence of the disease in free regions (Bergmann et al., 2005).

The vaccines are prepared by large-scale growth of particular strains of FMDV in cell cultures, followed by inactivation with an aziridine such as binary ethyleneimine BEI (Bahnemann, 1975). Thereafter, the inactivated viral materials can be formulated with adjuvants into ready-to-use vaccine, or stored as antigen concentrates over liquid nitrogen for many years (Lombard et al., 2003). The immunity they induce will only protect against a limited range of field strains. This range is maximized by selecting vaccine strains that are as immunogenic and cross reactive as possible.

Vaccines in South America are formulated with oil adjuvant (Augé de Melo, 1982). They contain selected strains harmonized for use in the region and include O_1/Campos, A_{24}/Cruzeiro and most of the Southern Cone countries comprise also virus C_3/Indaial. The variant A/2001 is also included in vaccine formulations in Argentina. In principle, these strains were able to give a satisfactory immunological coverage when systematic vaccination was applied.

Nevertheless, changes in antigenicity and immunogenicity, which can occur particularly in endemic settings, are of utmost importance for control programs, since the degree of protection of the vaccinated population depends not only on the potency of the vaccine and revaccination schemes applied, but also on the relatedness between the vaccine and the field strain.

Historically the disease had a worldwide distribution although nowadays is mainly present in Africa and Asia. In South America, and as a result of a coordinated regional eradication plan implemented in 1988, at present most of the countries/regions have their status recognized by the World Organization for Animal Health (OIE) as FMD-free either with or without vaccination. An overall decrease in clinical cases of over 95% has been registered for the region. Endemic countries are restricted to Ecuador, where only FMD type O episodes have been reported since the 2003, and Venezuela where FMD types O and A have been acting yearly (Maradei et al., 2011; Malirat et al., 2012).

Particularly in the Southern Cone of South America, the status of free of FMD by the OIE was obtained in the late 1990s when the following countries were recognized as FMD free where vaccination is practiced: Uruguay, 1994; Argentina, 1997; Paraguay, 1997. In 1998 a zone comprising the southern states of Brazil acquired the status of FMD-free zone where vaccination is practiced, and in 2000, the state of Mato Grosso do Sul also gained this status. Uruguay stopped vaccination in 1994 and became free without vaccination in 1996. Argentina and Paraguay discontinued vaccination in 1999 and Argentina was recognized free without vaccination in 2000 (Bergmann et al., 2005). Later in the year 2000, some outbreaks of serotype O put this region in an emergency status. Brazil (Rio Grande do Sul state), Argentina (Corrientes and Misiones provinces) and Uruguay (Artigas department) had sporadic episodes between July and October (Correa Melo et al., 2002). Moreover emergencies of serotype A viruses occurred in 2000–2001 in Argentina (Konig et al., 2007), Uruguay and Brazil (Rio Grande do Sul state). Afterwards, type O virus reemerged in Paraguay in 2002 and 2003, locations of Canindeyu and Pozo Hondo, respectively, the latter one spreading to Bolivia (departments of Chuquisaca and Potosı). During October–December, 2005, 33 FMD type O outbreaks were registered in the state of Mato Grosso do Sul, Brazil, and in February, 2006, one type O episode in the province of Corrientes, Argentina (Malirat et al., 2007). A new outbreak occurred in September 2011 in San Pedro, Paraguay, with an epidemiologically linked event recorded in January 2012 (OIE, 2012).

The emergency situations experienced have prompted the need for a deep characterization of strains presently circulating in endemic regions, as well as of those emerging in already free areas. Molecular characterization and phylogenetic analysis of relevant epidemiological viruses and of strains re-emerging in already-free areas of the Southern Cone of South America has been described (Mattion et al., 2004; Konig et al., 2007; Malirat et al., 2007). More recently, molecular epidemiology studies of strains circulating in the Andean region of South America were also carried out for serotypes O and A (Malirat et al., 2011, 2012). However, information on the immunogenic characteristics of these strains and particularly on estimation of the cross-protection afforded by the FMD vaccines containing the South American strains against an antigenically related but not identical field virus (vaccine matching) has been relatively insufficient. This information is decisive in order to verify to what extent currently used or stored vaccine strains are suitable to control the disease in endemic regions, in free areas applying preventive vaccination, or during emergency vaccination after introduction of the virus in free areas where attaining rapid immunity is of critical importance.

Complete characterization of viruses re-introduced in Argentina during the emergencies of type A in 2000–2001, including the antigenic and molecular characterization of the FMDV isolates, in vitro vaccine matching studies, in vivo heterologous challenge tests and the introduction of new vaccine strains and their performance during emergency and systematic vaccination campaigns, have been extensively described (Mattion et al., 2004). Also, the antigenic and genetic relatedness of the viruses circulating in Ecuador during the years 2009–2010 to the vaccine strain was assessed, including in vivo vaccine matching studies with representative virus samples (Maradei et al., 2011).

Very limited studies are available on the antigenic/immunogenic characterics of type O re-emerging viruses in the Southern Cone and none involved in vivo cross protection. This study describes the sequential steps followed to assess the antigenic and genetic relatedness of the virus circulating in San Pedro, Paraguay in 2011 to the vaccine strain. In vivo and in vitro vaccine matching studies were also carried out in order to establish to what extent the vaccine in use in the region is adequate to control the disease.

2. Materials and methods

2.1. Virus strains

Field samples from the FMD episode in San Pedro, Paraguay, 2011 (O/San Pedro/Par/11) and in Corrientes, Argentina, 2006 (O/Corrientes/Arg/06) were assayed directly from epithelium samples and/or after passages in baby hamster kidney (BHK-21) cells (clone 13). FMDV vaccine strain O_1/Campos/Brazil/58 (O_1/Campos) used throughout this study belongs to the SENASA reference collection. The geographic location of the O/San Pedro/Par/11 outbreak and of previous episodes recorded in the Southern Cone of South America between 1998 and 2006, included for comparison in part of this study, is depicted in Fig. 1.

2.2. Typing assays

Typing was performed by ELISA and complement fixation assays, as described previously (Alonso et al., 1992).

