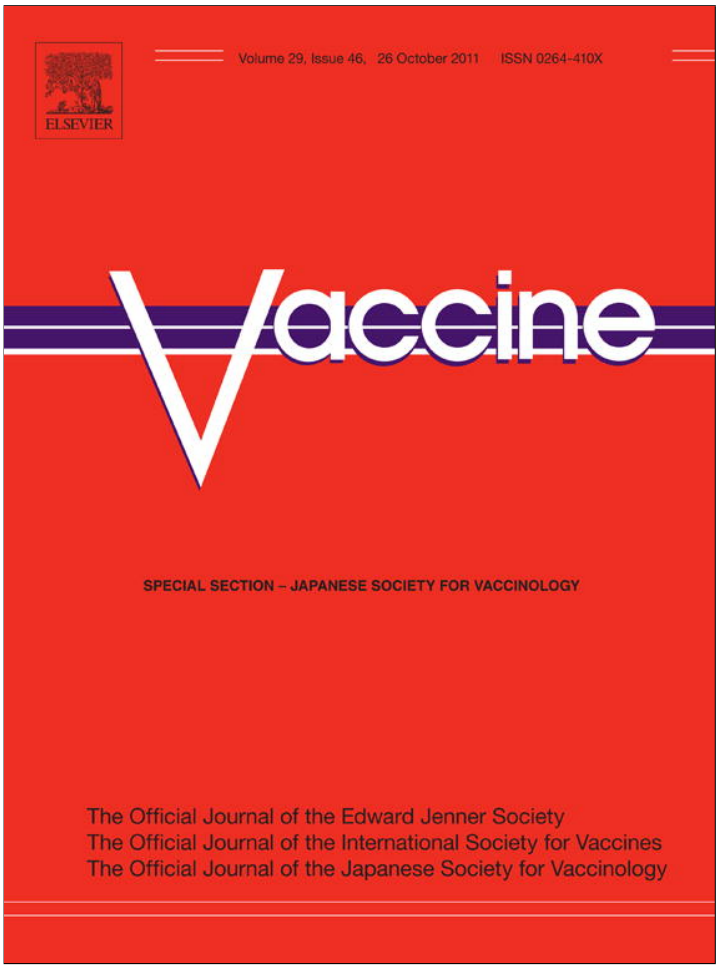

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Characterization of foot-and-mouth disease virus from outbreaks in Ecuador during 2009–2010 and cross-protection studies with the vaccine strain in use in the region

Eduardo Maradei[a], Claudia Perez Beascoechea[a], Viviana Malirat[b], Gustavo Salgado[c], Cristina Seki[b], Andrea Pedemonte[a], Paula Bonastre[a], Ricardo D'Aloia[a], José L. La Torre[b], Nora Mattion[b], Jorge Rodríguez Toledo[a], Ingrid E. Bergmann[b,*]

[a] *Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA), Avda. Fleming 1653, (1640) Martínez, Argentina*
[b] *Centro de Virología Animal (CEVAN), Instituto de Ciencia y Tecnología Dr. César Milstein, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Saladillo 2468 (1440), Buenos Aires, Argentina*
[c] *Laboratorios Veterinarios, Instituto de Medicina e Higiene Tropical "Izquieta Perez", Quito, Ecuador*

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ABSTRACT

During the years 2009 and 2010 relevant epidemic waves of foot-and-mouth disease (FMD) serotype O occurred in Ecuador, representing a great drawback for the last stages of the ongoing eradication program in South America. This study describes the molecular and antigenic characterizations of 29 isolates collected from various regions in the country and their relationship to the vaccine strain. The phylogenetic tree derived from sequences spanning the complete VP_1 protein showed that, despite the widespread origin of the viruses, they were all related among themselves and to previous isolates occurring in 2008, with around 10% difference with the vaccine strain O1/Campos. The high level of sequence conservation among different isolates in the various regions of Ecuador pointed to a common origin, suggesting animal movements as possible sources of viral spread. Monoclonal antibody profiling grouped the isolates in two major reactivity patterns which differed from that of the vaccine strain. Both profiles showed loss of reactivity with the same four MAbs, three of them with neutralizing properties. Additional sites were lost in the profile representing most of the 2010s viral samples. Levels of protective antibodies induced by the vaccine against the field strains assessed by in vitro vaccine matching studies also pointed to an increased temporal pattern of loss of a protective response. Moreover, results obtained with in vivo challenge in the protection against podal generalization test in cattle, clearly indicated lack of appropriate protection of the Ecuadorian field strains by the vaccine virus in use, which in the case of a 2010 variant was observed even after revaccination.

1. Introduction

Foot-and-mouth disease virus (FMDV) is a member of the *Picornaviridae* family, genus *Aphthovirus*, that causes a highly contagious vesicular disease of cattle and other cloven-hoofed animals [1,2]. The virus possesses a single-stranded positive RNA molecule of ca. 8200 nucleotides enclosed within an icosahedral capsid made of 60 copies each of four proteins VP_1, VP_2, VP_3, and VP_4. As reported for many other RNA viruses, FMDV is highly variable [3–5]. The virus can be differentiated into seven immunologically distinct serotypes, O, A, C, Asia 1, South African Territories (SAT) 1, SAT 2 and SAT 3, and intratypic variants (subtypes) [2,6]. 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 [7–9]. Serotypes O, A and C have been recorded in South America.

Although mortality due to the disease is very low and mostly restricted to young animals, drastic 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 [10,11], provokes huge economic consequences when outbreaks occur in disease free regions [12,13] and is considered one of the most important constrains to international trade of livestock and animal products.

Vaccination is widely used to control, eradicate and prevent FMD [14,15]. 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 [15]. The vaccines are prepared by large-scale

* Corresponding author. Tel.: +54 11 4686 6225; fax: +54 11 4687 2542/6735.
E-mail addresses: ibergmanncevan@centromilstein.org.ar, ingrid.bergmann@hotmail.com (I.E. Bergmann).

