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Genetic diversity of foot-and-mouth disease virus serotype A in Venezuela, (2001-2013)

Diversidad genética del virus de la fiebre aftosa serotipo A en Venezuela (2001-2013)

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ABSTRACT

Venezuela is the only southamerican country with endemic circulation of Foot-and-Mouth Disease Virus (FMDV) in all its territory. Molecular phylogeny (nucleotide sequences of the 1D-Vp1 gene) was used for characterization of active A serotype FMDV between 2006-2013 (16 strains). Philogenetic analysis also included 23 strains isolated between 2006 and 2013 and 3 venezuelan historical relevance strains. Considering the 2001-2013 period, an extensive genetic diversity is observed and reflected in: a) coexistence of five genetic groups (denominated subgenotypes 1, 2, 3, 4 and 16 according to the southamerican A serotype clasification), distributed in two different genetic lineages denominated A and B (genetic divergence>20%); b) increasing in the diversification of the most prevalent genetic group (subgenotype 1); and c) persistence of a phylogenetically distant group of the vaccine strain (subgenotype 16). Considering the strains of this study 2006-2013, the subgenotype 1 (most prevalent), shows more genetic heterogeneity respect to 2001-2006, and is redetected an isolated, phylogenetically distant of the vaccine strain, that coincides with higher incidence of FMDV in Venezuela (2006-2008). The greater diversification of FMDV in Venezuela is related to immunological selection pressure (positive) exerted by vaccination (new variants with less homology to the vaccine), therefore it highlight the necessity to improve the effectiveness of vaccination campaigns and the attention to outbreaks of disease. This study describes the virus acting in the most recent period (2006-2013) in Venezuela and a data bank with FMDV type A 1D-Vp1 sequences (45 strains 2001-2013) is complemented.

Key words: phylogenetic analysis, 1D-Vp1 gene, FMDV, type A.

RESUMEN

Venezuela es el único país suramericano con circulación endémica del virus de la Fiebre Aftosa (VFA) en todo su territorio. Se utilizó filogenia molecular (secuencias nucleotídicas del gen 1D-Vp1) para caracterizar los VFA serotipo A activos entre 2006-2013 (16 cepas). El análisis filogenético incluyó además 23 cepas aisladas (2001-2007) y 3 cepas de relevancia histórica en al país. Considerando el periodo (2001-2013), se observa una extensa diversidad genética reflejada en: a) coexistencia de cinco grupos genéticos (denominados subgenotipos 1,2,3,4 y 16 acorde a clasificación del serotipo A de Suramérica), distribuidos en dos linajes genéticos diferentes denominados A y B (divergencia genética>20%), b) incremento en la diversificación del grupo genético más prevalente (subgenotipo 1) y c) persistencia de un grupo filogenéticamente distante de la cepa vacunal (subgenotipo 16). Al analizar las cepas de este estudio (2006-2013), se encontró que el subgenotipo 1 (más prevalente), muestra mayor heterogeneidad genética respecto a 2001-2006 y se re-detecta un aislado filogenéticamente distante de la cepa vacunal, que coinciden con la mayor incidencia de FA en Venezuela (2006-2008). Esta mayor diversificación está relacionada con la presión inmunológica de selección (positiva) ejercida por la vacunación (selección de nuevas variantes de menor homología a la vacuna), por tanto, resalta la necesidad de mejorar la efectividad de las campañas de vacunación y la atención de focos. Este estudio describe los virus activos del periodo más reciente (2006-2013) en el país y se complementa el banco de datos de secuencias 1D-Vp1 del VFA tipo A de Venezuela (45 cepas 2001-2013).

Palabras clave: análisis filogenético, gen 1D-Vp1, fiebre aftosa, serotipo A.