2.3. Monoclonal antibody profiling

Monoclonal antibodies (MAbs) used in this study were obtained and characterized as described previously (Seki et al., 2009). Reactivity with reference strains and field isolates was performed by ELISA (Seki et al., 2009). Briefly, viruses to be analyzed were trapped by a type-specific rabbit serum and reacted with each MAb. The reactivity was developed by incubation with an anti-mouse serum conjugated with horseradish peroxidase and the substrate/chromophore mixture H_2O_2/ABTS (2,2-azino-bis3 ethyl-benzothiazoline-6 sulfonic acid diammonium salt, Sigma, USA). A blank with no virus was included in each test.

A panel of 22 MAbs for FMDV strains O_1/Campos (1H10, 1B9-3, 17, G8, 2B3, 3H10), O1/Caseros/Argentina/67 (2-6F,

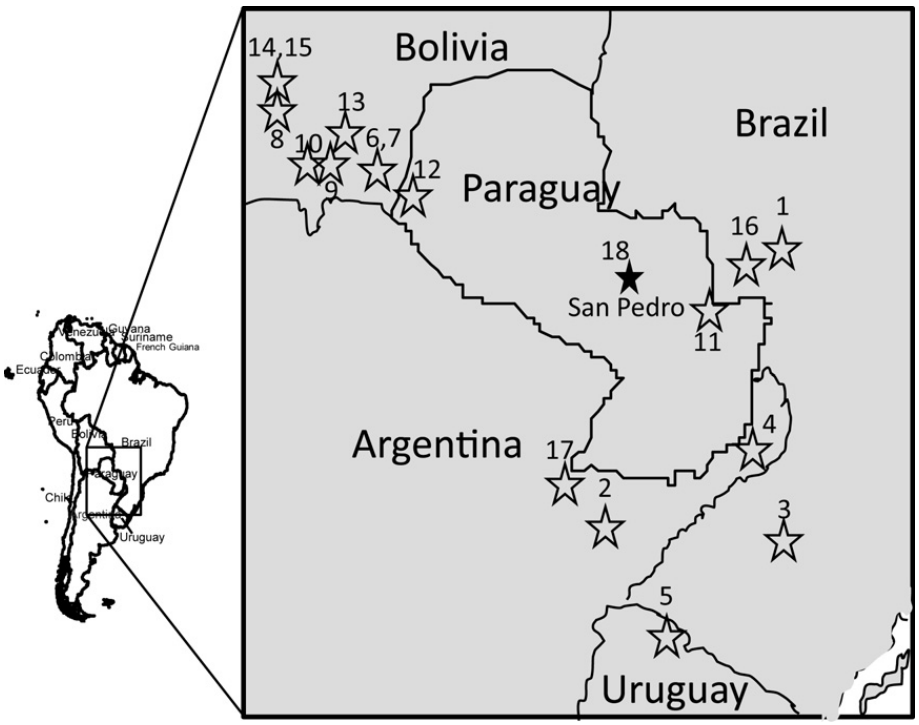

Fig. 1. Geographic location of FMD outbreaks in the Southern Cone of South America. The stars indicate the locations of the type O outbreaks recorded in the Southern Cone of South America between the years 1998 and 2006 [10,20], as well as the location of the O/SanPedro/Par/2011. 1: O/MS/Bra/98; 2: O/Corrientes/Arg/00; 3: O/RS/Bra/00; 4: O/Misiones/Arg/00; 5: O/Artigas/Uru/00; 6: O/Tarija/Bol/00(a); 7: O/Tarija/Bol/00; 8: O/Potosi/Bol/01; 9: O/Tarija/Bol/01(a); 10: O/Tarija/Bol/01; 11: O/Canindeyu/Par/02; 12: O/Pozo Hondo/Par/03; 13: O/Chuquisaca/Bol/03; 14: O/Potosı/Bol/03(a); 15: O/Potosı/Bol/03; 16: O/MS(1a)/Bra/05; 17: O/Corrientes/Arg/06; 18: O/SanPedro/Par/2011.

3, 74, 8G, 69) and O/Taiwan/97 (3A1, 3D1, 4B2, 1A11, 3A2, 2F8, 1B3, 2D4, 1B9, 2C9, 3G10) were used (Seki et al., 2009).

OD values obtained with each MAb after subtracting their corresponding blank values were plotted. Although antigenic profiles are usually shown as bars, in this case and as performed in our previous works, we plotted linear antigenic profiles, which allowed a better comparison of different samples in the same graph. Coefficients of correlation of ELISA reactivity for the field viruses O/Corrientes/Arg/06 and O/San Pedro/Par/11 against the reference strain O_1/Campos and between both emergency viruses were determined as described previously (Seki et al., 2009). Briefly, mathematical calculations were applied to obtain a match factor by plotting the absorbance values of the samples to be related. Linear regression was used to fit the best straight line, and the correlation coefficient was calculated. If the antigenic profiles are identical, the plotted points will fall on a quasi straight line (the correlation coefficient will be close to 1). If the antigenic profiles differ, the points will be widely scattered (the correlation coefficient will be close to 0).

2.4. Nucleotide sequencing

Procedures for RNA extraction, amplification and sequencing conditions applied to determine the sequence of the complete VP_1-coding region of the viral isolates were as already published (Malirat et al., 2007). Briefly, RNA was directly extracted from epithelium samples using QIAmp Viral RNA kit™ (QIAGEN), according to the manufacturer's protocol, after inactivation of the infectious material with Trizol™ reagent (Invitrogen). Reverse transcription of total RNA was conducted with 50 ng of random primers, 200 units of Superscript III Reverse Transcriptase™ (Invitrogen), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithyothreitol and 0.6 mM of each dNTPs in 25 μl final volume, incubating at 42 °C for 60 min, and 70 °C, 15 min. Primers used to amplify and sequence the complete VP_1-coding region rendered an amplification fragment of 790 bp. Their sequences are: 5′-AATTACA-CATGGCAAGGCCGACGG-3′ (forward), and 5′-GAAGGGCC-CAGGGTTGGACTC-3′ (reverse). Amplification reaction mix was prepared in a final volume of 50 μl containing: 5 μl cDNA, 0.5 μM of each primer, 2.5 units of Platinum *Taq* DNA Polymerase™ (Invitrogen), 0.2 mM each dNTP (dATP, dCTP, dGTP, dTTP) and 1.5 mM $MgCl_2$ in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.1% Triton X-100. The thermal profile applied in a programmable thermocycler GeneAmp PCR system 9700™ (Applied Biosystems) was as follows: 5 min at 94 °C, 30 cycles of 94 °C for 1 min, 60 °C for 45 s and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. After PCR, the amplified products were purified from 1% agarose gels with QIAEX II Gel Extraction kit™ (QIAGEN) and the recovered material was quantified by band intensity comparison with DNA mass and molecular weight marker (Invitrogen) in 1% agarose gel electrophoresis.