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growth of FMDV in cell cultures, followed by inactivation with BEI [16]. Thereafter the inactivated viral materials are formulated with oil adjuvants into ready-to-use vaccine [17,18].

Vaccines in South America contain serotypes O and A, and most of the Southern Cone countries include also serotype C. They are formulated using selected strains harmonized for use in the region and include O1/Campos, A24/Cruzeiro and C3/Indaial. 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 homology between the vaccine and the field strain.

Taking into account the present FMD situation in South America, where most countries were recognized by the World Organization of Animal Health (OIE) as FMD-free with or without systematic vaccination (either in specific areas or in the whole territory), characterization of viruses emerging in endemic areas is of critical importance. In this regard, during the years 2009 and 2010 most FMD episodes in the region took place in Ecuador (serotype O) and Venezuela (serotype A), the two South American countries which still remain endemic.

During the past decades, antigenic and genetic characterizations of FMDV in South America has been mainly carried out for strains in the Southern Cone [19–24]. More recently, molecular characterization and phylogenetic analysis of relevant epidemiological viruses and of strains re-emerging in already-free areas of this region has been described [9,25,26]. Complete characterization of viruses re-introduced in Argentina during the outbreaks in 2000–2001, including the antigenic and molecular characterizations of the FMDV isolates, the introduction of new vaccine strains and their performance during emergency and systematic vaccination campaigns, have been extensively described [9].

In contrast, characterization of strains circulating in the Andean region of South America is rather limited. Indeed, although basic antigenic characterization including mainly typing and subtyping of viruses of epidemiological relevance in this region has been carried out [20], information on the genetic and immunogenic characteristics of these strains has been quite insufficient. Recently, efforts have been made towards the genetic analysis of viruses that have been circulating during the past decade [27]. However, very limited information is available on the antigenic analysis of circulating strains and particularly on estimation of the cross-protection afforded by a FMD vaccine made from a particular virus strain against an antigenically related but not identical field virus (vaccine matching). 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 or in free areas applying preventive vaccination, or for emergency vaccination after introduction of the virus in free regions where attaining rapid immunity is of critical importance.

During the year 2009, frequent monthly epidemics of FMDV were reported throughout most provinces of Ecuador. In 2010, new epidemic waves occurred across the country. The reported control measures were mainly oriented to ring vaccination campaigns and control of animal movements, using a bivalent FMDV vaccine, containing both O1/Campos and A24/Cruzeiro strains. Nevertheless, the annual pattern of FMDV occurrence observed reflected low levels of herd immunity against the active circulating strains.

This study describes the sequential steps followed to assess the antigenic and genetic relatedness of the viruses circulating in Ecuador during the years 2009–2010 to the vaccine strain. Vaccine matching studies were also carried out with representative viruses in order to establish to what extent the vaccine strain in use is adequate to control FMD.

2. Materials and methods

2.1. Virus strains

Field samples from the FMD episodes in Ecuador were assayed either as epithelial tissue extracts or as first passage in baby hamster kidney (BHK-21) cells (clone 13). Twenty-nine field samples from episodes occurring in different provinces of Ecuador were assessed, 19 of them collected during the year 2009 and 10 in 2010 (Table 1). The geographical location of the Ecuadorian Provinces is depicted in Fig. 1. FMDV vaccine strain O1/Campos/Brasil/58 (O1/Campos) was provided by SENASA and included in the analysis.

2.2. Typing assays

Typing was performed by ELISA and complement fixation assays (CF), as described previously [28,29]. Briefly, CF was carried out as follows: 0.2 ml antiserum to each FMD serotypes O, A and C, diluted at a predetermined optimal dilution in borate-saline solution (BSS) were placed in a tube. Thereafter, 0.2 ml of test sample suspension were added, followed by 0.2 ml of a complement dilution containing 4 units of complement. The test system was incubated at 37 °C for 30 min prior to the addition of 0.4 ml 2% standardized sheep red blood cells (SRBC) in BSS sensitized with rabbit anti-SRBC. The reagents were incubated at 37 °C for further 30 min and the tubes were subsequently centrifuged and read. Samples with less than 50% haemolysis were considered positive. For the ELISA procedure, an indirect sandwich test was used: different rows in multiwell plates were coated with rabbit antisera to each O, A and C serotypes of FMDV and New Jersey and Indiana of Vesicular Stomatitis Virus. Test sample suspensions were added to each of the rows, and

Fig. 1. Map of Ecuador. The map indicates the geographical location of the Ecuadorian provinces, including those referred in Table 1. The star indicates the location of the Santo Domingo's livestock fair.

Table 1
Designation and origin of foot-and-mouth disease type O viruses analyzed in this work.

Identification number[a]	Geographical location		Collection date	GenBank Accession number
	Province	County	Day/month/year	
12-09	Los Ríos	Valencia	3/4/2009	JN005890
16-09	Esmeraldas	Atacames	4/27/2009	JN005891
39-09	Napo	Chaco	6/25/2009	JN005892
50-09	Tsáchila	Santo Domingo	6/24/2009	JN005893
65-09	Pichincha	Quito	6/4/2009	JN005894
76-09	Carchi	Tulcan	6/5/2009	JN005895
78-09	Tsáchila	Santo Domingo	6/6/2009	JN005896
83-09	Napo	Quijos	6/6/2009	JN005897
85-09	Pichincha	Mejía	6/9/2009	JN005898
86-09	Guayas	El Empalme	6/5/2009	JN005899
89-09	Tungurahua	Pillaro	6/10/2009	JN005900
109-09	Pichincha	Cayambe	6/15/2009	JN005901
126-09	Cotopaxi	Salcedo	6/18/2009	JN005902
145-09	Pichincha	Pedro Vicente Maldonado	6/26/2009	JN005903
148-09	Chimborazo	Guano	6/26/2009	JN005904
153-09	Manabí	Portoviejo	6/30/2009	JN005905
169-09	Imbabura	Ibarra	7/6/2009	JN005906
178-09	Pichincha	Mejia	7/14/2009	JN005907
188-09	Bolívar	Guaranda	7/31/2009	JN005908
10-10	Tsáchila	Santo Domingo	3/27/2010	JN005909
15-10	Pichincha	Los Bancos	4/29/2010	JN005910
21-10	Los Ríos	Babahoyo	5/27/2010	JN005911
23-10	Tsáchila	Santo Domingo	5/30/2010	JN005912
28-10	Sucumbíos	Shushufindi	5/31/2010	JN005913
32-10	Cotopaxi	La Maná	6/3/2010	JN005914
34-10	Imbabura	Cotacachi	6/3/2010	JN005915
44-10	Bolivar	Guaranda	6/8/2010	JN005916
46-10	Napo	Chaco	6/1/2010	JN005917
58-10	Orellana	Sacha	6/17/2010	JN005918

