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Short communication

Differences in the virulence of two strains of Foot-and-Mouth Disease Virus Serotype A with the same spatiotemporal distribution

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ABSTRACT

During the 2000–2001 epidemic of Foot-and-Mouth Disease Virus (FMDV) in Argentina, two FMDV serotype A viruses were identified among others. Since different pathogenic properties between these virus strains were noticed in cattle, we evaluated several biological properties and features of FMDV A/Arg/00 and FMDV A/Arg/01 in order to compare these viruses in terms of virulence and pathogenicity. Our results indicate that FMDV A/Arg/00 grows less efficiently than FMDV A/Arg/01, exemplified by smaller sized plaques, retarded one-step growth curves and overall low viral yields. Also, FMDV A/Arg/00 displayed the lowest specific infectivity in suckling mice requiring 50-fold more infectious particles than FMDV A/Arg/01 to generate a LD50 in suckling mice. Finally, FMDV A/Arg/00 did not cause death in adult C57Bl/6 mice even at high doses (10^7–10^6 PFU) whereas FMDV A/Arg/01 resulted lethal in doses as low as 10^2 PFU. Overall, we were able to demonstrate that these virus strains differ from each other in terms of virulence and pathogenicity.

Foot-and-Mouth Disease Virus (FMDV) is the prototype member of the *Aphtovirus* genus within the family *Picornaviridae*. This virus is the causative agent of Foot-and-Mouth Disease (FMD), a highly contagious vesicular disease of cloven-hoofed animals, including cattle, swine, goat and sheep, responsible for severe economic losses worldwide (Rowlands, 2003; Sobrino and Domingo, 2004). FMDV exists in the form of seven serotypes: A, O, C, Asia1, SAT1, SAT2 and SAT3 (Bachrach, 1968; Pereira, 1981). The virus particle consists of a single-stranded, positive-sense RNA of about 8200 bases and 60 copies, each of four structural proteins (1A, 1B, 1C and 1D) (Jackson et al., 2003; Rueckert and Wimmer, 1984). The genome contains a 5′-untranslated region of approximately 1100 nucleotides, followed by a single open reading frame that encodes the viral polyprotein (Palmenberg, 1990) and a 3′-untranslated region containing a poly(A) tract (Grubman and Baxt, 2004).

Despite the fact that a number of questions about FMDV biology have been addressed, the mechanisms underlying the pathogenicity and virulence of the virus remain poorly understood (Carrillo et al., 2005; Mason et al., 2003). Studies of host/virus interaction and comparative analysis of viruses with different properties may allow the identification of genome regions which are critical for these aspects of the viral biology (Beard and Mason, 2000; van Rensburg et al., 2004).

In Argentina, within the context of the National FMD Control Programme (Sutmoller et al., 2003), vaccination against FMD was halted in 1999 and in May 2000 the OIE recognized the country as free of FMD without vaccination. Despite this achievement, in July 2000 outbreaks of the disease reappeared affecting mainly cattle, especially animals under 2 years of age. During the epidemic, two FMDV serotypes were identified, A and O. Virus serotype O was responsible for only a few outbreaks at the beginning of the episode, while serotype A circulated until January 2002 (Mattion et al., 2004). Two serotype A virus strains were identified, FMDV A/Argentina/00 (FMDV A/Arg/00) and FMDV A/Argentina/01 (FMDV A/Arg/01), which belonged to two different lineages (Konig et al., 2007). Interestingly, different pathogenic properties between these virus strains were noticed in cattle (unpublished results). FMDV A/Arg/00 showed low virulence, causing mild clinical signs both in the field and in experimental trials. Only few and small lesions were found in infected animals. This strain was mainly present in year 2000 when 124 outbreaks were reported, it had low dissemination rate and was rapidly controlled. On the other hand, FMDV A/Arg/01 was highly virulent and caused severe lesions in the affected animals in the field as well as in experimental challenge trials. Moreover, calf deaths due to viral infection were reported. This virus showed extended spread in 2001, when 2436 outbreaks were reported, and resulted more difficult to be controlled (Mattion et al., 2004).