The nucleotide sequences were determined from 20 to 60 ng of the purified amplicons, using the BigDye® Terminator v3.1Cycle Sequencing Kit (Applied Biosystems), following the manufacturer's procedure. Cycle sequencing was performed in a thermocycler (96 °C, 1 min, followed by 30 cycles of 96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min). The reaction product was purified by ethanol/EDTA precipitation; the recovered material was dyed. For reading, the dyed samples were resuspended in formamide 10%, as recommended for use in a 3500 Genetic Analyzer machine (Applied Biosystems). The sequence determined in this study has been submitted to the GenBank database (Accession number JX514427).

2.5. Phylogenetic analysis

Sequences were edited manually to avoid misreading of peak dyes on an IBM compatible personal computer. Sequence alignment and phylogenetic analysis were performed using the program MEGA, version 5.05 (Tamura et al., 2011). Twenty-four different evolutionary models were tested using Bayesian Information Criterion, and including Akaike Information Criterion and Likelihood Ratio Test, to identify the optimal evolutionary model. The results of this analysis indicated that the Kimura 2-parameter model, using a discrete Gamma distribution to adjust the non-uniformity of evolutionary rates among sites (K2+G) best fit the sequence data. Using this model, maximum-likelihood unrooted trees were constructed using the MEGA software with evolutionary distances calculated using the Kimura 2-parameter method and a bootstrap re-sampling analysis performed with 1000 replicates.

2.6. Serum neutralization assays (VN)

Micro-neutralization tests were carried out according to the method described previously (OIE, 2008), using BHK-21 c13 cell suspensions. Bovine sera from 18 to 24 month-old cattle vaccinated or revaccinated with oil-adjuvanted monovalent vaccines against O_1/Campos or polyvalent vaccines including the O_1/Campos strain were collected at various times after vaccination (DPV) or revaccination (DPRV). The test was performed as a two-dimensional neutralization assay, and antibody titers were calculated as the $\log_{10}$ of the reciprocal antibody dilution required for 50% neutralization of 100 TCID 50 of virus (OIE, 2008).

2.7. Determination and interpretation of r_1 values

A pool of five serum samples from cattle vaccinated with a monovalent vaccine containing O_1/Campos vaccine strain, with a total antigenic mass of 20 μg of 146 S/dose, collected 27 days after vaccination was used for the two-dimensional neutralization assays (see Section 2.6). The sera were tested in 6 (O/Corrientes/Arg/06) or 7 (O/San Pedro/Par/11) independent assays for antibody titers against the homologous FMD vaccine strain and the heterologous O/San Pedro/Par/11 and O/Corrientes/Arg/06 field isolates. The relationship between strains was estimated according to the r_1 value (r_1: reciprocal serum titer against heterologous virus/reciprocal serum titer against homologous virus).

The interpretation of the results was as described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the OIE (OIE, 2008). r_1 values greater than 0.3 indicate that the field isolate is sufficiently similar to the vaccine strain and that the use of the vaccine is likely to confer protection against challenge with the field isolate. Conversely, values less than 0.3 suggest that the field isolate is so different from the vaccine strain that the vaccine is unlikely to protect.

2.8. Assessment of expectancy of protection (EPP)

EPP estimates the likelihood that cattle would be protected against a challenge of 10,000 infective doses after vaccination (OIE, 2008). Sera from 16 cattle vaccinated with a full dose of a commercial tetravalent vaccine containing the O_1/Campos vaccine strain were used for the two-dimensional neutralization assays (see Section 2.6). The sera were derived from the animals used for the in vivo trials (see Section 2.10).

The panel of sera was tested for antibody titers to the homologous FMD vaccine strain and the field isolate. The VN/EPP was determined from the average serological titer obtained, by reference to predetermined tables of correlation between serological titers and clinical protection, established for the vaccine strain. An EPP $\geq$75% is an indication that the vaccines will protect against the homologous vaccine strain (PANAFTOSA, 2001).

2.9. Vaccine formulation and potency assessment

FMDV strains were propagated in BHK-21 cl 13 suspension cell cultures. Infected tissue culture supernatants were collected, clarified and inactivated twice with binary ethyleneimine (BEI) (Bahnemann, 1975). Inactivated supernatants were concentrated and partially purified using polyethylene glycol 6000. Vaccines were prepared in water-in-oil emulsions as described (Mattion et al., 1998). Vaccine potency was assayed by EPP using liquid phase blocking competitive ELISA (lpELISA), performed as previously described (Maradei et al., 2008). The ELISA/EPP estimation was established from the mean antibody titer by reference to predetermined tables of correlation between lpELISA titers and clinical protection obtained with the vaccine strain. An ELISA/EPP $<$75% is an indication that the vaccines will give a low protection against the field strain (Maradei et al., 2008).

2.10. Protection against Podal Generalization (PPG) test

PPG trials were carried out as described previously (Mattion et al., 2004; Goris et al., 2008). Briefly, Hereford breed cattle, aged 18–24 months and free from FMDV antibodies, were used for the trials. They belonged to the FMD-free zone in Argentina, the South Patagonia Region, where vaccination is not practiced. A full dose of a commercial tetravalent vaccine containing the strain O_1/Campos was used. Two groups of 16 animals were used. One group was vaccinated and challenged 79 days later (animals 322–337). The second group was vaccinated, revaccinated at 30 DPV and challenged 79 days after revaccination (cattle 4951–4966).