[a] According to: Laboratorios Veterinarios, Instituto de Medicina e Higiene Tropical "Izquieta Perez", Quito, Ecuador.

appropriate controls were also included. Guinea-pig antisera to each of the serotypes of FMDV were added next, followed by rabbit anti-guinea-pig serum conjugated to peroxidase. A color reaction on the addition of enzyme substrate and chromogen indicated a positive reaction.

2.3. Monoclonal antibody profiling

Monoclonal antibodies (MAbs) used in this study were obtained and characterized as described previously [30]. Reactivity with reference strains and field isolates were performed by ELISA [30]. Viral samples identified as 65, 78 and 86, collected in the year 2009, were not assessed due to insufficient material. Briefly, viruses of different origins 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-bis-3-ethyl-benzothiazoline-6 sulfonic acid diammonium salt, Sigma, USA). A blank with no virus was included in each test.

A panel of 20 MAbs for FMDV strains O1/Campos (1H10, 1B9-3, 17, G8, 2B3, 3H10), O1/Caseros (3, 74, 69, 2-6F) and O/Taiwan (3A1, 3D1, 4B2, 1A11, 3A2, 1B3, 2D4, 1B9, 2C9, 3G10) were used [30].

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 we plotted linear antigenic profiles which allowed a better comparison of different samples in the same graph.

Coefficients of correlation of ELISA reactivity for each sample against the reference strain O1/Campos and against field viruses 169-2009 and 46-2010 were determined as described previously [30]. Briefly, mathematical calculations were applied to obtain a match factor by plotting the absorbance values of the unknown sample against the reference strain or against a selected field strain. 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, PCR amplification and sequencing conditions applied to determine the sequence of the complete VP1-coding region of the viral isolates were performed as described by Malirat et al. [26]. Briefly, RNA was extracted from epithelium samples using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription of total RNA was conducted with 50 ng of random primers, 50 units of Superscript II Reverse Transcriptase (Invitrogen), 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol 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′-AATTACACATGGCAAGGCCGACGG-3′ (forward), and 5′-GAAGGGCCCAGGGTTGGACTC-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 *Thermus aquaticus* 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. Thermal cycling was performed in a thermocycler GeneAmp PCR system 9700 (Applied Biosystems) 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 Wizard SV Gel and PCR Clean-up System (Promega) 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 Big Dye Terminator kit 3.1 (Applied Biosystems), following the manufacturer's procedure. Cycle sequencing was performed in a thermocycler (40 cycles of 94 °C, 45 s; 50 °C, 30 s; 60 °C, 4 min); after the extension, products were purified and afterwards resolved on an ABI Prism 3500 Genetic Analyzer sequencing machine. The sequences determined in this study have been submitted to the GeneBank database. Accession numbers are shown in Table 1.

2.5. Phylogenetic analysis

Sequences were edited manually to avoid misreading of peak dyes on an IBM compatible personal computer and aligned using the program BioEdit, version 5.0.2.1. Pairwise comparisons were performed by giving each base substitution equal statistical weight, and unrooted trees were constructed according to sequence relatedness using the neighbor-joining method as implemented in the computer program MEGA, version 5 (NJ/p-distance) [31]. Bootstrap resampling analysis was performed with 1000 replicates. Additionally, 24 different evolutionary models were tested using akaike information criteria (AIC) and a likelihood ratio test (LRT) by means of the program MEGA, to identify the optimal evolutionary model. The results of this analysis indicated that the TN3+G+I model (Tamura–Nei model using a discrete gamma distribution with 5 rate categories and by assuming that a certain fraction of sites are evolutionarily invariable) best fit the sequence data [32]. Using this model, maximum-likelihood unrooted trees were constructed using MEGA software. The general topology of these trees was consistent with those derived from the NJ/p-distance analysis: that is, the major clades and the relationships among sequences were maintained (data not shown).

2.6. Virus neutralization assays (VN)

Micro-neutralization tests were carried out according to the method described previously [33], using BHK-21 c13 cell monolayers. Bovine sera from 18 to 24 month-old cattle vaccinated or revaccinated with oil-adjuvanted monovalent vaccines against O1/Campos or polyvalent vaccines including the O1/Campos strain were collected 30 days after vaccination (DPV) or revaccination (DPRV) at 30 days after the first vaccination. 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 [34].

2.7. Determination and interpretation of r_1 values

Three serum samples from cattle vaccinated with a monovalent vaccine containing O1/Campos vaccine strain, with a total antigenic mass of 20 μg of 146S/dose, were used for the cross-neutralization assays (see Section 2.6). The sera used had VN titers $\geq$1.9 with the homologous virus. The sera were tested in three independent assays for antibody titers to the homologous FMD vaccine strain and the representative field isolates O Ecuador 169-2009 and 46-2010. 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), calculated from the average r_1 values obtained for each of the serum samples.

