Veterinary Microbiology 158 (2012) 82–94

Contents lists available at SciVerse ScienceDirect

Veterinary Microbiology

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

Molecular epidemiology of foot-and-mouth disease virus type A in South America

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ARTICLE INFO

Article history:
Received 13 December 2011
Received in revised form 6 February 2012
Accepted 9 February 2012

Keywords:
Foot-and-mouth disease type A virus
Molecular epidemiology
Sequence analysis
Europe–South America FMDV topotype

ABSTRACT

A databank of 78 VP_1 complete sequences of type A foot-and-mouth disease virus (FMDV) from South American isolates was constructed. Forty-nine samples corresponded to FMDV that circulated between the years 1999–2008, mainly in Venezuela, where most type A outbreaks have occurred lately and twenty-nine to strains historically relevant for the continent. The phylogenetic analysis showed that all South American FMDV belonged to the Euro-SA topotype. Sixteen subgenotypes could be identified, based on a 15% nucleotide divergence cut-off criterion: eight are extinguished, three were active until the year 2002 and the remaining five circulated in Venezuela during the years 2001–2007, illustrating the potential for FMDV diversification under appropriate selective pressure. The last emergencies reported in already-free areas of Colombia in 2004 and 2008 were closely related to isolates acting in Venzuela. Evidence of positive selection over codon 170, within the immunogenic site 4 of VP1 protein, was recorded. A codon deletion in amino acid position 142, within the G–H loop, was found in some isolates within subgenotypes 14, 15 and 16. Conversely amino acid deletion 197 was restricted to all isolates within a particular genetic cluster. The present work is the first comprehensive phylogenetic analysis of FMDV type A in South America, filling a gap of knowledge with respect to both, historical and acting viruses. The results provided evidence that supports the ecosystem dynamics in the region, and also served as an input to establish genetic links of emergencies in already-declared free areas, highlighting the need for strengthening control activities.

1. Introduction

Foot-and-mouth disease virus (FMDV) is a member of the *Aphthovirus* genus, *Picornaviridae* family, which causes a highly contagious vesicular disease in cattle and other cloven-hoofed animals. The disease impacts negatively in the livestock industry of countries where it is endemic, or in the event of re-introduction in already-free regions (Huang et al., 2000; Correa Melo et al., 2002).

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doi:10.1016/j.vetmic.2012.02.009

The emergency situations experienced by many foot-and-mouth disease (FMD)-free countries or regions worldwide (Paton et al., 2009) have prompted the need for a deep characterization of strains circulating in endemic regions. This information gives support to source hypotheses, knowledge that is of utmost importance at the moment of designing contingency and eradication plans, and also helps building a wide picture of the evolving nature of the virus and its biological and immunological characteristics.

Identification of genetic relationships among circulating viral strains has been aided mostly by nucleotide sequencing and phylogenetic studies. The complete nucleotide sequence coding for VP_1 protein, that contains the major immuno-dominant G–H loop (Bittle et al., 1982) and the RGD-integrin binding motif (Fox et al., 1989), has been extensively used for molecular epidemiology studies on FMD, mainly for being the most variable among the capsid polypeptides (Carrillo et al., 2005). These studies have helped establishing epidemiological links among viral isolates, giving input to follow geographical movement of strains, and facilitating the identification of viral sources (revised in Klein, 2009).

The disease has historically 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 (Fig. 1). 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 year 2003, and Venezuela where FMD types O and A have been acting yearly (PAHO/WHO, 2011a,b).

Molecular epidemiology studies available for South American strains had been restricted to selected strains or single epizootics and mainly focused on isolates from the Southern Cone (König et al., 2001; Araujo et al., 2002; Mattion et al., 2004; Bergmann et al., 2005; König et al., 2007; Malirat et al., 2007). Recently, phylogenetic analysis of FMD type O viruses circulating in the Andean region of South America and the comparison with other continental and worldwide representative strains, were published (Malirat et al., 2011; Maradei et al., 2011), which filled a gap of knowledge for this particular type. In contrast, limited information is available on type A circulating strains and/or on the genetic evolution of this virus in the continent.

This paper reports a comprehensive molecular epidemiology study of type A FMDV in South America, based on complete VP_1 nucleotide sequencing. The analysis was performed over a substantial number of lately circulating isolates and epidemiologically relevant strains.

2. Materials and methods

2.1. Viruses

The designation and origin of viruses examined in this study together with FMDV sequences available from Genbank and used in this analysis are listed in Table 1.

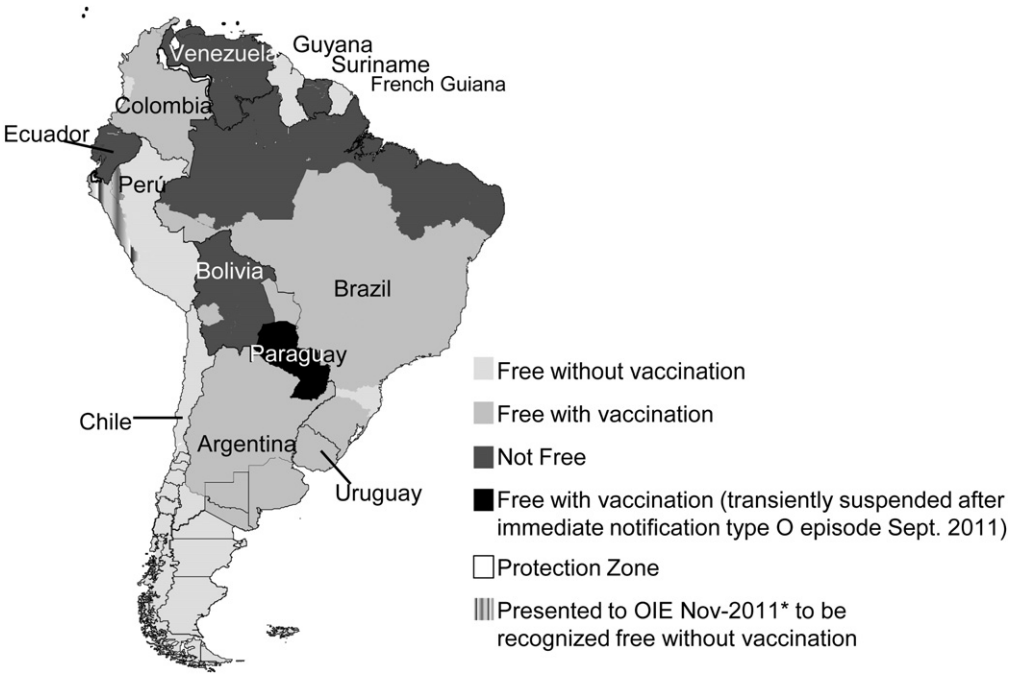

Fig. 1. OIE foot-and-mouth disease status in South America, 2011. Free zones with or without vaccination as recognized by OIE during the last General Session, 2011, and the Protection Zones are indicated.

Table 1
Designation and origin of foot-and-mouth disease type A viruses studied.