After vaccination, animals remained in isolated experimental premises and during the challenge period were kept in controlled pens, under biosecurity conditions. The viral isolate O/San Pedro/Par/11 was used for challenge by inoculation of 10,000 suckling mouse lethal dose 50% (SMLD 50%) by the intradermolingual route, after vaccination or revaccination. Two unvaccinated cattle were included in the trial as controls. Seven days after challenge, the animals were examined for podal lesions of FMD. Animals were considered unprotected when typical FMD lesions developed at least in one foot. All the unvaccinated control animals must show podal lesions caused by the disease. According to the Argentine Animal Health Service (SENASA) Resolution no. 351/06 (SENASA, 2006) a vaccine batch is approved for licensing if at least 12 out of the 16 animals are found to be protected. A vaccine batch must be retested if 10–11 vaccinated cattle are protected against challenge, and a vaccine batch is rejected if only 9 or less vaccinates show absence of lesions on the feet (Maradei et al., 2008). The challenge with live virus was carried out in the BSL3A facilities of the Instituto Nacional de Tecnología Agropecuaria (INTA) located in Castelar, Province of Buenos Aires, according to biosecurity and animal welfare federal regulations (SENASA, 2006).

3. Results

3.1. Antigenic characterization

Conventional serological tests (CF, ELISA) typed the FMDV isolate from the 2011 emergency in San Pedro, Paraguay as serotype O.

Further antigenic characterization was performed using a panel of MAbs generated against reference serotype O strains, represented by viruses O_1/Caseros and O_1/Campos. Monoclonal antibodies developed against strain O/Taiwan were also included (Seki et al., 2009). Reactivity by ELISA of the O/San Pedro/Par/11 isolate, the O/Corrientes/Arg/06 virus collected in Corrientes, Argentina during the previous type O FMD emergency in free regions of the Southern Cone in the year 2006, and the prototype vaccine strain O_1/Campos was tested against a panel of 22 MAbs.

Through the study of the MAbs profiling (Fig. 2) and the individual coefficient of correlation values it was possible to establish a viral reactivity profile of both emergency isolates with reduced match with the vaccine strain O_1/Campos and a rather similar, although not identical, correlation between them. When compared with the O_1/Campos vaccine strain, the O/San Pedro/Par/11 and O/Corrientes/Arg/06 isolates exhibited correlation coefficient values of 0.6 and 0.7, respectively. Evaluation of the O/San Pedro/Par/11 against the O/Corrientes/Arg/06 revealed a coefficient of correlation of 0.9, suggesting that the San Pedro isolate is closely related to the Corrientes virus.

The analysis of the reactivity with the individual MAbs included in the panel established clear-cut differences between the O/San Pedro/Par/11 and O/Corrientes/Arg/06 isolates with the vaccine strain O_1/Campos. Whereas the reference strain O_1/Campos had a high level of reactivity

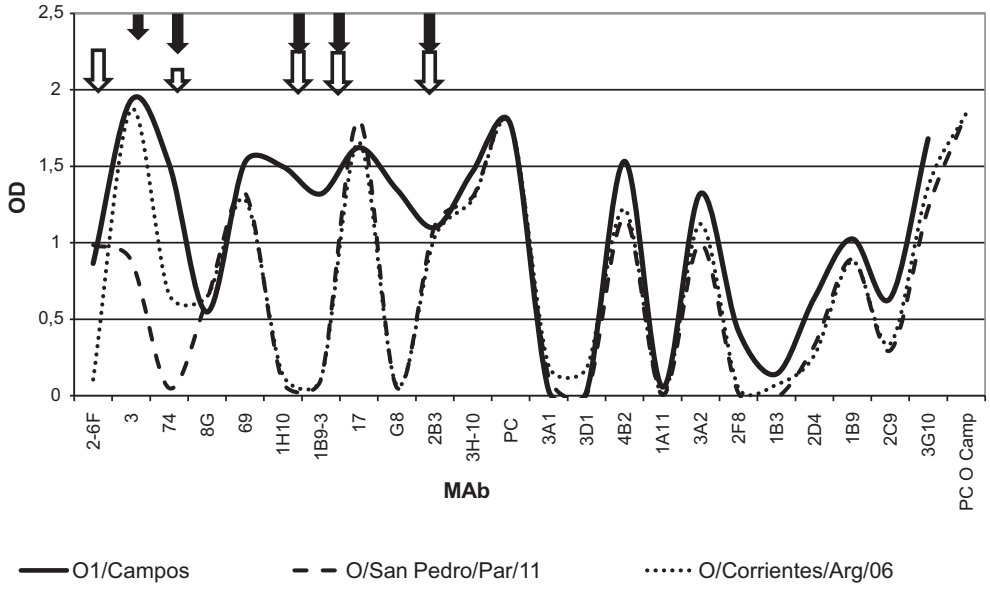

Fig. 2. MAbs profiling of field isolates from Southern Cone of South America. Samples were analyzed by ELISA using a panel of 22 MAbs raised against O_1/Campos (1H10, 1B9-3, 17, G8, 2B3, 3H10), O1/Caseros/Argentina/67 (2-6F, 3, 74, 8G, 69) and O/Taiwan/97 (3A1, 3D1, 4B2, 1A11, 3A2, 2F8, 1B3, 2D4, 1B9, 2C9, 3G10), indicated on the *x*-axis. Differences in ELISA reactivity between the field viruses and the vaccine strain are indicated by the arrows: black arrow, O/San Pedro/Par/11; white arrow, O/Corrientes/Arg/06. Complete or partial loss of reactivity when compared to the vaccine strain is shown by large or short arrows, respectively.

with MAbs 1H10, 1B9-3, G8 and 74, the last two of them having the capacity to in vitro neutralize the strain of origin, the O/San Pedro/Par/11 and O/Corrientes/Arg/06 viruses showed no reactivity with MAbs 1H10, 1B9-3 and G8, and a partial drop of reactivity with MAb 74, in the case of virus O/Corrientes/Arg/06, which is completely lost for virus O/San Pedro/Par/11. Additional differences, not shared by both emergency viruses when comparing them with the reference strain O_1/Campos, pointed out slight variations between them: virus O/Corrientes/Arg/06 lost the reactivity with MAb 2-6F and strain O/San Pedro/Par/11 revealed a diminished reactivity with MAb 3, resulting in a total of 3 differences between the two emergency viruses.