In the present report we evaluated the biological properties and features of FMDV A/Arg/00 and FMDV A/Arg/01 in order to compare them in terms of virulence and pathogenicity

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expanding the description of the different behavior of these two viruses.

FMDV A/Arg/00 (prototype strain MC38796 from General Villegas) and FMDV A/Arg/01 (prototype strain MC267 from Trenque Lauquen) were obtained from the National Institute for Animal Health (SENASA, Argentina). Viruses were isolated from infected animals from 2000 and 2001 outbreaks respectively, and propagated once in BHK-21 cells (Mattion et al., 2004). A second cell passage of these viruses was used for plaque assays, viral growth curves and mice inoculation experiments.

We compared the plaque morphology of these prototype viruses in BHK-21 cells; plaque assays were performed as previously described, cell monolayers were fixed and stained at 48 h post infection (hpi) (Tami et al., 2003). Both virus strains displayed plaques of clear morphology, but with significant different sizes. The diameters (mm) of 40 individual lytic plaques were measured and compared statistically (*t*-Student, $p < 0.05$) using Statistix 7 for Windows. The plaques produced by FMDV A/Arg/01 were significantly larger (mean diameter 4.125 ± 0.857 mm) than those produced by FMDV A/Arg/00 (mean diameter 2.575 ± 0.73 mm). In addition, using an optical microscope, we were able to observe lytic plaques in monolayers infected with FMDV A/Arg/01 within 20 hpi, whereas only a negligible cytopathic effect (CPE) was detected in FMDV A/Arg/00 infected cells (data not shown).

The FMDV 2000 and 2001 strains were distinguished in one-step-growth studies. In these experiments the virus titers (PFU/ml) and the amount of intracellular viral RNA were measured at different times after infection. Briefly, 24-well plates containing 2×10^5 BHK-21 cells/well were infected with FMDV A/Arg/00 or FMDV A/Arg/01 at a multiplicity of infection of 10, at 37 °C for 60 min. Then, extracellular virus was inactivated by the addition of phosphate buffered saline pH 4.5 for 5 min and cells were washed three times with DMEM-Hepes (DMEM, HyClone, supplemented with 1% antibiotics and 25 mM Hepes pH 7.5). Monolayers were covered with DMEM-Hepes supplemented with 1% fetal calf serum and incubated at 37 °C for additional 8 h. At different times post infection, supernatants were removed and cells were collected in 0.5 ml of RSB-NP40 (Tris 10 mM pH 7.4, $NaCl_2$ 10 mM, $MgCl_2$ 1.5 mM, NP40 0.01%). Finally, the isotonicity of extracts was restored by the addition of an equal volume of 2× DMEM-Hepes. Viral suspensions were clarified by centrifugation at 9000 × *g* for 10 min at 4 °C and kept at −80 °C until used. Viral titers were determined by plaque assays on BHK-21 cells (Tami et al., 2003). In parallel, cell monolayers infected as described before were collected in TRIzol© (Invitrogen) and intracellular RNA was obtained following manufacturer's instructions; total RNA in each sample was measured using a NanoDrop nd-1000 spectrophotometer. cDNA was synthesized in the presence of MMLV reverse transcriptase (Promega) using 0.5 μg of Random Primers (Biodynamics). Finally, a real time-PCR assay was performed in an ABI Prism 7000 sequence detection system (Komurian-Pradel et al., 2001; Oleksiewicz et al., 2001) using a Sybr® Green PCR master mix kit (Applied Biosystem) and a standard amplification profile with the primers Epi5 (TTCGAGAACGGCACGGTCGGAC, forward primer) and Epi2 (TCAGGGTTGCAACCGACCGC, reverse primer), to obtain a 270 bp fragment from the 3D region of FMDV genome.