Virus designation	Geographical location			Date collected	Database accession number	Reference
	County	State	Country	Day/month/year		
A. Field viruses						
A/Argentina/00	General Villegas	Buenos Aires	Argentina	August-2000	JQ082906	This work[b]
A/Rivadavia/BsAs/Arg/01	Rivadavia	Buenos Aires	Argentina	March-2001	JQ082907	This work[b]
A/Chapaleufu/LaPampa/Arg/01	Chapaleufu	La Pampa	Argentina	March-2001	JQ082908	This work[b]
A/T.Lauquen/BsAs/Arg/01	Trenque Lauquen	Buenos Aires	Argentina	March-2001	JQ082909	This work[b]
A/Treita_y_Tres/Uru/01	NR	Treinta y Tres	Uruguay	April-2001	JQ082910	This work
A/Colonia/Uru/01	NR	Colonia	Uruguay	April-2001	JQ082911	This work
A/S.doLivramento/RS/Bra/01(a)	Santana do Livramento	Rio Grande do Sul	Brazil	May-2001	JQ082912	This work
A/S.doLivramento/RS/Bra/01(b)	Santana do Livramento	Rio Grande do Sul	Brazil	May-2001	JQ082913	This work
A/S.doLivramento/RS/Bra/01(c)	Santana do Livramento	Rio Grande do Sul	Brazil	May-2001	JQ082914	This work
A/Sta_Cruz/Bol/00	Pailon/Chiquitos	Santa Cruz	Bolivia	5/30/2000	JQ082915	This work
A/Sta.Cruz/Bol/01(a)	Cotoca/A.Ibanez	Santa Cruz	Bolivia	5/7/2001	JQ082916	This work
A/Sta.Cruz/Bol/01(b)	La Cruceña/A.Ibanez	Santa Cruz	Bolivia	5/10/2001	JQ082917	This work
A/Cochabamba/Bol/01	Tiraque/Cercado/	Cochabamba	Bolivia	6/7/2001	JQ082918	This work
A/Oruro/Bol/01	Sorocachi/Cercado	Oruro	Bolivia	7/23/2001	JQ082919	This work
A/Potosi/Bol/01	Havaya/A.Ibanez	Potosi	Bolivia	8/7/2001	JQ082920	This work
A/Beni/Bol/02	Yucumo/Ballivian	Beni	Bolivia	3/30/2002	JQ082921	This work
A/Tibu/N. de Santander/Col/04	Tibu	Norte de Santander	Colombia	July-2004	JQ082922	This work
A/N.de Santander/Col/08 (a)[a]	Sardinata	Norte de Santander	Colombia	6/6/2008	JQ082923	This work
A/N.de Santander/Col/08 (b)[a]	Sardinata	Norte de Santander	Colombia	6/6/2008	JQ082924	This work
A/N.de Santander/Col/08 (c)[a]	Sardinata	Norte de Santander	Colombia	6/6/2008	JQ082925	This work
A/N.de Santander/Col/08 (d)[a]	Sardinata	Norte de Santander	Colombia	6/6/2008	JQ082926	This work
A/N.de Santander/Col/08 (e)[a]	Sardinata	Norte de Santander	Colombia	6/6/2008	JQ082927	This work
A/N.de Santander/Col/08 (f)[a]	Sardinata	Norte de Santander	Colombia	6/6/2008	JQ082928	This work
A/Ecuador/02	NR	Pichincha	Ecuador	June-2002	JQ082929	This work
A/Perú/99	Lurín	Lima	Perú	7/16/1999	JQ082930	This work
A/Perú/00	Chosica	Lima	Perú	1/19/2000	JQ082931	This work
A/Tachira/Ven/01	Panamericano	Táchira	Venezuela	6/13/2001	JQ082932	This work
A/Bolivar/Ven/01	Padre Chien	Bolivar	Venezuela	12/5/2001	JQ082933	This work
A/Mérida/Ven/02	Alberto Adriani	Mérida	Venezuela	11/28/2002	JQ082934	This work
A/Bolivar/Ven/03	Padre Chien	Bolivar	Venezuela	5/14/2003	JQ082935	This work
A/Barinas/Ven/03	Torunos	Barinas	Venezuela	7/3/2003	JQ082936	This work
A/Mérida/Ven/03 (a)	Febres Cordero	Merida	Venezuela	12/9/2003	JQ082937	This work
A/Mérida/Ven/03 (b)	Alberto Adriani	Merida	Venezuela	12/12/2003	JQ082938	This work
A/Táchira/Ven/04 (a)	Garcia de Hevia	Táchira	Venezuela	1/23/2004	JQ082939	This work
A/Táchira/Ven/04 (b)	Garcia de Hevia	Táchira	Venezuela	2/11/2004	JQ082940	This work
A/Barinas/Ven/04 (a)	E. Zamora	Barinas	Venezuela	3/10/2004	JQ082941	This work
A/Táchira/Ven/04 (c)	Garcia de Hevia	Táchira	Venezuela	4/1/2004	JQ082942	This work
A/Merida/Ven/04	Alberto Adriani	Merida	Venezuela	5/19/2004	JQ082943	This work
A/Yaracuy/Ven/04	Veroes	Yaracuy	Venezuela	7/12/2004	JQ082944	This work
A/Falcon/Ven/04	M. Iturriza	Falcon	Venezuela	8/13/2004	JQ082945	This work
A/Barinas/Ven/04 (b)	Barinas	Barinas	Venezuela	8/26/2004	JQ082946	This work
A/Táchira/Ven/04 (d)	Garcia de Hevia	Tachira	Venezuela	9/24/2004	JQ082947	This work
A/Apure/Ven/04	Paez	Apure	Venezuela	11/18/2004	JQ082948	This work
A/Apure/Ven/05	Mantecal	Apure	Venezuela	2/3/2005	JQ082949	This work
A/Mérida/Ven/05 (a)	Jaji	Merida	Venezuela	4/6/2005	JQ082950	This work
A/Mérida/Ven/05 (b)	Obispo Ramos de L.	Mérida	Venezuela	4/20/2005	JQ082951	This work
A/Mérida/Ven/05 (c)	Obispo Ramos de L.	Merida	Venezuela	4/29/2005	JQ082952	This work
A/Apure/Ven/06	NR	Apure	Venezuela	6/13/2006	JQ082953	This work
A/Portuguesa/Ven/07	Guanarito	Portuguesa	Venezuela	30/01/2007	JQ082954	This work

Virus designation	Country	Year collected	Database	Reference
B. South American collection viruses				
A24/Cruzeiro/Bra/55-PANAFTOSA (Vaccine strain)	Brazil	1955	JQ082960	This work
A13/Brasil/58	Brazil	1958	JQ082955	This work
A16/Belem/Bra/59	Brazil	1959	JQ082956	This work
A17/Guarulhos/Bra/59	Brazil	1959	JQ082957	This work
A25/Argentina/59	Argentina	1959	JQ082961	This work
A18/Zulia/Ven/62	Venezuela	1962	JQ082958	This work
A19/Suipacha/Arg/62	Argentina	1962	JQ082959	This work
A26/Argentina/66	Argentina	1966	JQ082962	This work
A27/Colombia/67	Colombia	1967	JQ082963	This work
A29/Peru/69	Perú	1969	JQ082964	This work
A32/Venezuela/70	Venezuela	1970	JQ082965	This work
A24/Bra/70	Brazil	1970	JQ082966	This work
A/Ecuador/75	Ecuador	1975	JQ082967	This work
A/San Martín/Per/75	Perú	1975	JQ082968	This work
A79/Bage/Bra/76	Brazil	1976	JQ082969	This work

Table 1 (*Continued*)

Virus designation	Country	Year collected	Database	Reference
A79/Cacapava/Bra/76	Brazil	1976	JQ082970	This work
A79/Pedregulho/Bra/76	Brazil	1976	JQ082971	This work
A27/Cundinamarca/Col/76	Colombia	1976	JQ082972	This work
A/Ecuador/79	Ecuador	1979	JQ082973	This work
A/RS/Bra/81	Brazil	1981	JQ082974	This work
A/SaoCarlos/Bra/84	Brazil	1984	JQ082975	This work
A/Colombia/84	Colombia	1984	JQ082976	This work
A/Sabana/Col/85	Colombia	1985	JQ082977	This work
A/Venezuela/89	Venezuela	1989	JQ082978	This work
A/RS/Bra/97	Brazil	5/8/1997	JQ082979	This work
A/Antioquia/Col/97	Colombia	15/09/1997	JQ082980	This work
A/Cordoba/Col/97	Colombia	15/09/1997	JQ082981	This work
A/Cundinamarca/Col/97	Colombia	15/09/1997	JQ082982	This work
A_2001_(Vaccine strain)	Argentina	2001	JQ082983	This work