3.2. Sequencing and phylogenetic analysis

Results from phylogenetic analysis of the complete VP_1 gene sequence (Fig. 3) of the O/San Pedro/Par/11 virus showed that it clustered together with viruses responsible for emergencies in the Southern Cone between the years 2000 and 2006. This group shows a mean divergence of 8%, and represents a unique lineage assembling all viruses causing the emergencies in this region, separated from the lineages including Andean strains.

This variant showed the closest genetic distance in the VP_1 gene, between 94.8% and 93.2%, with viruses collected in Tarija, and Potosí, Bolivia, between the years 2000, 2001, with isolate from Rio Grande do Sul, Brazil, 2000, with the strains of Pozo Hondo, Paraguay, and Chuquisaca and Potosí, Bolivia in the year 2003, and with the virus from Corrientes, Argentina, in the year 2006.

When compared with the Paraguayan field virus, the strain used for the vaccine formulation, O_1/Campos, recorded values of 18.6% nucleotide sequence differences and was placed in a different group. In average all viruses from the Southern Cone group present a divergence value of 17.8% with respect to the strain O_1/Campos, used for vaccine formulation.

3.3. In vitro vaccine matching studies

Vaccine matching studies were carried out in order to infer to what extent the vaccine strain O_1/Campos was able to protect the O/San Pedro/Par/11 field isolate.

A two-dimensional virus neutralization test with sera from animals vaccinated with the O_1/Campos vaccine strain was used to assess serological relationship of the O/San Pedro/Par/11 isolate against the vaccine strain (r_1 values). Virus O/Corrientes/Arg/06 was also included in the analysis. Studies were carried out with pools of five medium to high titer serum samples, as recommended, 27 days after vaccination (Mattion et al., 2009). These sera were confronted with the O_1/Campos vaccine strain, and with the field isolates. The neutralizing titer and the r_1 values were obtained as described in Section 2. As can be seen in Table 1, average neutralization titers with the homologous virus O_1/Campos were 2.24 and 2.40, respectively, for the assays with samples O/San Pedro/Par/11 and O/Corrientes/Arg/06, while titers averaged 1.32 and 1.64 for the samples O/San Pedro/Par/11 and O/Corrientes/Arg/06, respectively. Results for r_1 were calculated for each individual trial. Average values were 0.13 and 0.18 for samples O/San Pedro/Par/11 and O/Corrientes/Arg/06, respectively, indicative of poor relatedness between the field viruses and the vaccine strain.

In addition, EPPs of the field strains O/San Pedro/Par/11 and O/Corrientes/Arg/06 were evaluated by VN tests using panels of 16 sera from vaccinated cattle collected at 30 and 79 DPV and at 79 DPRV (revaccination performed at 30