The interpretation of the results was based on [35]. 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 a single or boosted vaccination [34]. Sera from 14 vaccinated cattle or 17 revaccinated animals were used for the cross-neutralization assays (see Section 2.6). A full dose of a commercial trivalent vaccine containing the strain O1/Campos was used. The panel of sera was tested for antibody titers to the homologous FMD vaccine strain and the representative field isolates. The VN/EPP was determined from the serological titer obtained, for each individual serum, by reference to predetermined tables of correlation between serological titers and clinical protection, established for the vaccine strain. The mean VN/EPP was then calculated from the VN/EPP for each individual serum. An EPP $\geq$ 75% is an indication that the vaccines will protect against the field strain [36].

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) [16]. Inactivated supernatants were concentrated and partially purified using polyethylene glycol 6000 (PEG-6000). Vaccines were prepared as water-in-oil emulsions as described [37]. Vaccine potency was assayed by EPP using liquid phase blocking competitive ELISA (lpELISA), performed as previously described [38]. 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 [38].

2.10. Protection against podal generalization (PPG) test

PPG trials were carried out as described previously [9,39]. Briefly, 36 Hereford breed cattle, aged 18–24 months and free from FMDV antibodies, were used for the trials. They were sourced from the FMD-free zone in Argentina, the South Patagonia Region, where vaccination is not practiced. After vaccination, animals remained in isolated experimental premises and during the challenge period were kept in controlled pens, under biosecurity conditions. Monovalent vaccines containing an antigenic mass of 20 μg of 146S of the vaccine strain O1/Campos were used. This vaccine had been previously tested by PPG at 30 DPV, resulting in more than 75% protection when challenged with the homologous strain O1/Campos. A group of 32 animals were vaccinated, 16 of which were revaccinated 30 days later. The viral isolate 46-2010, collected in the Province of Napo, was used for challenge by inoculation of 10,000 suckling mouse lethal dose 50% (SMLD 50%) by the intradermolingual route, 30 days after revaccination or vaccination. Two unvaccinated cattle were included in each 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 [40] 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 [38]. The challenge with live virus was carried out in the BSL3A facilities of the Instituto Nacional de Tecnología

Table 2
Coefficient of correlation values for FMDV field strains against the indicated viruses, determined by ELISA using the panel of 20 MAbs shown in Fig. 2.

Virus	Coefficient of correlation values		
	O1/Campos	169-2009	46-2010
O1/Campos	1.00	0.56	0.49
12-2009	0.6	0.94	0.57
16-2009	0.57	0.96	0.59
39-2009	0.58	0.99	0.67
50-2009	0.54	0.99	0.69
76-2009	0.58	0.99	0.71
83-2009	0.56	0.99	0.70
85-2009	0.60	0.97	0.62
89-2009	0.60	0.99	0.69
109-2009	0.54	1.00	0.67
126-2009	0.58	0.99	0.68
145-2009	0.59	0.99	0.70
148-2009	0.59	0.99	0.71
153-2009	0.55	0.97	0.60
169-2009	0.56	1.00	0.68
178-2009	0.53	1.00	0.67
188-2009	0.56	0.99	0.70
10-2010	0.44	0.93	0.60
15-2010	0.51	0.95	0.67
21-2010	0.49	0.68	0.99
23-2010	0.49	0.67	1.00
28-2010	0.50	0.65	0.98
32-2010	0.47	0.68	1.00
34-2010	0.49	0.67	1.00
44-2010	0.47	0.68	0.99
46-2010	0.49	0.68	1.00
58-2010	0.53	0.68	0.97

Agropecuaria (INTA) located in Castelar, Province of Buenos Aires, according to biosecurity and animal welfare federal regulations [40].

3. Results

3.1. Antigenic characterization

Conventional serological tests (CF, ELISA) typed all of the 29 FMDV isolates from 2009 to 2010 outbreaks in the various regions in Ecuador as serotype O.

Further antigenic characterization was performed using a panel of MAbs generated against reference serotype O strains, represented by viruses O1/Caseros and O1/Campos. Monoclonal antibodies developed against strain O/Taiwan were also included [30]. Reactivity by ELISA of 16 FMDV field strains collected in 2009, 10 samples recovered in 2010, and the prototype vaccine strain O1/Campos was tested against a panel of 20 MAbs.

Through the study of the MAbs profiling (Fig. 2) and the individual coefficient of correlation values (Table 2) it was possible to establish the emergence of two clearly different viral reactivity profiles with reduced match with the vaccine strain O1/Campos, indicating that they had undergone important antigenic variations upon circulation in the field. Group 1 included the viruses active in the year 2009 and the first two viruses isolated in 2010 and group 2 comprised the rest of the viruses collected in the year 2010. Strains within groups showed a similar reactivity pattern and presented coefficient of correlation values among themselves close to 1, indicating that they were in a range of high homology. Between both groups, coefficient of correlation values ranged from 0.57 to 0.71. When compared with the O1/Campos vaccine strain, both groups exhibited values ranging between 0.44 and 0.60.

The analysis of the reactivity with the individual MAbs included in the panel established clear-cut differences between Ecuadorian isolates and the vaccine strain O1/Campos. Whereas the reference strain O1/Campos had a high level of reactivity with MAbs 1H10, 17, G8 and 74, the last three of them having the capacity to in vitro neutralize the strain of origin, O1/Campos, the field viruses showed no reactivity with these MAbs. Within the isolates recovered in the year 2010, samples 10 and 15 revealed a profile similar to the predominant reactivity pattern of the variants isolated in 2009. The rest of the viral samples collected in 2010, in addition to the lack of reactivity with MAbs 1H10, 17, G8 and 74, also presented lack of reactivity with MAbs 2B3, 3H10, 2D4 and 1B9.

3.2. Sequencing and phylogenetic analysis

The complete nucleotide sequence of the VP_1-coding region was determined for all the isolates collected in 2009 and 2010. These sequences were aligned and compared to the viruses responsible for the previous episodes occurring in the Andean region between the years 2002–2008, as well as to other type O relevant strains of the continent, including the virus used for vaccine formulation, O1/Campos. Representative strains of topotypes exogenous to South America were also included. A phylogenetic tree generated using the neighbor-joining method is shown in Fig. 3.