Virus designation	Country	Year collected	Database	Reference
C. Already published sequences				
A10/Argentina/61	Argentina	1961	V01130	Boothroyd et al. (1982)
A/Arg/68	Argentina	1968	AJ308694	König et al. (2001)
A/Bahia Blanca/Arg/71	Argentina	1971	AJ308695	König et al. (2001)
A76/Argentina/76	Argentina	1976	AJ409219	König et al. (2001)
A/Argentina/79	Argentina	1979	K03345	Weddell et al. (1985)
A/25_de_Mayo/Arg/87	Argentina	1987	AJ306220	Direct submission
A/Castellanos/Arg/87	Argentina	1987	AJ306222	Direct submission
A/Utracan/Arg/87	Argentina	1987	AJ306221	Direct submission
A/Córdoba/Arg/90	Argentina	1990	AJ308697	König et al. (2001)
A/Ayacucho/Arg/90	Argentina	1990	AJ308696	König et al. (2001)
A/San Ignacio/Arg/90	Argentina	1990	AJ308698	König et al. (2001)
A/Rivadavia/Arg/91	Argentina	1991	AJ308699	König et al. (2001)
A/Corrientes/Arg/92	Argentina	1992	AJ308701	König et al. (2001)
A/Pehuajo/Arg/92	Argentina	1992	AJ308702	König et al. (2001)
A/Cordoba/Arg/92	Argentina	1992	AJ308700	König et al. (2001)
A/Venceslau/Bra/76	Brazil	1976	M12905	Cheung et al. (1984)
A79/Brasil/79	Brazil	1979	AY593788	Carrillo et al. (2005)
A/SaoPaulo/Bra/94(A3)	Brazil	1994	NA	Araujo et al. (2002)
A/SaoPaulo/Bra/94(A4)	Brazil	1994	NA	Araujo et al. (2002)
A/SaoPaulo/Bra/94(A5)	Brazil	1994	NA	Araujo et al. (2002)
A/SaoPaulo/Bra/95(A1)	Brazil	1995	NA	Araujo et al. (2002)

Virus designation	Topotype	Linage	Database	Reference
D. Representative strains/isolates for type A FMDV topotypes along with an accession number as referred by the OIE/FAO World Reference Laboratory for Foot-and Mouth Disease (http://www.wrlfmd.org/fmd_genotyping/prototypes.htm)				
A/KEN/42/66	AFRICA	G-I	n/a	Knowles et al. (unpub.)
A/EGY/1/72	AFRICA	G-II	EF208756	Knowles et al. (unpub.)
A_{21}/Lumbwa/KEN/64	AFRICA	G-III	AY593761	Carrillo et al., 2005
A/SUD/3/77	AFRICA	G-IV	n/a	Knowles et al. (unpub.)
A/NGR/2/73	AFRICA	G-V	n/a	Knowles et al. (unpub.)
A/GHA/16/73	AFRICA	G-VI	n/a	Knowles et al. (unpub.)
A/UGA/13/66	AFRICA	G-VII	n/a	Knowles et al. (unpub.)
A_{23}/Kitale/KEN/64 (KEN/46/65)	?	G-VIII	AY593766	Carrillo et al., 2005
A_{11}/GER/29 (AGB)	?	A_{11}	EU553852	Valarcher et al., 2008
A_{22}/IRQ/24/64	ASIA	A_{22}	AY593763	Carrillo et al., 2005
A/IRN/2/87	ASIA	Iran-87	EF208770	Knowles et al. (unpub.)
A/IRN/1/96	ASIA	Iran-96	EF208771	Knowles et al. (unpub.)
A/IRN/22/99	ASIA	Iran-99	EF208772	Knowles et al. (unpub.)
A/IRN/1/2005	ASIA	Iran-05	EF208769	Knowles et al. (unpub.)
A_{15}/Bangkok/TAI/60	ASIA	A_{15}	AY593755	Knowles et al. (unpub.)
A/TAI/118/87	ASIA		EF208777	Knowles et al. (unpub.)
A_{12}/119/Kent/UK/32	EURO-SA	A_{12}	M10975	Robertson et al. (1985)
A/Alem/ARG/81	EURO-SA	A-81	AJ306219	König et al. (2001)

NR: not registered.

[a] Oesophageal-pharyngeal fluid.

[b] VP_1 complete sequences were determined and resulted identical to those published by König et al. (2007).

2.2. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

RNA was directly extracted from epithelium samples, oesophageal-pharyngeal (OP) fluid or infected cell culture supernatants using Trizol reagent (Invitrogen), according to the manufacturer's protocol, as previously described (Malirat and Bergmann, 2003).

Reverse transcription of the RNA (5 μl, 3–5 μg RNA) was carried out using 50 ng of random primers and

50 units of Superscript II Reverse Transcriptase (Invitrogen) and incubating at 42 °C for 60 min, followed by extension at 70 °C, 15 min, in a 25 μl reaction mix containing 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.

Oligonucleotide primers used in this study to amplify and sequence the complete VP_1-coding region, rendering an amplification fragment of 795 bp are: 5′-TACCAAATTACACACGGGAA-3′ (forward) and 5′-GAAGGGCCCAGGGTTGGACTC-3′ (reverse). Amplification was performed in a programmable thermocycler GeneAmp PCR system 9700 (Applied Biosystems) in a final volume of 50 μl of a reaction mixture 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. After denaturing 5 min at 94 °C, 30 cycles of denaturation step at 94 °C for 1 min, annealing step at 55 °C for 1 min, and chain-elongation step at 72 °C for 1.5 min were performed in each reaction mixture. After the last cycle, polymerization was continued at 72 °C for 5 min.

The amplified product was purified by band excision from 1% agarose gel followed by chromatography in affinity columns (Promega) and the recovered material was quantified by band intensity comparison with DNA mass and molecular weight marker (Invitrogen) in 1% agarose gel electrophoresis.

2.3. Nucleotide sequence determination

The nucleotide sequences were determined from 20 to 60 ng of the purified amplicon, 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). The reaction product was purified by exclusion chromatography (CentriSep columns, Princeton Separations); the recovered material was dyed. For reading, the dyed samples were diluted in formamide 10%, as recommended for use in an ABI Prism 3100 Avant Genetic Analyzer sequencing machine. The sequences determined in this study have been submitted to the EMBL/GenBank/DDBJ databases; accession numbers are shown in Table 1. Sequences were edited manually to avoid misreading of peak dyes on an IBM compatible personal computer and aligned using the program BioEdit, version 7.0.5.3 (Hall, 1999).

2.4. Phylogenetic study

Twenty-four 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 result of this analysis indicated that the K2 + G + I model (Kimura 2-parameter, modelled by 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 (Posada and Crandall, 2001). Unrooted trees were constructed according to sequence relatedness using the neighbor-joining method (NJ), with evolutionary distances calculated using the Kimura two-parameter (K2) method (Kimura, 1980), as implemented in the computer program MEGA, version 5.05 (Tamura et al., 2011). Bootstrap resampling analysis was performed with 1000 replicates.