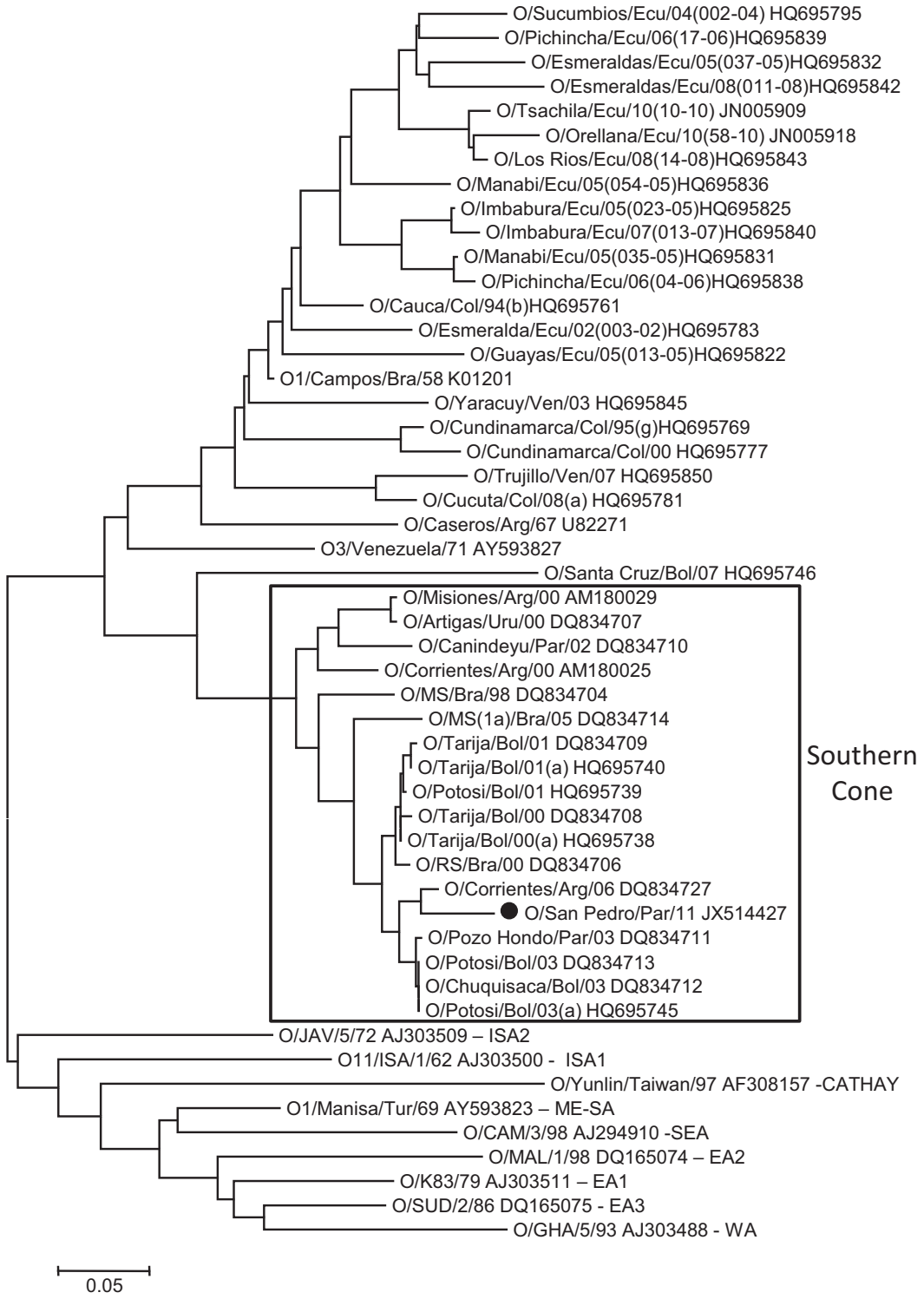

Fig. 3. Phylogenetic tree showing the genetic relationships of FMD virus type O isolated in Paraguay. The genetic distances were calculated based on the comparison of the 633 nucleotides of the VP_1 gene. The unrooted tree was constructed computing the evolutionary distances by the Kimura 2-parameter method, using the Mega 5.0 program as described in Section 2. A distance of 5% is depicted by the scale. (●) Isolate collected in San Pedro, Paraguay. GenBank accession numbers are indicated at the end of the taxon labels.

DPV), as described in Section 2 (Table 2). As can be seen with the panel of sera collected 30 days after vaccination, average titers for the vaccine virus reached values of 2.19 and 2.04, corresponding to EPPs of 94.90 and 91.65, for the assay with the virus O/San Pedro/Par/11 and O/Corrientes/Arg/06, respectively. The VN titers of both field viruses dropped considerably when compared to the vaccine strain. Titers of only 1.29 and 1.32 were obtained for samples O/San Pedro/Par/11 and O/Corrientes/Arg/06, respectively, giving an EPP of 48.87% and 50.52%, values, which are not likely to give satisfactory protection.

When assessed with sera collected at 79 DPV, the O/San Pedro/Par/11 and the O/Corrientes isolates gave average VN titers of 1.70 and 1.87, respectively, corresponding to

Table 1
VN titers and their corresponding r_1 values for strains O/San Pedro/Par/11 and O/Corrientes/Arg/06 using FMDV O1/Campos vaccination.

Test	Virus				
	O1/Campos	O/Corrientes/Arg/06		O/San Pedro/Par/11	
	VN titer	VN titer	r_1	VN titer	r_1
VN1	2.67	1.66	0.10		
VN2	2.38	1.7	0.21		
VN3	2.28	1.71	0.27		
VN4	2.45	1.62	0.15		
VN5	2.14	1.33	0.15	1.08	0.09
VN6	2.49	1.79	0.20	1.63	0.14
VN7	2.28			1.22	0.09
VN8	2.47			1.64	0.15
VN9	1.91			0.91	0.10
VN10	1.98			1.32	0.22
VN11	2.4			1.43	0.11
Average (VN1–VN6)	2.40	1.64	0.18		
Std. deviation	0.18	0.16	0.06		
Average (VN5–VN11)	2.24			1.32	0.13
Std. deviation	0.23			0.27	0.05

Table 2
VN titers and their corresponding EPP estimations for strains O/San Pedro/Par/11 and O/Corrientes/Arg/06 using FMDV O1/Campos vaccination.

Virus	VN titer			EPP (%)		
	30 DPV	79 DPV	79 DPRV	30 DPV	79 DPV	79 DPRV
O1/Campos	2.19	2.29	2.39	94.9	96.28	97.3
O/San Pedro/Par/11	1.29	1.7	1.86	48.87	78.15	85.84
O1/Campos	2.04	2.29	2.39	91.65	96.28	97.3
O/Corrientes/Arg/06	1.32	1.87	1.95	50.52	86.63	89.4

Table 3
Protection of O1/Campos vaccinated cattle against challenge with O/San Pedro/Par/11 (O/SP) virus and VN/EPPs.

Bovine	30 DPV		79 DPV			Bovine	79DPRV		
	VN titer		PPG	VN titer			PPG	VN titer	
	O/SP	O1/Campos	O/SP	O/SP	O1/Campos		O/SP	O/SP	O1/Campos
322	1.27	1.94	NP	1.34	1.94	4951	P	1.87	2.58
323	1.25	2.18	P	1.49	2.46	4952	P	1.87	2.46
324	1.29	2.39	P	2.02	2.31	4953	NP	1.64	1.49
325	1.22	2.27	P	1.57	2.48	4954	NP	1.64	1.75
326	1.36	2.02	NP	1.57	2.34	4955	P	1.57	2.48
327	1.21	2.05	P	1.57	2.14	4956	P	1.94	2.77
328	1.24	2.21	P	1.79	2.46	4957	P	2.17	2.43
329	1.32	2.31	P	1.79	2.34	4958	P	1.72	2.31
330	1.32	2	NP	1.57	2.24	4959	P	1.94	2.58
331	1.25	2.38	P	2.02	2.44	4960	P	2.32	2.66
332	1.21	1.94	P	1.79	2.4	4961	P	1.94	2.62
333	1.25	1.61	NP	1.41	1.58	4962	P	1.79	2.34
334	1.38	2.44	P	1.64	2.44	4963	P	1.57	2.44
335	1.4	2.39	P	1.72	2.17	4964	P	1.94	2.48
336	1.33	2.38	P	2.09	2.41	4965	P	1.87	2.46
337	1.28	2.45	P	1.79	2.45	4966	P	1.94	2.45
			12/16 = 75%				14/16 = 87.5%		
Mean titer	1.29	2.19		1.70	2.29			1.86	2.39
EPP (%)	48.87	94.9		78.15	96.28			85.84	97.3

P: protected; NP: non-protected.

EPP values of 78.15% and 86.63% in both cases, estimated to confer adequate protection. Mean titer for the vaccine strain was 2.29 corresponding to an EPP of 96.28%.

Additional evaluations were carried out with 16 sera collected 79 days after revaccination (revaccination performed 30 days after the first vaccination). As can be seen, the average VN titer for the O_1/Campos vaccine strain was 2.39 corresponding to an EPP of 97.30%, while for the field samples average VN titers were 1.86 and 1.95, for the O/San Pedro/Par/11 and O/Corrientes/Arg/06 viruses, respectively, corresponding to EPPs of 85.84% and 89.4%, projected to give adequate protection.