All viruses collected in the years 2009–2010 belonged to one unique lineage within the Euro-South American topotype, with identities among them of over 95% in the VP_1-coding region. Viruses collected in 2009 recorded over 98% identity among themselves. The first isolate collected in 2010 (O/Ecu/10/2010) showed higher identity in the VP1 gene with most variants circulating in 2009 (over 99%) than to those circulating in 2010 (about 95%). Isolate O/Ecu/15/2010 presented 96% relatedness with 2009 viruses and 97% with the isolates from 2010. All the rest of the viruses collected in 2010 were almost identical, with circa 100% identity among them and showed on average 96% relatedness with the 2009 isolates.

When compared with strains that have been circulating since the year 2002 in Ecuador, they showed the closest genetic distance with a virus which belonged to a unique representative of a subgroup described in a previous study [27], isolated in May 2008 in Los Ríos. The 2009 variants presented a higher degree of identity (>98.7%) with Los Ríos isolate than those circulating in 2010 (96.7%). Overall average nucleotide differences with the rest of the samples from Ecuador were above 9%.

When compared with the strain used for the vaccine formulation, O1/Campos, the Ecuadorian viruses recorded values of 8–11% nucleotide sequence differences and were placed in a different group.

Comparisons with representative isolates from emergencies in the Southern Cone recorded between the years 2000–2006 showed that presently circulating Andean strains clustered in a different lineage, with differences of around 17%.

Although the sequences showed an important degree of identity with an already existing circulating virus in 2008, most viruses isolated in 2009, and all samples collected in 2010, registered a codon deletion. This observation is exceptional for serotype O viruses, and results in the loss of one amino acid in position 139 in the corresponding original alignment in most samples from 2009, and in position 144 of most of the 2010 samples. This latter position is immediately upstream the RGD motif.

3.3. In vitro vaccine matching studies

Vaccine matching studies were carried out in order to evaluate to what extent the vaccine strain O1/Campos was able to protect the Ecuadorian field isolates.

A two-dimensional VN test with sera from animals vaccinated with the vaccine strain O1/Campos was used to assess serological reactivity of selected field isolates against the vaccine strain (r_1 values). Studies were carried out with 3 medium to high titer sera, as recommended [41]. These sera were tested with the O1/Campos

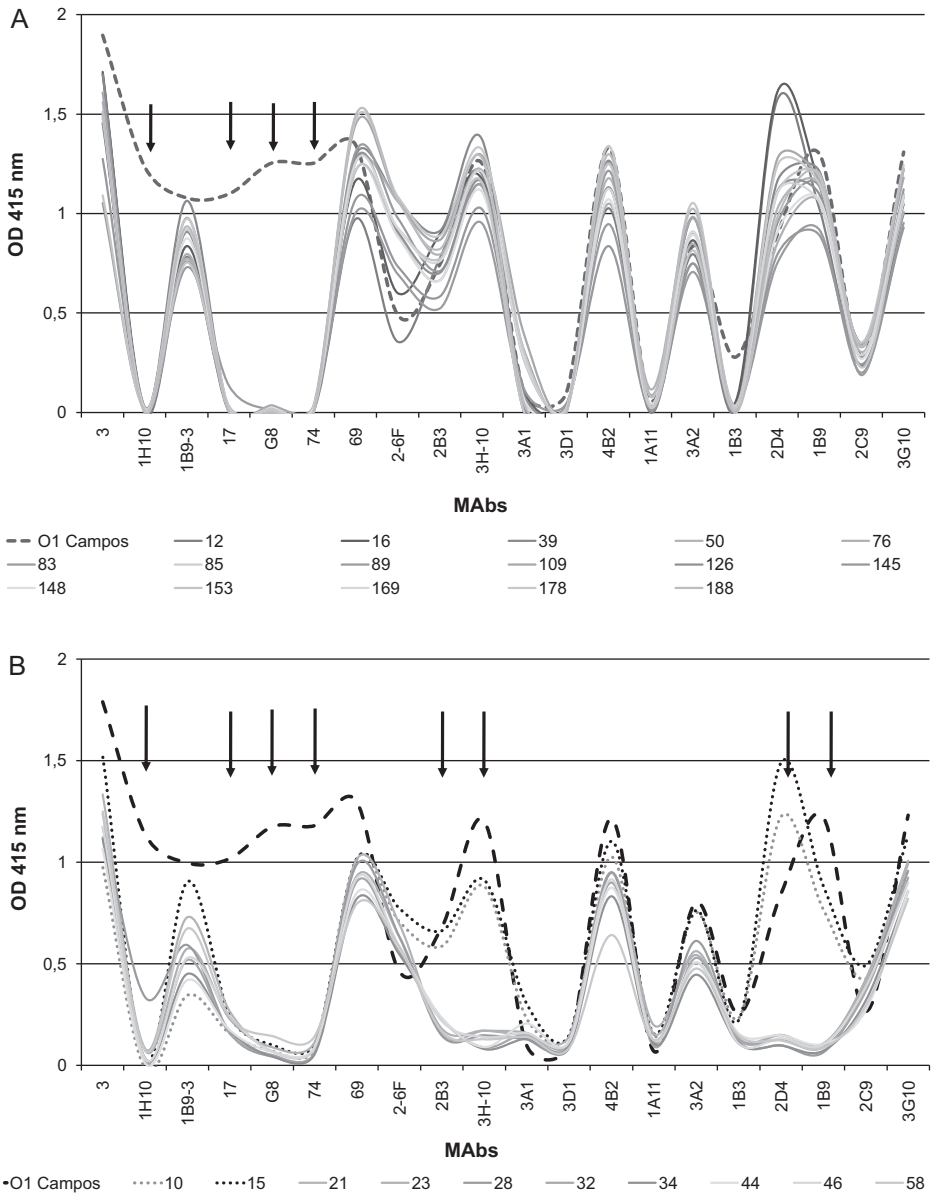

Fig. 2. MAbs profiling of field isolates from the 2009 (A) and 2010 (B) outbreaks in Ecuador. Field samples were analyzed by ELISA using a panel of 20 MAbs for serotype O strains indicated on the *x*-axis. The corresponding reactivity correlation coefficients values for these strains are shown in Table 2. The arrows indicate the differences in ELISA reactivity between the field isolates and the vaccine strain. Supplementary file 1 shows outbreak viruses individually.

vaccine strain and with the virus isolates 169-2009, collected in 2009 in the Province of Imbabura, and 46-2010 collected in the Province of Napo. The neutralizing titer and the r_1 values were obtained as described in Section 2.