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INTRODUCTION

Foot-and-mouth disease (FMDV) is a highly contagious acute viral disease of cloven-hoofed animals, which temporarily affects livestock productivity, and its cross-border nature, restricts trade in animals and animal products in international markets (OIE, 2017). In endemic countries it generates high costs represented primarily by the biannual vaccination of the national herd (Castro, 2005). The FMD virus (FMDV) has a single stranded positive sense RNA, member of *Aphthovirus* genus, *Picornaviridae* family, and is classified immunologically in seven serotypes (O, A, C, Asia 1, SAT1-2-3) (Alexandersen *et al*., 2003) and numerous subtypes that exhibit epidemiological and degree of cross protection differences (Paton *et al*., 2005). Serotype A is considered extremely diverse genetically, the most antigenically diverse among Eurasian serotypes (Knowles and Samuel, 2003) and in which genetic recombination occurs more often than in the rest of the serotypes (Tosh *et al*., 2002a, Jackson *et al*., 2007). Of the capsid proteins, Vp1 is considered to be highly polymorphic, the most immunogenic (Grubman and Baxt, 2004) and subject to selective pressure (Carrillo *et al*., 2005). Venezuela is the only South American countries with endemic/epidemic circulation throughout its territory (PANAFTOSA, 2016) and serotype A has always been the most prevalent, although serotype O is also present and serotype C has never been reported.

Differences in 1D(Vp1) gene sequence is the base for genetic classification, and for phylogeny and epidemiology molecular studies (Samuel and Knowles, 2001; Tosh *et al*, 2002b; Mohapatra *et al*., 2011), important in terms of surveillance for molecular detection of viral variants phylogenetically distant from the vaccine strain (antigenic prediction) (Paton *et al*., 2005), introduction of exotic variants to a country product of imports and illegal trafficking of animals, and tracing its source (Knowles and Samuel, 2003; Klein *et al*., 2007; König *et al*., 2007). In endemic countries, is important to study the field virus circulating through appropriate sampling and eventual immediate genotyping. Studies by Malirat *et al*. (2012), included venezuelan isolated detected in the period 2001-2007, and have classified the serotype A from South America in 16 subgenotypes (using a cutoff of 15% genetic divergence), among which, 1, 2, 3, 4, and 16 corresponding to venezuelan isolates. The aim of this study was to characterize phylogenetically the FMDV serotype A actives in Venezuela in the most recent period (2006-2013), based on nucleotide sequences of the complete 1D region (Vp1).

METHODOLOGY

Samples were characterized by 1D complete gene sequencing as described by Word Reference Laboratories (Knowles and Samuel, 1998; Malirat and Bergmann, 2003).

Clinical samples: epithelial bovine samples belonging to the collection of Laboratorio Nacional de Referencia de Enfermedades Vesiculares-Instituto Nacional de Investigaciones Agrícolas (INIA-Maracay-Venezuela) that were collected from 2006 to 2013 at different farm located in several states.

Viruses strain: three historical and epidemiologically relevant venezuelan strains and 23 venezuelan strains detected in the period 2001-2007, were included on the phylogenetic analysis. Also eight type A strains classified as topotype Euro-SA (Europe) and not Euro-SA (Africa, Asia and Europe) and strains of serotype Asia 1. The designation and origin of FMDV isolates studied are listed in Table 1.

Extraction of Virus RNA: It was extracted from 50-100 mg of epithelial tissue using TRIzol reagent (Invitrogen™).

Reverse Transcription of Virus RNA: It was made starting from 5 µl of viral ARN as template, using Kit ImProm-II Reverse Transcription system® PROMEGA, and addition of dithiothreitol (DTT) 0,1 M.

PCR Amplification of Reverse Transcribed RNA and cycle sequencing: The viral genomic regions encoding VP1 and 3D polymerase were amplified using pairs primer specific for serotype based on published sequences (Knowles and Samuel, 1998) and tested in circulating FMDV strain of South America (Malirat and Bergmann, 2003; Clavijo *et al*., 2003; Malirat *et al*., 2008; Malirat *et al*., 2012). The 1D(Vp1) PCR products were purified with the Qiaquick PCR Purification kit1 (Qiagen) and submitted to the Unidad de Estudios Genéticos y Forenses – InstitutoVenezolano de

Table 1. Viruses used for genetic analysis of the coding sequencesVp1 (1D).