2.5. Positive selection analysis

Selection pressure study was performed applying Maximum Likelihood analysis of natural selection codon-by-codon carried out by the HyPhy software package (Pond et al., 2005), implemented in the MEGA 5.05 program. dN and dS estimates were produced using the joint Maximum Likelihood reconstructions of ancestral states under a Muse-Gaut model (Muse and Gaut, 1994) of codon substitution and general time reversible model of nucleotide substitution. For estimating Maximum Likelihood values, a tree topology was automatically computed. A cut-off *p*-value of 0.05 was used.

3. Results

3.1. FMDV type A occurrence in South America: viral isolates studied

Table 2 summarizes the number of FMD type A reported outbreaks in South America between 1991 and 2010. As can be seen, outbreaks had diminished in the past 10 years in almost all countries. During 2001 an FMD epizootic affected already-free regions in the Southern Cone (Argentina, Uruguay and south of Brazil) resulting in more than 4190 reported outbreaks. Control measures applied, including stamping out, ring vaccination, restriction of animal movements and others, led to an effective control of the episodes in the sub-region. Since 2003 most type A outbreaks have concentrated in Venezuela (192 out of the 197 reported).

To perform the phylogenetic study, the complete VP_1-coding region was determined from a total of 49 field samples, collected between the years 1999–2008 (Table 1A), and 29 strains corresponding to the South American strain collection (oldest isolate from 1955) (Table 1B). Previously published sequences corresponding to 21 South American isolates (Table 1C) and 18 representative strains of FMDV type A topotypes (Table 1D), were also included in the analysis.

Field samples include:

i) 23 Venezuelan isolates collected during 2001–2007,
ii) seven Colombian isolates obtained in the years 2004 (one sample) and 2008 (six samples) from outbreaks in free territories located in border areas,
iii) seven samples collected in Bolivia between 2000 and 2002,
iv) one sample from the last type A outbreak recorded in Ecuador in 2002,
v) two samples from Perú, collected during the last type A outbreaks, one in 1999 and one in 2000,
vi) nine samples representing the A-2001 epidemic in the Southern Cone of the continent: four isolates from

Table 2
FMD type A reported outbreaks in South America between 1991 and 2010.

Year	Argentina	Bolivia	Brazil	Colombia	Ecuador	Perú	Venezuela	Uruguay	TOTAL
1991	60	2	18	113	5	0	16	0	214
1992	72	0	72	82	0	3	7	0	236
1993	4	5	182	33	0	1	3	0	228
1994	0	3	15	40	0	0	5	0	63
1995	0	19	99	79	0	0	3	0	200
1996	0	1	18	81	5	15	1	0	121
1997	0	4	5	17	34	0	1	0	61
1998	0	6	1	11	14	0	17	0	49
1999	0	18	2	8	2	15	4	0	49
2000	0	18	6	1	8	48	4	0	85
2001	2126	81	15	0	8	0	4	2057	4291
2002	1	1	0	0	4	0	9	0	15
2003	0	0	0	0	0	0	55	0	55
2004	0	0	0	2	0	0	29	0	31
2005	0	0	0	0	0	0	10	0	10
2006	0	0	0	0	0	0	25	0	25
2007	0	0	0	0	0	0	33	0	33
2008	0	0	0	3	0	0	27	0	30
2009	0	0	0	0	0	0	10	0	10
2010	0	0	0	0	0	0	3	0	3

Only countries where outbreaks were reported during the period are listed. Data were gathered from published references (PAHO/WHO, 2000, 2009, 2010, 2011a,b).

Argentina (one A-2000 and three A-2001), two from Uruguay and three from Brazil.

The 29 epidemiologically relevant strains studied constituted part of the South American strain collection held at the Pan-American Foot-and Mouth Disease Center PAHO/WHO. The constitution of this collection, as well as the definition of relevant isolates collected between 1955 and 1984, has been reviewed by Alonso Fernandez (1983). A brief description of the collection viruses included in this study is presented in Table 3.

The complete nucleotide sequence of the VP_1-coding region was determined directly from epithelium or from the 3rd BHK-21 cell passage in the case of field samples or collection strains, respectively.

3.2. Molecular epidemiology of FMDV type A in South America

The phylogenetic analysis (Fig. 2) and the pairwise distances (data not shown) indicated that all FMD type A viruses from South America belonged to the EURO-SA topotype, with difference values that ranged between 18% and 32% with respect to African and Asian reference topotype strains.

From the topology of the constructed tree, and considering a cut-off value of 15% difference in the VP_1-coding region, as proposed in previous publications (Tosh et al., 2002; Mohapatra et al., 2011), 16 different subgenotypes or genetic groups could be distinguished that have circulated in the continent. Divergence average values among them ranged from 15% to 25%, and homology within group values varied between 86% and 98% (Table 4). Some of these subgenotypes had already been identified in previous papers (Tosh et al., 2002; Mohapatra et al., 2011).

Eight of the 16 groups (numbers 5, 7, 8, 9, 10, 12, 14 and 15) included only isolates recovered more than 27 years ago, indicating that they are extinguished. Some of them were composed of only one representative. For example

Table 3
Brief description of the strains of the South American collection, included in this study (extracted from Alonso Fernandez, 1983).

1 – Most of the isolates first recognized as new subtypes (identified by the number immediately following the type definition, for example A_{24}) that appeared in the continent until the middle 70s, when the criterion to classify new subtypes was changed during the International Symposium on Variants and Immunity, held in Lyon in 1967:
(a) A_{13}, A_{16}, A_{17}, A_{19}: all of them isolated between 1958 and 1964 from partially immune bovines in slaughterhouses, inoculated to produce vaccine antigens;
(b) A_{18}: Venezuelan field subtype isolated only during years 1962 and 1963;
(c) A_{24}: field subtype first isolated in Brazil and representative of the continent;
(d) A_{25} and A_{26}: subtypes sporadically isolated in the Southern Cone between 1965 and 1967;
(e) A_{27}: Andean subtype, serologically related to A_5, frequently isolated since the year 1967;
(f) A_{29}: Only identified in Peru in 1969;
(i) A_{32}: Venezuelan representative isolate since 1969;
(j) A_{10}: European subtype exceptionally isolated in South America(considered exotic for the region).
2 – Antigenic groups A79 and A81, present in the Southern Cone. The former is constituted of strains A/Venceslau/Bra/76, A79 /Bage/Bra/76, A/Argentina/79 and A79/Brazil/79; and the latter one by strains A/Uruguay/79, A/RS/Brazil/81 (Uruguaiana) and A/Argentina/81 (Alem). The A81 group has been described to be the end effect of the A79 epidemic.
3 – Antigenic groups A84 and A85, and A89, the former two first detected in Colombia in the years 84 and 85, and the latter one in Venezuela. These viruses define Andean antigenic groups and have been added to subtypes A27 and A32 to perform a serologic classification of appearing isolates.
4 – The strain A/Sao Carlos Brazil 1984 was identified in Sao Paulo, Brazil, 1984, and eventually controlled by intensified epidemiological surveillance and strategic vaccination with A24 and A/Venceslau vaccines.
5 – Viruses from the nineteenth decade, obtained both in the Southern Cone region (A/RS/Bra/97) and in the Andean region (in Antioquia, Cordoba and Cundinamarca states of Colombia) isolated in 1997.
6 – Strains currently used for vaccine formulation (A_{24} Cruzeiro, A2001).