Consistent with the results obtained during the plaque size assays, the kinetics of progeny virus production were different between the two viruses. One-step growth curves showed that FMDV A/Arg/00 grew less and slower than FMDV A/Arg/01 (Fig. 1A). At 4 hpi the viral titer for FMDV A/Arg/00 (2×10^4 PFU/ml) was approximately 50-fold lower than that observed for FMDV A/Arg/01 (9.8×10^5 PFU/ml). At this time, the FMDV A/Arg/01 titer reached a plateau (2×10^6 PFU/ml), while the highest titer of FMDV A/Arg/00 was reached by 6 hpi (1×10^5 PFU/ml). Nevertheless, similar levels of intracellular vRNA were observed in cell monolayers infected with both viruses (Fig. 1B). From the data shown in Fig. 1, it can be estimated that in FMDV A/Arg/01 infected cells the ratio of infectivity to intracellular viral RNA at 4 hpi was 40-fold higher than the ratio calculated for FMDV A/Arg/00. These results suggest that the viral growth differences observed in BHK-21 cells would not be due to events occurring between the early steps of viral replication and the synthesis of RNA.

In order to evaluate their pathogenicity, FMDV A/Arg/00 and FMDV A/Arg/01 were inoculated in CF-1 suckling mice (Campbell, 1970; Skinner, 1951). Briefly, groups of 12 newborn mice were injected intramuscularly with 50 μl of five serial tenfold dilutions of each virus and simultaneously, viral inoculums were titrated by plaque assay. Dead animals were scored up to 7 days after inoculation and the 50% Lethal Doses (LD_{50}) were determined by the method of Reed and Muench (1938). In each case, the specific infectivity was determined as the ratio PFU/LD_{50} (Rieder et al., 1993). In order to confirm the presence of each virus in the infected mice, RNA was extracted from brain tissue using Trizol, cDNA was synthesized and a PCR from the 3D region of FMDV genome was performed as described earlier. The resultant 270 bp fragments

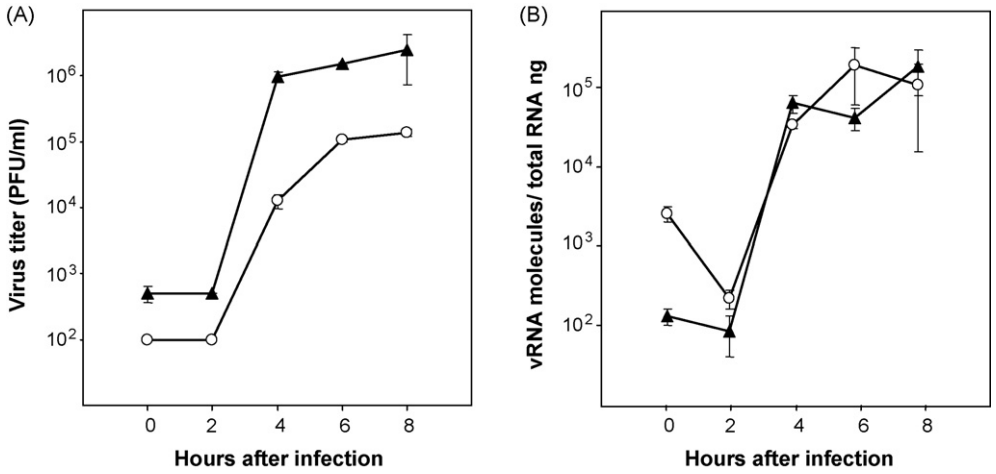

Fig. 1. Growth kinetics of FMDV A/Arg/00 and FMDV A/Arg/01. BHK-21 cells were infected and harvested at different times post infection. (A) Single-step growth curves obtained by plaque assay titration of virus present at each time. Samples were taken in duplicates and standard deviations are shown in the graphic. (B) RNA production curves were obtained by real time RT-PCR assay from Trizol resuspended monolayers. Viral RNA was relativized to total extracted RNA. Samples were taken in triplicates and standard deviations are shown in the graphic. (○) FMDV A/Arg/00 and (▲) FMDV A/Arg/01.