Denomination	Geographical origin		Collection date	Reference	Gene Bankaccession
	State	County			
Recent FMDV isolates analyzed in this work (2006-2013)					
A/Mérida/Ven/06[a]	Mérida	Andres Bello	15-02-06	This work	KX150527
A/Barinas/Ven/06a	Barinas	Ezequiel Zamora	09-10-06	This work	KX150523
A/Apure/Ven/06b	Apure	José Antonio Páez	31-10-06	This work	KX150522
A/Barinas/Ven/06b	Barinas	Barinas	08-01-06	This work	KX150524
A/Mérida/Ven/06b	Mérida	Obispo Ramos de Lora	15-11-06	This work	KX380578
A/Barinas/Ven/06c	Barinas	Nicolás Pulido	25-11-06	This work	KX150525
A/Táchira/Ven/07a	Táchira	San Cristóbal	07-05-07	This work	KX150526
A/Barinas/Ven/07	Barinas	Barinas	01-08-07	This work	KX150528
A/Táchira/Ven/07c	Táchira	Libertad	13-09-07	This work	KX150529
A/Bolívar/Ven/07a	Bolívar	Manuel Carlos Piar	28-09-07	This work	KX150530
A/Monagas/Ven/08a	Monagas	Maturín	01-04-08	This work	KX150531
A/Mérida/Ven/08	Mérida	Alberto Adriani	20-05-08	This work	KX150532
A/Bolívar/Ven/08	Bolívar	Sifonte	10-06-08	This work	KX150533
A/Barinas/Ven/11[a]	Barinas	Andrés Eloy Blanco	20-10-11	This work	KX150534
A/Barinas/Ven/13a	Barinas	Barinas	01-04-2013	This work	KU234721
A/Barinas/Ven/13b	Barinas	Barinas	01-04-2013	This work	KX380577
Venezuelan FMD isolates sequences previously published (2001-2007)					
A Bolívar Ven 01	Bolívar	Padre Chien	05-12-2001	Malirat et al., (2012)	JQ082933
A Bolívar Ven 03	Bolívar	Padre Chien	14-05-2003	Malirat et al., (2012)	JQ082935
A Mérida Ven 02	Mérida	Alberto Adriani	28-11-2001	Malirat et al., (2012)	JQ082934
A Mérida Ven 03[a]	Mérida	Febres Cordero	09-12-2003	Malirat et al., (2012)	JQ082937
A Mérida Ven 03b	Mérida	Alberto Adriani	12-12-2003	Malirat et al., (2012)	JQ082938
A Mérida Ven 04	Mérida	Alberto Adriani	19-05-2004	Malirat et al., (2012)	JQ082943
A Mérida Ven 05[a]	Mérida	Jají	06-04-2005	Malirat et al., (2012)	JQ082950
A Mérida Ven 05b	Mérida	Obispo Ramos de L	20-04-2005	Malirat et al., (2012)	JQ082951
A Mérida Ven 05c	Mérida	Obispo Ramos de L.	29-04-2005	Malirat et al., (2012)	JQ082952
A Táchira Ven 01	Táchira	Panamericano	13-06-2001	Malirat et al., (2012)	JQ082932
A Táchira Ven 04[a]	Táchira	García de Hevia	23-01-2004	Malirat et al., (2012)	JQ082939
A Táchira Ven 04b	Táchira	García de Hevia	02-11-2004	Malirat et al., (2012)	JQ082940
A Táchira Ven 04c	Táchira	García de Hevia	01-04-2004	Malirat et al., (2012)	JQ082942
A Táchira Ven 04d	Táchira	García de Hevia	24-09-2004	Malirat et al., (2012)	JQ082947
A Barinas Ven 03	Barinas	Torumos	03-07-2003	Malirat et al., (2012)	JQ082936