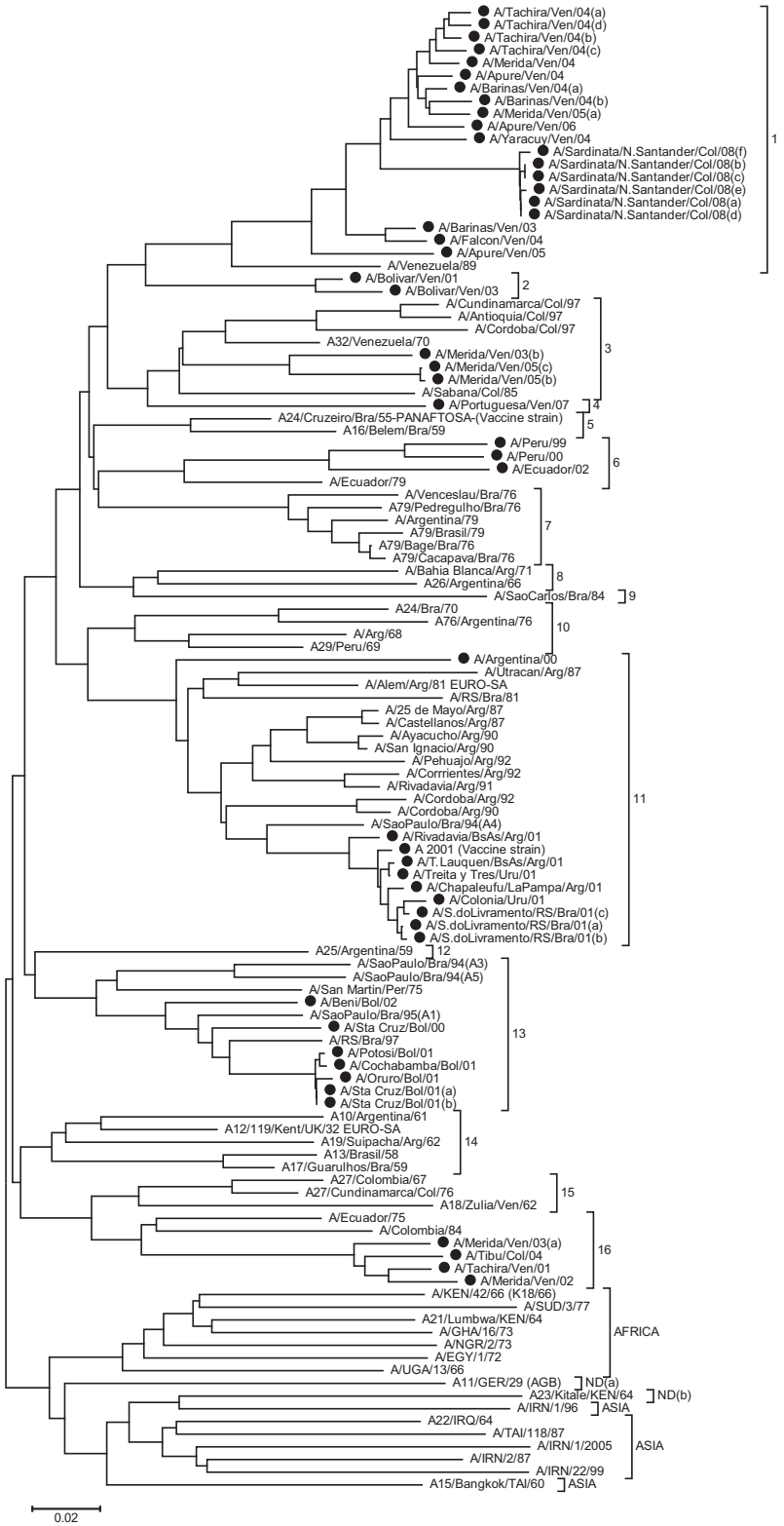

Fig. 2. Phylogenetic tree showing the genetic relationships of South American type A FMDV isolates. Distances were calculated upon the Kimura-two (K-2) parameter model, based on the comparison of the complete region coding for VP_1 protein. A Neighbor-Joining tree was constructed using the Mega 5.05 program. Scale bar indicates nucleotide substitutions per site. Sixteen different lineages (labeled 1–16) are indicated, as well as type A topotypes different from the Euro–SA topotype (Asia, Africa or non-determined topotypes a or b). (●) Isolates collected since 1999.

Table 4
Estimates of average percent homology (A) within and (B) between subgenotypes. Analyses were conducted using the Kimura 2-parameter model in the MEGA 5.05 program. The rate variation among sites was modeled with a gamma distribution (shape parameter = 5).

A. Average evolutionary divergence (percentage) within subgenotypes

Subgenotype	Percent average within group divergence
1	5.0
2	2.5
3	11.4
4	–
5	6.0
6	9.9
7	4.0
8	13.8
9	–
10	10.9
11	9.2
12	–
13	8.2
14	12.7
15	10.5
16	10.4

B. Average evolutionary divergence (percentage) between subgenotypes

Euro-SA topotype

Subgenotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	15.6															
3	21.2	16.8														
4	18.7	17.9	16.0													
5	18.8	14.8	14.5	16.4												
6	21.8	18.6	20.0	21.0	16.6											
7	19.8	16.8	20.3	18.2	15.4	18.9										
8	21.1	18.4	20.7	21.0	15.3	20.7	19.7									
9	22.3	19.9	22.9	22.2	18.9	23.0	20.8	18.5								
10	21.4	18.4	20.4	20.2	16.3	20.4	18.3	19.5	21.7							
11	23.1	17.3	20.3	20.6	19.1	22.4	19.8	21.2	22.3	16.7						
12	21.9	18.3	19.4	19.7	16.5	21.5	18.5	19.2	22.8	17.5	19.7					
13	22.4	16.5	20.0	20.2	17.9	21.2	17.9	21.8	23.2	20.2	20.0	16.4				
14	21.9	18.3	19.1	19.1	16.2	21.2	19.5	20.2	24.6	19.3	19.4	16.1	16.8			
15	25.1	21.4	21.1	23.2	18.5	24.1	21.9	19.6	23.8	19.3	20.0	17.6	19.0	16.9		
16	25.1	22.5	22.3	22.1	22.0	22.6	24.0	23.6	24.9	22.0	20.8	21.4	21.4	19.7	17.2	

Exogenous topotypes

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Africa	ND(a)	ND(b)	Asia
Africa	24.9	23.4	25.1	23.4	23.3	25.7	23.3	24.3	25.2	23.0	24.1	21.6	22.5	21.6	21.7	24.4				
ND(a)	27.7	25.1	25.8	25.3	21.8	22.9	22.9	26.3	25.9	24.3	25.6	20.5	22.0	20.6	24.2	23.4	23.0			
ND(b)	28.2	26.0	27.7	27.6	26.4	26.2	23.0	27.1	28.6	25.7	27.8	23.8	24.0	24.3	23.4	26.6	23.3	25.3		
Asia	25.7	23.9	26.5	27.3	23.9	26.4	24.3	26.0	28.1	25.3	25.4	22.6	22.4	23.1	23.6	24.9	24.0	23.4	23.4	

subgenotypes 9 and 12 are constituted exclusively of strains A/SaoCarlos/Bra/84 and A25/Argentina/59, respectively. Others were constituted of more than one isolate, in some cases closely related, as in subgenotype 7 with over 96% homology among them. More heterogeneous subgenotypes were also found: for example group 14 clustered some of the serologic subtypes detected for the continent (A_{10}, A_{13}, A_{17} and A_{19}), which presented approximately 87% genetic homology among them. In general groups were composed of isolates collected at relatively short time intervals (4–6 years), sometimes in different countries (i.e. group 7). Conversely, different subgenotypes had co-circulated in the same country (for example groups 5 and 14 in Brazil). Most of the historic South American strains collected until 1989 and considered for the analysis, were placed within these eight extinguished subgenotypes, including vaccine strains, for example A_{24}/Cruzeiro/Bra/55, in group 5 used in most South American countries as the solely type A variant in the vaccine formulation, or A/Argentina/79 in group 7, which was used in the formulation of vaccines up to the year 2001 in Argentina.