As can be seen in Table 3, average neutralization titers with the homologous virus O1/Campos were 1.99 and 2.05, respectively for the assays with samples 169-2009 and 46-2010, while for these viruses and in that order titers averaged 1.24 and $\geq$0.95. Values for r_1 were calculated for each individual serum. Average values were 0.20 and 0.08 for samples 169-2009 and 46-2010, respectively, indicative of low level of neutralization of the variants prevalent in the field by the vaccine strain O1/Campos. A similar r_1 value, 0.09, was obtained with sample 23, collected in the year 2010 in the province of Tsáchila (data not shown).

In addition, EPPs of the field strains 169-2009 and 46-2010 were evaluated by VN tests using the panel of 17 sera from cattle revaccinated with the vaccine strain O1/Campos, as described in Section 2 (Table 3). Average titers for the vaccine virus reached values of over 2.26, while titers of only 1.9 and 1.44 were obtained for samples 169-2009 and 46-2010, respectively, giving an EPP of over 94% for the O1/Campos vaccine strain, and 83.48% and 59.24% for viruses 169-2009 and 46-2010, respectively. In the case of virus 169-2009, additional tests were carried out with 14 sera collected 30 days after first vaccination. As can be seen, in this case the average VN titers obtained for vaccine strain O1/Campos was 1.78 corresponding to an EPP of 81.78%, while for sample 169-2009 the average VN titer was only 1.16 corresponding to an EPP of 38.31%. These results indicate poor protection by the vaccine strain in use, which was particularly low for the virus 46-2010, even after revaccination.

3.4. *In vivo* vaccine matching studies

In order to evaluate the degree of protection induced by the available vaccines against the field viruses isolated in Ecuador in 2009–2010, a monovalent vaccine was used to assess protection