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Venezuelan FMD isolates sequences previously published (2001-2007)					
A Barinas Ven 04[a]	Barinas	Ezequiel Zamora	10-03-2004	Malirat et al., (2012)	JQ082941
A Barinas Ven 04b	Barinas	Barinas	26-08-2004	Malirat et al., (2012)	JQ082946
A Falcón Ven 04	Falcón	M. Iturriza	13-08-2004	Malirat et al., (2012)	JQ082945
A Yaracuy Ven 04	Yaracuy	Veroes	12-07-2004	Malirat et al., (2012)	JQ082944
A Apure Ven 04	Apure	Páez	18-11-2004	Malirat et al., (2012)	JQ082948
A Apure Ven 05	Apure	Mantecal	03-02-2005	Malirat et al., (2012)	JQ082949
A Apure Ven 06	Apure	NR	13-06-2006	Malirat et al., (2012)	Q082953
A Portuguesa Ven 07	Portuguesa	Guanarito	30-01-2007	Malirat et al., (2012)	JQ082954
South American isolates sequences collection FMDV					
A_{32}Ven/iso36	Bolívar	-	1970	Carrillo et al., (2005)	AY593775
A/Venezuela/89	--	-	1989	Malirat et al., (2012)	JQ082978
A_{18}/Zulia/Ven/62	Zulia	-	1962	Malirat et al., (2012)	JQ08295
A_{24}Cruzeiro/Br/55	Vaccinestrain strain	-	1955	Malirat et al., (2012)	JQ082960
Continental FMDV isolates sequences					
Asia 1/IND 328/2004	India	West Bengal	09-03-2004	Valarcher et al, (2009)	FJ785303
Asia 1/IND 389/2004	India	Gujarat	2004	Valarcher et al, (2009)	FJ785304

Investigaciones Científicas (UEGF-IVIC) for cycle direct sequencing. Details of the primers used in the PCR amplification and sequencing are shown in Table 2.

Bioinformatics analysis: The obtained sequences were edited by visual inspection using the Program Bioedit, Version 7.2.0© (Hall,1999). The multiple nucleotide sequence alignments were generated using DNAman Program version 2.1 and Phylogenetic analyzes were conducted using MEGA version 5.0.

RESULTS AND DISCUSSION

The disease in Venezuela have had two epizootic outbreaks for both serotypes (A and O) in recent years (2002-2003 and 2007-2008) and a significant reduction in incidence since 2009. The latest serotype A virus reported was detected in 2013, which had the highest incidence (78.76% of cases) [Figure 1]. A total of 16 nucleotide sequences of VP1 coding region of serotype A were analyzed. Phylogenetic analysis showed that isolates detected in period 2006-2013 were closely related to strain of subgenotypes 1, 3, 4 and 16 described by Malirat *et al*. (2012), and to historical and epidemiological relevant venezuelan strains (3 isolates), indicating that all of them correspond to endogenous viral variants (Figure 2).

No viruses closely related to vaccine strain were detected, showing that there was no inadvertent release of virus to the field from the vaccine used in the country (A_{24}/Cruzeiro/Br55). The analysis of circulating viral variants in the entire period (2001-2013), showed that they are grouped into five major genetic clusters, designated as subgenotypes 1, 2, 3, 4 and 16 according to the nomenclature described by Malirat *et al*. (2012) for South American serotype A strain. Two separate genetic lineages were distinguished (lineages with > 20% nucleotide divergence) and designated as A and B, one represented by the subgenotype 16 and the other represented by the remaining isolates (subgenotypes 1, 2, 3, 4).

Table 2. Oligonucleotide primers used for RT-PCR and cycle sequencing of foot-and-mouth disease viruses

Primer designation*	**Sense**	**Localization**	**Primer sequence (5' 3')**	**Productlength (bp)**	**Reference sequency**	**Reference use in South America**
1C $_{562}$	Forward	1C	TACCAAATTACACACGGGAA	795 bp	*Knowles and Samuel, 1998*	*Malirat et al., 2008, Malirat et al., 2012*
FMD-2A$_{34}$ (NK72)	Reverse	2A	GAAGGGCCCAGGGTTGGACTC		"	"
ADir	Forward	1C	TACCAAATTACACACACGGGAA	863-866	*Knowles and Samuel, 1998*	*Malirat y Bergman, 2002*
FMD-2B$_{58}$ (NK61) o (LMR)	Reverso	2B	GACATGTCCTCCTGCATCTG		"	"
7401	Forward	3D	GCAGTGACGC-CATGAACATC	540	*Tami et al., 1998*	*Clavijo et al., 2003*
7941	Reverse	3D	CCTGCCACGGAGATCAACTT		"	"