None of the historical strains from South America that did not belong to extinct subgroups: A_{32}/Venezuela/70 (subgenotype 3), A/Ecuador/75 (subgenotype 16), A/Ecuador/79 (subgenotype 6), A/San Martin/Per/75 (subgenotype 13), A/Colombia/84 (subgenotype 16), A Sabana/Col/85 (subgenotype 3), A/Venezuela/89 (subgenotype 1) and A/Alem/Arg/81 (subgenotype 11), showed homology values over 94% with respect to recent isolates (Fig. 2).

In overall, no direct correlation could be established between the historical serologic classification of FMD type A viruses and the genetic subgenotypes defined through phylogenetic analysis. Strains A_{24}/Cruzeiro/Bra/55 and

A_{24}/Bra/70, both serologically classified as subtype A_{24} belong to two different subgenotypes, 5 and 10, respectively. However, in some cases the genetic classification matches the serologic grouping: strains A/Venceslau/Bra/76, A/Bagé/Bra/76, A/Argentina/79 and A/Brasil/79 within subgenotype 9 also constitute a single serologic group (Alonso Fernandez et al., 1987).

3.3. Phylogenetic analysis of type A strains circulating during 1999–2008

As mentioned previously, 49 samples have been analyzed of isolates recovered between the years 1999–2008 in South America. All these samples were distributed within South American subgenotypes 1, 2, 3, 4, 6, 11, 13 and 16, showing between group divergence distances as high as 25% (i.e. average distance value between groups 1 and 16).

Subgenotypes 1, 2, 3, 4 and 16 contained isolates recovered from Venezuela between the years 2001–2007. Group 6 contained the last type A isolates from Peru and Ecuador, subgenotype 11 clustered the isolates responsible for the vast emergency in 2001 in the Southern Cone whereas group 13 included viruses isolated in Bolivia between the years 2000–2002 (Fig. 2).

The five subgenotypes present in Venezuela between 2001 and 2007 depict the co-circulation of variants in this country, in some cases with remarkable genetic divergences. For example, isolates A/Merida/Ven/03(a) and A/Merida/Ven/03(b) recovered 1 week apart from the same state, belonged to two different groups (16 and 3) and presented a divergence value of 23%.

Subgenotype 1 appeared as a rather homogeneous group, with within group average identity of 95%. It included the highest number of recent Venezuelan samples: 14 of the 23 isolates from six different states of Venezuela (Barinas, Táchira, Mérida, Yaracuy, Falcon and Apure) recovered during 2003–2006. The isolates responsible for the type A emergency in Colombia in 2008, near the Venezuelan border (Sardinata county, Norte de Santander state), are also localized in this subgenotype. The six samples recovered during this emergency showed the closest relationship (95%) with 2004 and 2005 Venezuelan viruses: A/Barinas/Ven/04(a), and A/Merida/Ven/05(a). Within this subgenotype, strain A/Venezuela/89 is one of the historically representative strains of the Andean Region, and has been used for serological classification (subtyping) using complement fixation tests. This strain presented an average distance value of 10% with respect to circulating strains in the same group.

Group 3 was constituted of rather different isolates, presenting within group homology value of 89%. It was composed of three Venezuelan isolates: two very closely related recovered from the same county nine days apart in April 2005, and 8% different from the third one, recovered sixteen months before. Within this group, two Andean reference historic strains: A/Sabana/Col/85 and A_{32}/Venezuela/70 were also localized. They presented a difference of 9% between them and average distance values of 15% and 10%, respectively, with the other isolates in the same group. Additionally, three Colombian isolates of the late 90s also clustered within this subgenotype.

Subgenotype 4 was composed of a unique representative sample collected in Venezuela in 2007.

Group 16, encompassed circulating strains from Venezuela, collected between the years 2001–2003. This subgenotype also included the strain A/Tibu/Col/04, responsible for the FMDV emergency in Colombia in 2004, which showed a close genetic link (at least 95% identity) with the Venezuelan isolates. The reference historic strains appearing in this group, A/Colombia/84 and A/Ecuador/75, presented divergence values of 15% and 14%, respectively, with regard to recent isolates of the same subgenotype.

Subgenotype 6 was composed only of three strains recovered in 1999, 2000 and 2002 from the last outbreaks registered in Peru and Ecuador, which showed a relative close relationship among them (92–96% identity). The Andean reference strain A/Ecuador/79 also belonged to this subgenotype and presented divergence values of 12–14% with respect to strains circulating in 1999–2002 in the same group.

Isolates that caused the type A re-emergence in 2000–2001 in the Southern Cone, all clustered together within group 11. A2001 viruses were closely related among them (average 99% homology) and with respect to the other viruses in the group, they were more related to an isolate in Sao Paulo, Brazil, in 1994 (approximately 94% homology). The A2000 strain was more distant in the branch, with average homology of 86% with other viruses in the group. Reference strain A/Argentina/81 was also included in this group and showed homology values between 88% and 92% with respect to the strains responsible of the 2000–2001 epidemics.

All 7 samples studied from Bolivian isolates recovered between 2000 and 2002, which was the last year of reported type A outbreaks in this country, clustered together within subgenotype 13. These isolates were approximately 94% related to a strain recovered from Rio Grande do Sul state, in Brazil, in 1997 (A/RS/Bra/97) and more distantly related (92–88%) to strains that have circulated in the middle '90s in the Brazilian State of São Paulo (Araujo et al., 2002). Strain A/San Martin/Per/75, from the virus collection, was also localized in this group and showed a divergence value of over 10% with respect to the Bolivian strains in the same group.

3.4. VP_1 amino acid composition

In terms of amino acid composition, comparison of the determined and available sequences showed that 79 out of the 100 South American type A viruses presented only point mutations without any deletion within the 639 nucleotides coding for VP_1 protein.

The 21 isolates that presented lack of at least 1 amino acid in the VP_1 protein (Fig. 3) were distributed as follows:

(a) Deletion of codon 425–427 (amino acid position 142) was registered in eleven isolates scattered through subgenotypes 14, 15 and 16. This deletion was detected both in historic isolates (A_{10}/Argentina/61, A_{18}/Zulia/

Ven/62, A_{19}/Suipacha/Arg/62, A_{27}/Colombia/67, A/Ecuador/75, A_{27}/Cundinamarca/Col/76, A/Colombia/84) as well as in more recent strains (A/Tachira/Ven/01, A/Merida/Ven/02, A/Merida/Ven/03(a) and A/Tibu/Col/04).

(b) Nine isolates presented a deletion in codon 589–591 (amino acid position 197), all of them belonging to subgenotype 13, and grouped in a single cluster (isolates A/SaoPaulo/Bra/95(A1), A/RS/Bra/97, A/StaCruz/Bol/00, A/Potosi/Bol/01, A/Oruro/Bol/01, A/Cochabamba/Bol/01, A/StaCruz/Bol/01(a), A/StaCruz/Bol/01(b) and A/Beni/Bol/02).

(c) Only one isolate, A/Corrrientes/Arg/92 from subgenotype 11, showed a two-adjacent codon deletion (nucleotide positions 466–471, corresponding to amino acids 156 and 157, already reported by König et al., 2007).

Significant evidence for positive selection was found in residue 170 ($p = 1 \times 10^{-4}$) (Fig. 4) behind a background of strong purifying selection, when the deduced VP_1 amino acid sequences of type A isolates from South America were examined.