3.4. In vivo vaccine matching studies

In order to further evaluate the degree of protection induced by the available vaccines against the field virus isolated in San Pedro in 2011, a commercial vaccine containing viruses O_1/Campos, A_{24}/Cruzeiro, A/2001 and C_3/Indaial was used to assess protection from challenge with the O/San Pedro/Par/11 virus, as described in Section 2.

Protection data (Table 3) showed that cattle vaccinated with one dose of commercial tetravalent vaccine containing the O_1/Campos vaccine virus induced 75% protection (12 animal protected/4 unprotected) against challenge with the virus O/San Pedro/Par/11, 79 days after vaccination, and 87.5% (14 animals protected/2 unprotected) for the revaccinated group, 79 days after revaccination. The potency of the vaccine used in the trials was confirmed by evaluating lpELISA/EPP from the vaccinated animals against the homologous vaccine virus, giving an EPP of 96.7%.

In Table 3 the VN titer and the corresponding protection outcome is detailed for individual animals. The logit regression curves used for potency test for virus O_1/Campos established a VN titer of 1.65 for the homologous virus to attain 75% expectancy of protection which is considered the pass mark for appropriate protection (PANAFTOSA, 1994) with sera at 30 days postvaccination. Although the limit of the serum titer that correspond to antibody pass-level for protection is difficult to establish universally for the various conditions (heterologous viruses, revaccination, different days after vaccination, vaccine potency, etc.), within the 32 animals challenged with the heterologous virus, all of the 20 animals with serum titers ≥ 1.65 were protected and every one of the 6 non-protected animals showed serum titers < 1.65. None of the 16 animals used for the studies at 79 DPV reached adequate protection level at 30 DPV.

4. Discussion

The re-introduction of FMD in a previously disease-free region of the Southern Cone of South America had a severe impact on the local and export trade of susceptible animals and their products. A deep characterization of the strains re-appearing in this region, their genetic distribution/evolution and particularly, the assessment of the probable efficacy of the vaccine strain presently in use to control FMD is of utmost importance. The latter information is decisive in order to verify to what extent currently used or stored vaccine strains are suitable to control the disease in endemic regions or in free areas applying preventive vaccination, or for emergency vaccination after introduction of the virus in free regions without vaccination in which case attaining rapid immunity is of critical importance. When necessary, appropriate vaccine strain updating is an important element in the control of FMD and is necessary for the application of vaccination programs as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions.

Although extensive genetic characterization of the viruses emerging sporadically in free regions of the Southern Cone of South America between 2000 and 2006 was performed (Malirat et al., 2007), limited information was available on the antigenic characteristics of the viruses. The present study is the first report of the antigenic/immunogenic characterization of type O viruses re-emerging in already free areas of the Southern Cone of South America, describing procedures for assessment of vaccine matching, including in vivo challenge studies, and focusing particularly to the strain representing the episode in San Pedro, Paraguay in 2011. The results described could improve the understanding of the observed re-emergencies that took place in this region between 2000 and 2011, despite reporting simultaneously satisfactory availability of vaccines for the vaccination program and adequate vaccine coverage.

The first studies were oriented to the genetic characterization of the O/San Pedro/Par/11 virus comparing it with other epidemiological relevant regional and extra-regional strains and with the O_1/Campos vaccine virus. Phylogenetic analysis based on the complete VP_1 sequence indicated that all viruses responsible for re-emergencies that occurred within a period of 11 years in various FMD-free regions in the Southern Cone of South America clustered together in a unique lineage approximately 18% different from the one of the vaccine strain, and differs from those including viruses presently circulating in the Andean region, reflecting the different livestock circuits and epidemiological scenarios. The maintenance of a variant that, although appearing sporadically, remains active and evolves during at least 11 years, supports the idea that active virus is present only in restricted niches and pops up once in a while when it reaches a population with low immune levels against the field virus. This situation in a free region where systematic vaccination is applied under strict management not only reinforces the requirement to strengthen active surveillance oriented to identify putative viral niches but also refreshes the need to thoroughly assess the vaccination program effectiveness, including the evaluation of the immune coverage conferred by the vaccine virus against the acting variant.

Sequencing results are quite appropriate for epidemiological follow up, being relevant to provide information on the possible sources of strains causing the outbreaks and in our case suggested animal movements as responsible for the spread of the disease from potential viral niches. Moreover the results indicated quantitative and qualitative changes of the field viruses when compared to the

vaccine strain that could be responsible for a poor induction of protective antibodies by the vaccine strain in use. However, it is not possible at present to predict the impact of genetic/aminoacid changes on the antigenic behavior of the viruses (Paton et al., 2005). In fact, it has been reported that quite distantly related isolates may have similar antigenic characteristics (Samuel et al., 1988; Hernandez et al., 1992; Barnett et al., 2001). Conversely very close sequence homology may mask large antigenic differences (Mateu et al., 1990; Crowther, 1993; Mateu et al., 1996). Consequently further studies were undertaken to assess the extent of antigenic relatedness between the O/San Pedro/Par/11 virus and the vaccine strain.

The use of a panel of MAbs for a rapid and sensitive way of assessing antigenic differences was reported (Mattion et al., 2004; Seki et al., 2009; Maradei et al., 2011). Using a similar panel, assessment of the MAbs profiling and the individual coefficient of correlation indicated, in agreement with the phylogenetic analysis, low levels of antigenic relatedness between field strains and the reference vaccine strain used in the region. The results also point to some antigenic modifications under the field circumstances, suggesting that, either reversion is taking place, or two distinct viral groups are co-evolving in the niches (the O/San Pedro/Par/11 does not seem to directly derive from the O/Corrientes/Arg/06). The latter situation has already been observed for isolates responsible for the emergencies in the year 2000, when two different variants were identified by phylogenetic studies and by MAbs profiling (Mattion et al., 2004). The overall results, anticipating that significant changes with respect to the vaccine strain have taken place, indicated the need for assays aimed to help evaluate to what extent the currently in use vaccine strain O_1/Campos shall protect against the viruses in the field.