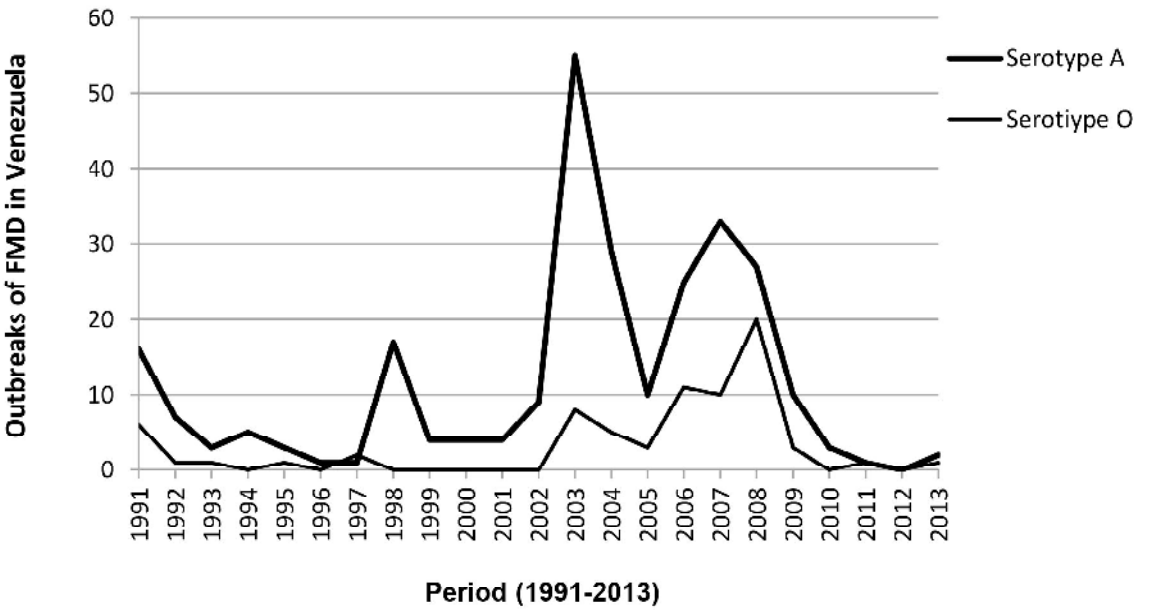

Figure 1. History outbreaks of FMD in Venezuela. Serotypes A and O. 1991-2013.

Although the FMDV entered to Venezuela from Argentina in 1950 (Piñate, 2000), there are very few sequences (Vp1) available from isolates detected in this period (Only 3 venezuelan isolates from 1962 to 1989). More phylogeographic evidence is required of previous isolated from Venezuela and South America, to display the roots of these genetic groups and to determine whether these lineages have separate origins (two introductions) or have a common venezuelan ancestor (come from the evolution of virus in Venezuela). Equally, we have considered these genetic groups as two different lineages based on genetic divergence in the region 1D(Vp1), but it is likely that if we analyzed a wider region as the P1 region, it could clarify phylogenetically if the genetic groups A and B are really so different. Subgenotype 1 from lineage A showed the closest phylogenetic relationship to the vaccine strain and the highest prevalence and, paradoxically, the subgenotype 16 from lineage B, being the most distant group to the vaccine strain (21% genetic divergence), showed the lowest prevalence (one isolated in 2008).