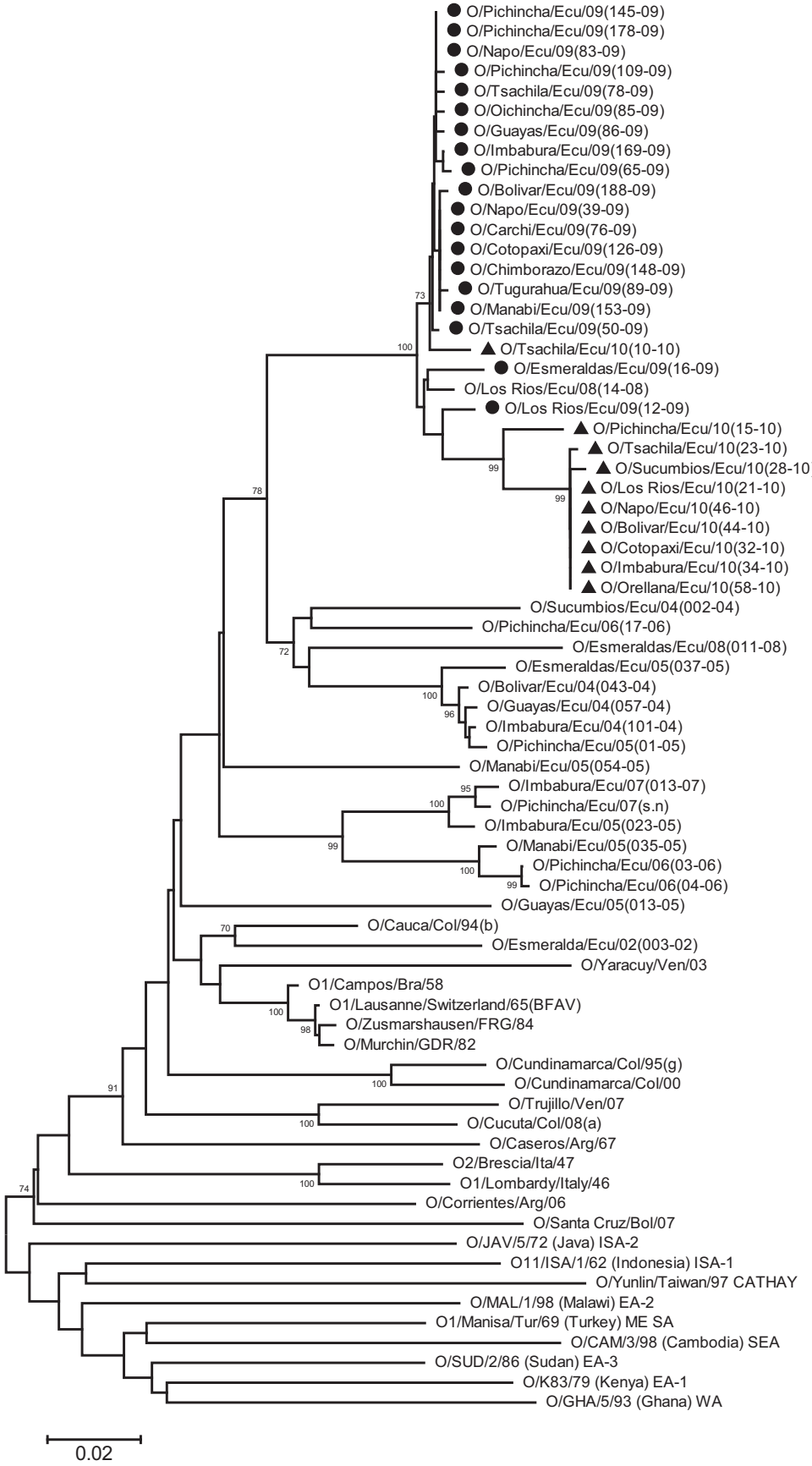

Fig. 3. Phylogenetic tree showing the genetic relationships of FMD virus type O isolates in Ecuador. The *p*-distances were calculated based on the comparison of the 633 nucleotides of the VP_1 gene. The neighbor-joining tree was constructed computing the evolutionary distances by the *p*-distance method, using the Mega 5.0 program. A distance of 2% is depicted by the scale. Bootstrap values >70 based on 1000 replicates are indicated. Isolates collected in (●) 2009 or (▲) 2010.

Table 3
VN titers and their corresponding r_1 values and VN/EPP estimations for selected field strains using FMDV O1/Campos vaccination.

	r_1			EPP (%)		
	VN titer[a]	r_1[a]	VN titer		EPP(%)[d]	
Virus	30 DPV	30 DPRV	30 DPV[b]	30 DPRV[c]	30 DPV	30 DPRV
O1/Campos	1.99		1.78	≥2.37	81.78	≥96.8
169-2009	1.24	0.20	1.16	1.9	38.31	83.48
O1/Campos	2.05		ND	2.27	ND	94.55
46-2010	≥0.95	0.08	ND	1.44	ND	59.24

DPV, days post-vaccination; DPRV, days post-revaccination; ND, not determined.
[a] Mean of 3 serum samples, each calculated from the mean of 3 assays.
[b] Mean of 14 sera.
[c] Mean of 17 sera.
[d] Mean of individual serum sample values.

from challenge with the Ecuadorian virus 46-2010, as described in Section 2.

Protection data showed that cattle vaccinated with one dose of monovalent O1/Campos vaccine induced only 6% protection (one animal protected/15 unprotected) against challenge with the virus O Ecuador 46-2010, and 18% (3 animals protected/13 unprotected) for the revaccinated group. The potency of the vaccine used in the trial was confirmed by evaluating lpELISA antibody titers of serum samples obtained prior to challenge from the vaccinated and revaccinated animals against the homologous vaccine virus. Average results indicated a satisfactory level of homologous protection, reaching EPPs of 91.3% and 99.2% for the vaccinated and revaccinated group, respectively (Table 4).

4. Discussion

This is the first report of the antigenic characterization of serotype O viruses circulating in Ecuador, describing procedures for assessment of vaccine matching, including in vivo challenge studies, specifically for strains circulating during the 2009–2010 epidemics. The results described here could improve the understanding of the observed epidemic waves registered in Ecuador during the past two years, despite having reported simultaneously the greatest historical availability of vaccines for the vaccination program, and over 90% coverage during the 6-month vaccination cycles.

Awareness of the strains prevailing, their genetic distribution/evolution and particularly, assessment of the probable efficacy of the vaccine strain in use to control the disease is of utmost importance. Moreover, when necessary, appropriate vaccine strain replacement/inclusion is an important element in the control of FMD and is necessary for the application of vaccination programs in FMD-affected areas, as well as for the establishment and maintenance of vaccine antigen reserves to be used in the event of new FMD incursions.

In this context, an algorithm of tests was performed. A first group of assays was oriented to establish the antigenic and genetic characteristics of the circulating viruses comparing them with other epidemiological relevant regional and extra-regional strains and with the O1/Campos vaccine virus. Studies indicated close similarities among the field viruses and gave a first approximation of their poor match with the vaccine virus. These results indicated that a second group of assays was needed, aimed to help evaluate to what extent the currently in use vaccine strain O1/Campos will protect against the viruses in the field, and also to give input for the selection of representative viruses from the 2009 and 2010 episodes for these studies.

Results from phylogenetic analysis of the complete VP_1 coding region from FMDV isolates responsible for outbreaks in Ecuador during the years 2009–2010, showed that they were closely related and clustered together with viruses already described in Ecuador in 2008 [27] with around 10% difference with the vaccine strain. It is interesting to note that out of the 3 lineages which circulated in the country since the year 2002, only the one containing the 2009–2010 viruses seemed to have been maintained over time, suggesting its higher evolutionary fitness. Moreover, this variant has spread rapidly over large distances, supporting a common origin for the different FMD outbreaks in the country. The recovery of almost identical isolates from distant geographical locations in a very short time period that has already been described [27], can be understood considering that each week a livestock fair is carried out in Santo Domingo location, province of Tsáchila, in the geographical center of Ecuador, where livestock from all over the country is gathered together for commercial trade. This suggests that animal movements could be an important risk parameter, reinforcing the need for strengthening the official controls carried out within the country.

Analysis of the virus amino acid sequences indicated changes with respect to the vaccine strain that could be relevant for antigen presentation, particularly a codon deletion. This molecular marker has been seen in other FMDV serotypes, but not in serotype O viruses. Further studies will be conducted to better understand the implications of such changes, particularly taking into account that this unexpected event appeared in different nucleotide positions within a short time period.

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 a relevant aspect for the spread of the disease. Moreover the results indicated quantitative and qualitative changes of the field viruses when compared to the vaccine strain that could be responsible for the poor induction of protective antibodies by the current vaccine strain. However, it is not possible at present to predict the impact of genetic/amino acid changes on the antigenic behavior of the viruses and consequently the genetic results should be taken with caution [42]. In fact, it has been reported that quite distantly related isolates may have similar antigenic characteristics [43–45]. Conversely very close sequence homology may mask large antigenic differences [46]. Therefore, further studies were oriented to a better understanding of the antigenic behavior of the strains.