The integrin-binding site Arg-Gly-Asp (RGD), was highly conserved in all 78 serotype A South American isolates sequenced in this work, except in 3 isolates: A/Merida/Ven/02 and A_{25}/Argentina/59, which presented an RDD triplet and A_{27}/Colombia/67, which changed to PGD.

4. Discussion

This work presents the first comprehensive phylogenetic study of FMDV type A isolates in South America, covering over half a century and including an important number of strains that circulated during the past decade, collected in endemic countries or during emergencies in already-free areas, as well as strains of historical relevance from the South American strain collection. The results presented allowed the identification of 16 different subgenotypes, either presently circulating or probably extinguished, when 15% genetic difference in the VP_1-coding region was considered as a cut-off. Some of the referred lineages had already been recognized in previous papers (Tosh et al., 2002; Mohapatra et al., 2011). None of these viruses showed a close relationship with vaccine strains, in contrast to what was reported in other occasions (Beck and Strohmaier, 1987; König et al., 2001).

Sequences with similar identification as some of the viruses in the South American collection have been published previously by other laboratories. When compared to the sequences reported in this work, differences in nine of the sequences could be accounted for by the different origin and/or history passage of the viruses studied in each laboratory. Small differences derived from different passage history do not impact in the molecular epidemiology analysis, as they will result in no more than 2% differences within the complete VP_1-coding region, which can be explained by the quasispecies nature of FMDV. In two cases, differences can only be explained by wrong identification of the sample or other manipulation error (Table S1).

All viruses placed in the 16 subgenotypes are indigenous and belong to the Europe–SA topotype. Although it has been described that some of the subgenotypes include viruses that have circulated in other regions, no evidence has been documented of introduction of FMDV from South America to Europe, Africa or Asia in the last 20 years (Valarcher et al., 2008; Mohapatra et al., 2011).

The phylogenetic analysis also highlighted the lack of correlation between serological and genetic data, as viruses belonging to the same subgenotype are indistinctly placed in the same or in different serologic subtypes or groups. This might be probably due to the fact that not all genetic changes impact uniformly in antigenic/immunogenic behaviour, highlighting the limitations faced until today in deriving immunological behaviour from genetic data (Maradei et al., 2011).

Eight of the 16 subgenotypes are now extinguished, as they are constituted only of isolates collected at least about 3 decades ago. Many viruses belonging to these groups represent the serologic subtypes recorded for the continent (identified by the number immediately following the type definition, for example A_{24}), or epidemiologically relevant viruses. Isolates of the South American strain collection placed within the non-extinguished subgenotypes, show percent homology values with respect to recently circulating viruses in accordance with the viral evolutionary rates described for FMDV.

With respect to the composition of the subgenotypes, some of them were constituted of only one representative (i.e. subgenotypes 4, 9, and 12). In the case of subgenotype 9 this could be explained by the history of the virus, as the epidemiological reports describe a transient appearance and a rapid control trough intensified epidemiological surveillance and strategic vaccination with strains A Cruzeiro and A Venceslau (Alonso Fernandez et al., 1987). The case of subgenotype 4, constituted of only one sample obtained in 2007 in Venezuela, may account for a different situation, and much more samples will be needed to analyze whether this apparently recent subgenotype is being maintained in the field. The need for more samples from the last years (from 2008 to date) is crucial, considering that Venezuela is the solely country in the continent in which type A cases have been consistently detected yearly during the last decade.

Some of the subgenotypes constituted by several isolates appear rather homogeneous (within group homology values of over 95%). This can result from highly related samples collected within short time intervals, as in subgenotype 7, or might represent similar isolates obtained in a wider time interval pointing to a more fitted genome, as in the case of group 1.

The phylogenetic analysis of strains that have circulated in the last decade showed that of the 8 recently acting subgenotypes, only one contain the isolates responsible of the re-emergence of FMDV type A in the Southern Cone. Previous papers have been published with respect to the type A 2000–2001 emergency in Argentina, in which two different type A groups were reported to be acting (König et al., 2007). We have placed both variants under the same subgenotype based not only in the cut-off proposed, but also in the observed topology of the tree. The last isolate within this subgenotype was reported more than 10 years ago, pointing to a probable extinction of this group, as a