Indirect estimates allow an inference of the protective capacity of the vaccines by measuring the immunological relatedness between virus strains evaluated with well known panels of sera of vaccinated animals collected at 30 DPV (r_1 values) as well as by estimating the likelihood that cattle would be protected against a challenge of 10,000 infective doses after vaccination (EPP calculations). The results showed r_1 values below the 0.3 threshold, indicating a low degree of relatedness between the O_1/Campos and both field viruses and suggesting that the vaccine strain is unlikely to effectively protect against the field isolates. In accordance with the r_1 results, EPP calculations also revealed for both field viruses a poor protective response by the vaccine prepared containing the strain O_1/Campos, when evaluated 30 days after vaccination.

However, an augmented protective response at 79 days after vaccination was observed with EPP values that are expected to give a satisfactory protection (using correlation tables constructed with VN titers of 30 DPV). Indeed both field isolates presented EPPs above the indicative value for an expected appropriate protection in primovaccinated cattle at 79 DPV. Although maximum immunity levels for homologous virus is usually attained between 30 and 45 DPV, there is deficient experimental data for heterologous immune/protective responses, which can be influenced by various mechanisms, such as somatic mutation, affinity and avidity of antibodies, which are still not well understood. As expected, both isolates increased the EPP when evaluated after revaccination.

Finally the direct "in vivo" cross-protection test, based on the challenging with the field virus of animals which were previously vaccinated with a commercial vaccine containing the reference vaccine strain, considered the gold standard test for vaccine matching (OIE, 2008), was performed. Taking into account that the results of such test would only be obtained after more than a month while the evaluation of the efficacy of the vaccine had to be made within days and were oriented to prophylaxis in regions with regular vaccination programs with normal strength vaccines, such heterologous challenge test was carried out according to the epidemiological needs in the country and the availability of already vaccinated cattle from the vaccine potency tests. In accordance with the EPP results, animals primovaccinated with a commercial tetravalent vaccine challenged at 79 DPV with the O/San Pedro/Par/11 isolate attained appropriate protection in vivo, response which was augmented after revaccination, as assessed by EPP and by PPG. This outcome is in agreement with the fact that through the slaughtering of diseased and in-contact animals and the strengthening of vaccination programs applied in Paraguay after recording a second epidemiologically linked event in the same location in January 2012, the spread of the outbreak was finally contained (OIE, 2011, 2012). Moreover, samplings performed to confirm absence of viral activity in free regions of the Southern Cone, including border areas, confirmed that the outbreak was restricted (SENASA, unpublished data).

It should be noted that the estimated EPP values must be taken with caution. Although calculation of EPP has been described to estimate vaccine matching (Paton et al., 2005), it is based on predetermined correlation tables associating antibody titers at 30 DPV with homologous protection against the vaccine strain, which may not be strictly valid for heterologous strains, different days after vaccination or revaccination, vaccine potency, etc. However it is interesting to note that using this table, at least for the conditions studied, all animals with titers above 1.65, considered the pass mark for 75% protection, were protected and in overall 81% of the animals would be correctly classified.

The choice of a vaccine strain to be used will depend very much on circumstances. In an emergency situation it will not be feasible to immediately develop a vaccine strain from a field isolate but it may be possible to supply a closely matched strain if required. The urgency of vaccine application will need vaccines formulated from antigens held in the banks. Antigen reserves have become a priority since the increased acceptance to reduce reliance on large-scale culling of animals to control FMD outbreaks being emergency vaccination increasingly accepted. It is neither economically nor logistically feasible to keep vaccine antigens in stock that are pertinent to any given outbreak situation. Therefore, vaccine reserves are established on a prioritized basis according to the likeliest perceived risk. In this regard, further studies need to point out the convenience of including a representative strain of the

Southern Cone emergent viruses in antigen banks. This selection should take into consideration not only if the strain has a broad antigenic/immunogenic spectrum, but also its capacity to adapt for vaccine production.

Vaccine matching requirements differ for emergency use and prophylaxis. For emergency use it may require a more exact match, principally when needed for completely naïve animals, when rapid responses are essential and when assessing the convenience of incorporating the new virus into antigen vaccine banks.

For the Southern Cone variant described in this work, the use of the O_1/Campos vaccine strain for prophylaxis seems to be adequate according to in vitro and in vivo tests at 79 DPV and after revaccination. However, considering the low r_1 values and EPP at 30 DPV, indicating poor protection, the convenience of including such a variant in an antigen bank for use in an emergency situation, required further studies, including an in vivo test at 30 DPV. Indeed, results obtained with an O_1/Campos experimental monovalent vaccine indicated that, in agreement with the in vitro tests, no protection could be observed in animals challenged 30 days after vaccination with the O/Corrientes/Arg/06 isolate, despite the fact that the vaccine used had appropriate level of potency as assessed by ELISA/EPP and by homologous challenge (manuscript in preparation). Further studies need to be conducted in order to give more input to justify the incorporation of this variant in antigen banks.

Tests measuring the protective effect of an FMD vaccine by an experimental set-up that mimics the field situation, e.g. by contact challenge, are difficult to standardize. There is no generally accepted procedure and the variability of in vitro (Robiolo et al., 2010) as well as of in vivo (Goris et al., 2008) tests is difficult to overcome. This limitation becomes even more noticeable when dealing with cutting edge results, as illustrated in this work in which results at 30 DPV point to poor protection, whereas at 79 DPV or after revaccination protection seems adequate. The dissemination of results for the different conditions seems important for decision making policies. This work reinforces the relevance of having all possible results to fit for the various purposes in which vaccine matching data could be applied.

Overall, the results of the indirect in vitro assays were quite in line with the ones observed in the in vivo challenge test. This is in agreement with recent reports on the confidence in indirect assessment of FMD vaccine matching carried out by virus neutralization tests for serotype A viruses (Mattion et al., 2009; Robiolo et al., 2010) and more recently for type O strains (Maradei et al., 2011). Concordance between viral challenge and indirect serological tests is of great relevance taking into account the strong consensus existing worldwide to improve animal welfare. In addition, the in vivo cross-protection test has other limitations such as its variability (Goris et al., 2008) and the time needed for the trials, which can be critical for the control of an outbreak. Consequently further validation and acceptance of indirect alternatives to in vivo vaccine matching merit consideration. From the perspective of the 3R (Refinement, Reduction, Replacement) concept, the results of this study favor further research into and acceptance of indirect alternatives to in vivo vaccine matching.

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