Extensive genetic diversity was represented by three observations: a) the existence of five different subgenotypes (based on a cutoff of 15% and observed topology tree); b) the presence of a subgenotype (subgenotype 16) phylogenetically distant from vaccine strain and from the remaining venezuelan isolates, and c) the increase of heterogeneity in the most prevalent genetic group (subgenotype 1). Studies by Malirat *et al*. (2012) described the subgenotype 1 as a rather homogeneous group based on their percentage of genetic homology within group (95%), with a significant number of isolates (14 isolates) collected in a short period of time (2003-2006). Contrary to this research, results of this work on the period 2006-2013, showed that in this subgenotype greater genetic diversity there was with respect to that observed in the period 2001-2006, as evidenced by the presence of multiple subclades with genetic distances that increase their value divergence, reflecting the possibility of classifying new subgenotypes from this genetic group by applying the cutoff of 15%. In the serotype A phylogenetic analysis of South America (Malirat *et al*., 2012), included only a unique isolated in 2006 and one from 2007 representing those years. This study has included a greater number of viral variants (6 isolates 2006, 4 of 2007 and 3 of 2008) from the years of highest incidence of the disease (Table 2 and Figure 2), enabling

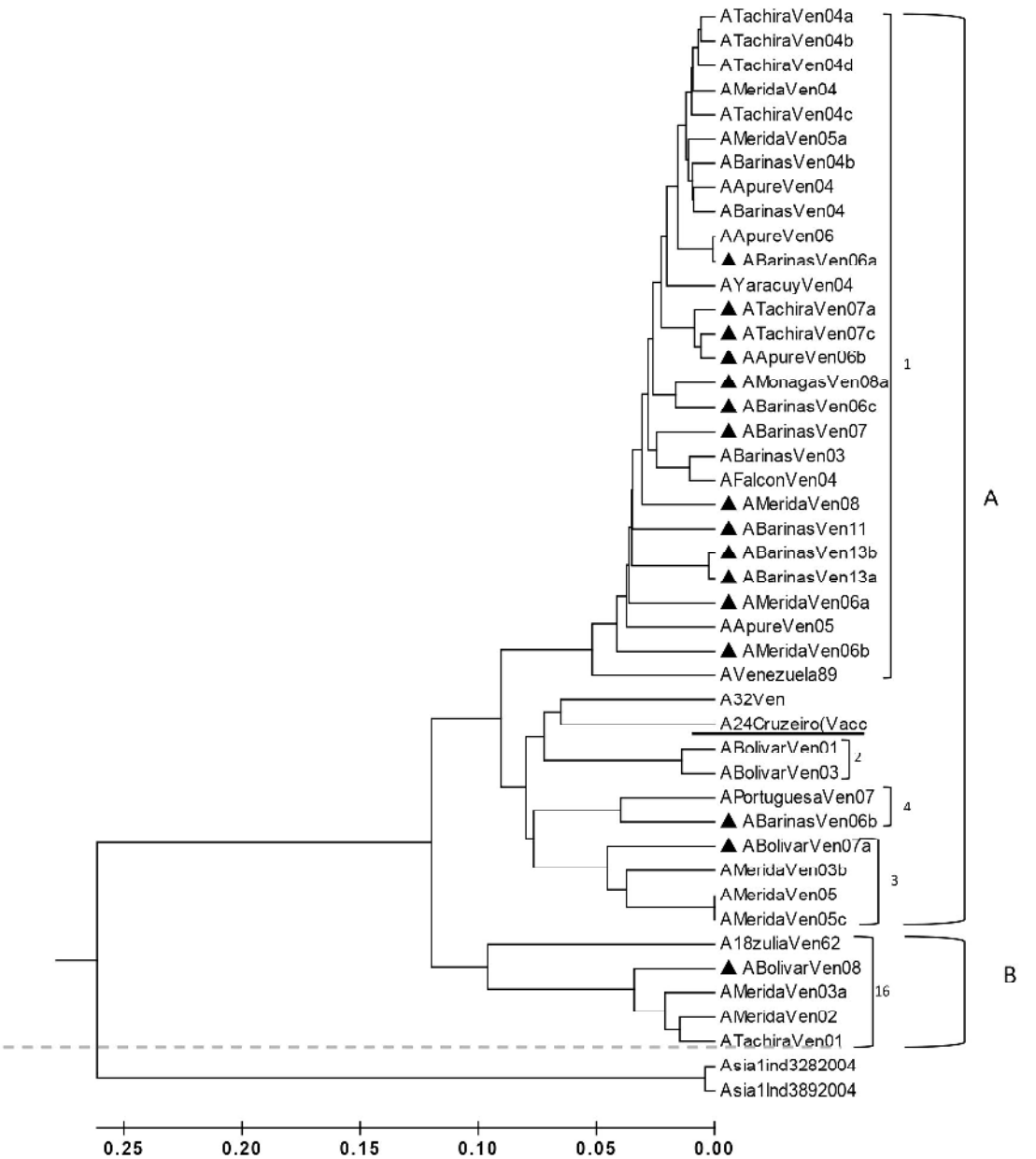

Figure 2. Phylogenetic tree based on the complete region 1D coding for VP1 protein of Venezuelan Serotype A FMDV isolated detected in period 2006-2013 and its genetic relationships with isolated acting in Venezuela in past. Distances were calculated upon the Kimura-two (K-2) parameter model and phylogenetic analyzes with UPGM method using MEGA version 5.0 program were conducted. Two different lineages distributed into five genetic groups (labeled subgenotypes 1,2,3,4 and 16 as described for Malirat et al., 2012 for South American type A FMDV) are observed. ( ) Isolates collected between 2006-2013.

a more close reconstruction of the evolutionary process of FMDV type A in Venezuela.

The events of greater diversification of subgenotype 1 from 2006 and re-detection of subgenotype 16 in 2008, coincided with the highest incidence of FMDV in Venezuela between 2006 and 2008 (Figure 1 and 2), which is related with a low immune status of the animals against FMDV in this period. At the same time, the low immune status of the animals allowed the FMDV replicated in a large population sizes (competition between different genetic variants) under the influence of immune selection positive pressure, which is exerted by the vaccination through the selection of