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                                  150         160         170         180         190         200         210
                                  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ....|....|  ...
A24/Cruzeiro/Bra/55_{5}           SGRRGDMGSL AARVVKQLPA SFNYGAIKAD AIHELLVRMK RAELYCPRPL LAIEVSSQDR HKQKIIAPAK QLL
A/Corrrientes/Arg/92{11}          SNRRGDLGAL AARVA--LPA SFNYGAIKAQ NIHELLVRMK RAELYCPRPL LAIEVSSQDR HKQRTIAPAK QSL
A/SaoPaulo/Bra/95(A1){13}         SGRRGDLGPL AARVAKQLPA SFNFGAIKAT TIHELLVRMK RAELYCPRPL LAIEVS-QDR HKQKIIAPAK QLL
A/StaCruz/Bol/00{13}              SGRRGDMGSL AARVAKQLPA SFNFGAIQAT TIHELLVRMK RAELYCPRPL LAMEVA-QDR YKQKIIAPAK QLL
A/RS/Bra/97{13}                   SGRRGDLGSL AARVAKHLPA SFNFGAIRAT NIHELLVRMK RAELYCPRPL LAVEAS-QDR YKQKIIAPAK QLL
A/Potosi/Bol/01{13}               SERRGDLGSL AARVAKQLPA SFNFGAIRAT HIHELLVRMK RAELYCPRPL LAIEVS-QDR HKQKIIAPAK QLL
A/Oruro/Bol/01{13}                PERRGDLGSL AARVAKQLPA SFNFGAIRAT HIHELLVRMK RAELYCPRPL LAIQVS-QDR HKQKIIAPAK QLL
A/Cochabamba/Bol/01 {13}          SERRGDLGSL AXRVAKQLPA SFNFGAIRAT HIHELLVRMK RAEXYCPRPL LAIEVS-QDR HKQKIIAPAK QLL
A/StaCruz/Bol/01(a){13}           SERRGDLGSL AARVAKQLPA SFNFGAIRAT HIHELLVRMK RAELYCPRPL LAIEVS-QDR HKQKIIAPAK QLL
A/StaCruz/Bol/01(b){13}           SERRGDLGSL AARVAKQLPA SFNFGAIRAT HIHELLVRMK RAELYCPRPL LAIEVS-QDR HKQKIIAPAK QLL
A/Beni/Bol/02{13}                 SGRRGDLGSL AARVAKQLPA SFNYGAIRAD TIHELLVRMK RAELYCPRPL LAIEVS-QDR HKQKIIAPAK QLL
A10/Argentina/61{14}              S-RSGDLGSI AARVATQLPA SFNYGAIQAQ AIHELLVRMK RAELYCPRPL LAIKVTSQDR YKQKIIAPAK QLL
A19/Suipacha/Arg/62{14}           P-GRGDVGPL AARVVKQLPA SFNYGAIKAT KIHELLVRMK RAELYCPRPL LAIEVVSQDR HKQKIIAPAK QLL
A18/Zulia/Ven/62{15}              P-RRGDMGAP AARVAKQLPA SFNYGAIRAE TIHELLVRMK RAELYCPRPI LAIEVSSQDR HKQKIIAPAR QLL
A27/Colombia/67{15}               Q-RPGDMGSL AARVAKQLPA SFNYGAIRAQ TIHELLVRMK RAELYCPRPL LAIEVSSQDR HKQKIIAPAK QLL
A27/Cundinamarca/Col/76{15}       Q-RRGDMGSL AARVAKQLPA SFNYGAIKAQ TIHELLVRMK RAELYCPRPL LAIEVSSQDR HKQKIIAPAK QLL
A/Ecuador/75{Gp16}                T-RRGDLGPL AARVAKQLPA SFNYGAIKAQ TIHELLVRMK RAELYCPRPL LAVEVSSQDR HKQKIIAPAK QLL
A/Merida/Ven/03(a) {16}           P-RRGDLGAL AARVASQLPA SFNYGAIRAQ TIHELLVRMK RAELYCPRPL LAVEVSSQDR HKQKIIAPAK QLL
A/Tibu/Col/04{16}                 T-RRGDLGAL AARIASQLPA SFNYGAIRAQ TIHELLVRMK RAELYCPRPL LAVEVSSQDR HKQKIIAPAK QLL
A/Tachira/Ven/01{16}              P-RRGDLGAL AARVASQLPA SFNYGAIRAQ TIHELLVRMK RAELYCPRPL LAVEVSSQDR YKQKIIAPAK QLL
A/Merida/Ven/02{16}               P-RRDDPGAL AARVASQLPA SFNYGAIRAQ TIHELLVRMK RAELYCPRPL LAVEVSSQDR YKQKIIAPAK QLL
A/Colombia/84{16}                 A-RRGDLGSL AARVANQLPA SFNYGAIKAQ AIHELLVRMK RAELYCPRPL LAVEVSSQGR HKQKIIAPAK HLL
A/GHA/16/73{AFRICA}               STRRGDLGPL AARVAAQLPA SFNYGALRAD TIRELLVRMK RAELYCPRPL LAVEVS-ADR HKQKIIAPAK QLL
A/IRN/2/87{ASIA}                  -ARRGDLGSL AARVAAQLPS SFNFGAIRAT TIHELLVRMR RAELYCPRPL LAMEVSAEGR HKQKIIAPAK QLL
A/IRN/22/99{ASIA}                 -SRRGDLGAL AARVAAQLPA SFNFGAIRAT NIHELLVRMK RAELYCPRPL LSTEVTTQDR HKQRIIAPAR QLL
A/TAI/118/87{ASIA}                -TRRGDLGSL AARVAAQLPA SFNFGAIRAT EIQELLVRMK RAELYCPRPL LAVEVSSQDR HKQKIIAPAK QLL
```

Fig. 3. Amino acid deletions in the VP_1 protein sequence of FMDV type A South American viruses. Dashes inside boxes represent amino acid deletions with respect to reference virus (upper line). Number between brackets at the end of the strain identification indicates corresponding subgenotype.

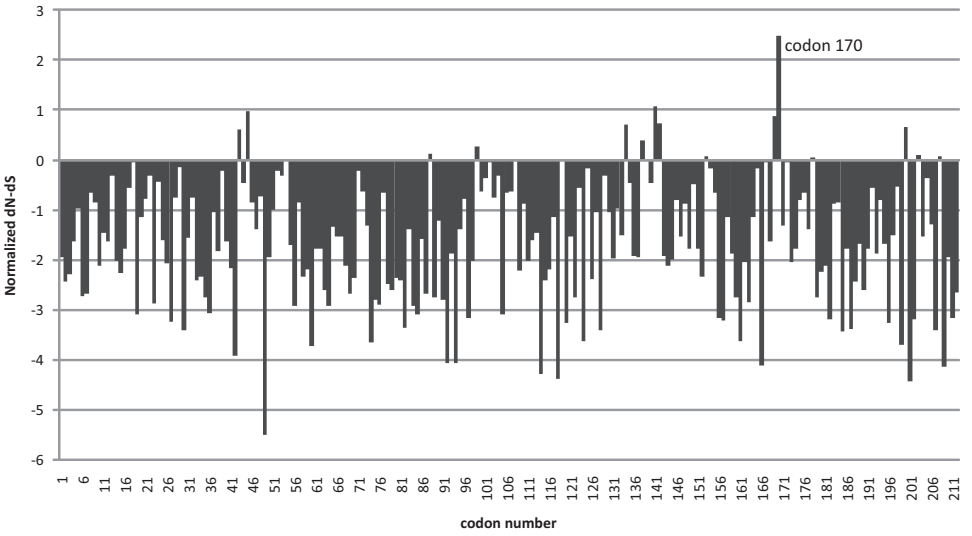

Fig. 4. Rate of synonymous and non-synonymous nucleotide substitutions/site from FMDV type A VP_1 sequences of South American viruses. Extent of positive and negative selection on individual codons was studied. The value above and below the abscissa, indicates the number of per-site non-synonymous substitutions (dN) over per-site synonymous ones (dS) in an individual codon. Zero value means dN = dS.

consequence of the rigorous control measures applied in the sub-region to restrain the emergency.

Detection of unique subgenotypes in particular areas (Southern Cone, Bolivia, and Ecuador/Peru) is in marked contrast to the identification of five subgenotypes co-circulating within one single country, as in the case of Venezuela. It has to be considered that identification of subgenotypes 1 and 2 as two separate ones may represent an overestimation as the difference between these two groups is only 15.2% and the choice of 15% as cut-off value may need re-definition.

All isolates collected during the type A emergencies in already declared free territories of Colombia near the Venezuelan frontier are closely related to viruses collected in Venezuela in previous periods. Strain A/Tibu/Col/04 showed at least 95% identity with isolates collected between the years 2001–2003 in Táchira and Mérida Venezuelan states, and the viruses recovered during the emergency in Norte de Santander, Colombia in 2008, showed the closest relationship (95%) with isolates recovered from Venezuela in 2004 and 2005. These data give support to the suggested origin of the outbreaks at the time, which pointed to movement of people, vehicles and/or animals across the border with Venezuela, as the main probable origin (OIE, 2008). The apparent time gap registered between the Colombian isolate in 2008 and the last virus from Venezuela belonging to the same subgenotype, that was recorded in 2006, is most probably due to lack of viral samples and information from the latter country in this period (PANAFTOSA, 2011b).

The observed co-circulation of various subgenotypes, and the molecular tracing of the emergencies indicating a link to high risk areas, together with the fact that Venezuela is the only country in South America recording type A isolates since 2003, reinforces the need for strengthening FMD control not only within this country, but also in the hot spots of border areas. The fact that different lineages can be observed co-circulating in Venezuela, within a similar time bracket in which only a unique lineage is detected during the emergencies in the Southern Cone, illustrates the potential for FMD diversification under limited selective pressure.

Selective pressure acting over FMDV evolution has been previously described (Haydon et al., 2001; Tosh et al., 2003). In this work, positive selection was determined for amino acid 170. This residue is located within immunogenic site 4 and therefore exposed to the immune system of the host (Baranowski et al., 2001). Moreover, positive selection pressure was described acting over the same amino acid site in Indian type A FMDV isolates (Tosh et al., 2003). The integrin-binding site Arg-Gly-Asp (RGD) was invariant in most isolates.

The deduced VP_1 amino acid sequences of all South American isolates studied showed a number of codon deletions. Deletion of amino acids within the G–H loop (aa 136–160) seems to be promiscuous (i.e. aa 142 is lacking in isolates from different subgenotypes, and one isolate from another subgenotype is lacking amino acids 156 and 157). A deletion within the G–H loop can also be observed even in isolates from Asian topotypes (aminoacid 141). Conversely, deletion of amino acid 197, within antigenic site 2 (Baranowski et al., 2001), is restricted to a unique cluster within subgenotype 13, and may constitute a marker for this cluster.

This work, which fills a gap of knowledge on FMDV type A in South America particularly with respect to both retrospective strains and those presently circulating in the region, serves as an input to establish genetic links of recent and putative emergencies, and helps building a comprehensive picture of the evolving nature of the virus in the continent.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2012.02.009.

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