The use of a panel of MAbs as a rapid and sensitive way of assessing antigenic differences was reported [30]. Using a similar panel and, in agreement with the sequencing data, low levels of antigenic relatedness between field strains and the reference vaccine strain used in the region was established. Assessment of the MAbs profiling and the individual coefficient of correlation identified two distinct groups, which showed an increased differentiation in time

Table 4
Protection against challenge with field virus 46-2010 of O1/Campos vaccinated cattle.

30 DPV				30DPRV			
Bovine	46-2010	O1/Campos[a]		Bovine	46-2010	O1/Campos[a]	
	PPG	lpELISA titer	%EPP		PPG	lpELISA titer	%EPP
572	NP	2.26	82.1	556	NP	2.82	95.8
573	NP	1.49	33.9	557	P	3.55	99.4
574	P	3.39	99.1	558	P	3.67	99.6
575	NP	2.65	93.3	559	NP	3.42	99.2
576	NP	2.49	89.8	560	NP	3.42	99.2
577	NP	2.13	76.0	561	NP	3.79	99.7
578	NP	3.34	99.0	562	NP	3.70	99.6
579	NP	2.91	96.7	563	NP	2.99	97.3
580	NP	2.95	97.0	564	NP	3.09	98.0
581	NP	2.81	95.6	565	P	3.61	99.5
582	NP	2.45	88.7	566	NP	3.32	98.9
583	NP	1.98	67.4	567	NP	3.51	99.4
584	NP	2.72	94.4	568	NP	3.41	99.2
585	NP	2.65	93.3	569	NP	2.96	97.1
586	NP	2.45	88.7	570	NP	3.46	99.3
587	NP	2.20	79.5	571	NP	3.69	99.6
Mean titer		2.55	91.3	Mean titer		3.40	99.2

P, protected; NP, non-protected; DPV, days post-vaccination; DPRV, days post-revaccination; second dose administered at 30 DPV.
[a] Confirmation of vaccine potency at the time of the trial by lpELISA/EPP.

with respect to the vaccine strain. Both groups lack reactivity with the same 4 MAbs, 3 of them with neutralizing properties, but the pattern of the viruses circulating in 2010 had additional differentiation markers. These results clearly indicate a rapid antigenic modification under the field circumstances.

Results of MAbs profiling and phylogenetic analysis of the viruses active in Ecuador indicated that significant changes could have taken place and consequently, that further and more specific vaccine matching studies were required. On this basis, we selected representative viruses of the 2009 and 2010 episodes, 169-2009 and 46-2010, and carried out indirect in vitro and direct in vivo vaccine matching tests. Indirect evaluations allow an estimation of the protective capacity of the vaccines based on the calculation of r_1 values and EPPs estimated from results of virus neutralization tests. The direct test is considered as the gold standard test for vaccine matching whereby animals which were previously vaccinated with the reference vaccine strain are challenged with the field virus [34].

The results showed r_1 values below the 0.3 threshold, suggesting that the vaccine strain is unlikely to protect against the field isolates. A lower r_1 value was found for the 2010 isolate when compared with the 2009 one, clearly indicating in accordance with the MAbs profiling, a temporal pattern that suggests a progressive loss of protective response by the vaccine prepared with the strain O1/Campos.

The VN results observed by using a larger panel of sera for the EPP calculation [42], also indicated a temporal tendency, with loss of protective response by the vaccine prepared with the strain O1/Campos, with average VN titers, and consequently EPP values, dropping considerably between 2009 and 2010. The 2009 isolate presented an EPP below the indicative value for an expected appropriate protection in primovaccinated cattle, although not in the revaccinated population. For viruses isolated in 2010, the EPP value was below the protective value even in revaccinated animals. 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 [42], it is based on predetermined correlation tables associating antibody titers with homologous protection against the vaccine strain, which may not be strictly valid under heterologous conditions. However, in overall, a curve for the new emerging strain would not be available.

Finally and taking into account all the tests performed, which suggested a loss in the effectiveness of the vaccine virus to protect the field isolates, the in vivo cross-protection test (PPG) was performed.

The results of PPG trials indicated very poor protection, even after revaccination. Since this test takes into account not only the cross reaction, but also the potency of the vaccines, it is valid to suppose that the same challenge in vaccinated and revaccinated animals with a vaccine with a higher potency could lead to different quantitative results. However it is unlikely to suppose that the results would change the main conclusion that was drawn from this study.

The emergence of antigenically distinct viruses resistant to neutralization due to selective pressure under sub-neutralizing conditions has been previously reported [47]. In this context, updating of vaccine strains is important in order to achieve levels of protection which can impair the selection of new variants resistant to neutralization.

Therefore, to induce a high level of protection in vaccinated animals, the inclusion of the new strain in the vaccine is highly convenient for a rapid and effective response. In fact, the replacement or inclusion of new variants in vaccine formulations has been previously documented [9,21]. For example, during the emergencies of serotype A viruses which occurred in already free regions of the Southern Cone of South America during 2000–2001, Argentina included type A 2001 in their vaccines for emergency vaccination, which helped attain a rapid control of the disease. This strain is still present in the vaccines used in this country and was included in international vaccine banks [9]. In contrast, Uruguay, having a considerable smaller cattle population than Argentina, managed to compensate the antigenic differences between the vaccine strain and the field virus by revaccinating the cattle population at 30 days after first vaccination [48]. However, in the Ecuadorian variants described herein the situation is more drastic since r_1 values, particularly for the 2010 isolates were even lower compared to what was reported for the A 2000–2001 strains [41]. Moreover, the in vivo challenge tests of the A serotype viruses causing the 2000–2001 epidemics, indicated acceptable levels of protection after re-vaccination with the reference vaccine strain A24/Cruzeiro [9], which is in contrast with the results obtained with the viral variants in Ecuador.

The results of the indirect in vitro assays were 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 [41,49]. 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 [39] 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.08.120.

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