those new variants of lower homology with the vaccine strain (escape mutants which are not effectively neutralized) (Cowan *et al*., 1974; McCahon, 1981; Domingo *et al*., 2003). This typically occurs in the presence of both, the best vaccine and convalescent animals (immune animals), although it has seen also favored by the existence of non-vaccinated or partially immunized cattle (Haydon *et al*., 2001; Grubman and Baxt, 2004).

Genetic variants of FMDV co-circulate and accumulate rapidly in the field (Domingo *et al*., 2003) and result in antigenic variation which increases over time (Tully and Fares, 2009) However the reduction in the incidence of the disease in recent years is suggestive that the vaccine in use is protective. But the extensive genetic diversity in the region 1D(Vp1) of type A venezuelan isolated highlights the importance of avoiding partial and insufficient immune coverage which allowing endemic circulation of the virus. Surveillance of both antigenic characterization (by serological methods such as vaccine matching) and population immunity studies are necessary. Equally, increasing surveillance in the field (attention to outbreaks of disease and opportune sampling) and improving the precise detection by laboratory are also necessary in order to obtain a more accurate estimation of the incidence of the disease in Venezuela.

CONCLUSIONS

Phylogenetic analysis of the venezuelan isolates detected in the period 2001-2013 revealed significant genetic diversity represented by the coexistence of two genetic lineages classified into five different subgenotypes, increase in the diversification of the most prevalent genetic group (subgenotype 1), and persistence of a phylogenetically distant vaccine strain group.

The greater diversification of FMDV type A in Venezuela coincides with the period of highest incidence of the disease in Venezuela (2006-2008), constitutes a risk for the eradication program, and made it necessary to strengthen the attention and control of outbreaks of disease.

The greater diversification of FMDV type A in Venezuela is related to the immunological selection pressure (positive) exerted by vaccination (selection of new variants with less homology to the vaccine) therefore it is necessary to improve the effectiveness of vaccination campaigns through the use of high quality vaccines with high values of coverage throughout the country.

This paper fills the gap of knowledge about viral variants acting in the most recent period in Venezuela (2006-2013) and therefore active in South America, and complements the formation of a database with FMDV type A 1D(Vp1) sequences of Venezuela (45 strains 2001-2013).

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