Table 1
Specific infectivity of FMDV A/Arg/00 and FMDV A/Arg/01 in suckling mice.

FMDV	Specific infectivity (PFU/LD_{50})	Inoculated dose (PFU/ml)	Lethal Dose (LD_{50}/ml)
A/Arg/00	47.56	1.17×10^6	2.46×10^4
A/Arg/01	0.94	6.67×10^5	7.1×10^5

LD_{50} values were determined by the method of Reed and Muench at seven days post inoculation. Lower PFU/LD_{50} values indicate that the virus is more infectious for suckling mice.

were sequenced and FMDV A/Arg/00 and FMDV A/Arg/01 were corroborated as the causal agents of suckling mice death. As can be observed in Table 1, both viruses were virulent for suckling mice but with different levels of lethality, since FMDV A/Arg/00 induced symptoms and death with about 50-fold-lower efficiency than FMDV A/Arg/01. Moreover, the time required for mouse death ranged from 2 to 7 days depending on the virus strain and the viral doses (Fig. 2A). All mice inoculated with 3.3×10^2 PFU of FMDV A/Arg/01 died within 3 days upon virus exposition, whereas a similar dose of FMDV A/Arg/00 (5.8×10^2 PFU) required 6 days to kill 100% of animals. Similarly, 30 PFU of FMDV A/Arg/01 killed all mice at the end of the experiment in contrast to the 40% of mice dead in the group that received 58 PFU of FMDV A/Arg/00. The results show that the infection with FMDV A/Arg/01 caused symptoms of disease and death faster than FMDV A/Arg/00, suggesting a differential pathogenicity in suckling mice between these virus strains.

Salguero et al. reported that it is possible to differentiate closely related viruses and viruses of the same serotype by their pathogenic properties using an adult mice model (Salguero et al., 2005; Sanz-Ramos et al., 2008). Therefore, we evaluated the pathogenicity of FMDV A/Arg/00 and A/Arg/01 in C57Bl/6 adult mice by intraperitoneal inoculation of serial tenfold dilutions of each virus to groups of four females of 9–10 weeks. For FMDV A/Arg/00 we injected doses from 10^3 to 10^7 PFU/mice and for FMDV A/Arg/01 doses ranged from 10^2 to 10^6 PFU/mice. As expected, the results shown in Fig. 2B confirm the differential behavior of the two viruses. At 48 hpi all mice inoculated with A/Arg/01 died, except for one mice belonging to the group inoculated with 10^2 PFU which did not show any sign of disease throughout the experiment. On the contrary, none of the mice infected with A/Arg/00 died; moreover, there were no signs of disease by the end of the experiment, at 7 dpi (Fig. 2B). Mice inoculated with 10^7 and 10^6 PFU of FMDV A/Arg/00 did show certain degree of apathy at 24 hpi, but by 72 hpi they were fully recovered.

In summary, we were able to distinguish FMDV A/Arg/00 and FMDV A/Arg/01 in terms of virulence and pathogenicity. The results reported here confirm previous observations notified by field veterinarians during 2000–2001 FMD outbreaks. FMDV A/Arg/00 grows less efficiently than FMDV A/Arg/01, exemplified by smaller sized plaques, retarded one-step growth curves and overall low viral yields. Likewise, FMDV A/Arg/00 displayed the lowest specific infectivity in suckling mice. Furthermore, animals injected with this virus showed clinical signs and died later in comparison with FMDV A/Arg/01. Finally, FMDV A/Arg/00 did not cause death of adult C57Bl/6 mice even at high doses (10^7–10^6 PFU) while FMDV A/Arg/01 resulted lethal in doses as low as 10^2 PFU.

Current available information indicates that FMDV utilizes different kinds of integrins for cell entry in the natural hosts (Baxt and Rieder, 2004; Neff et al., 1998). Nevertheless, it has been reported

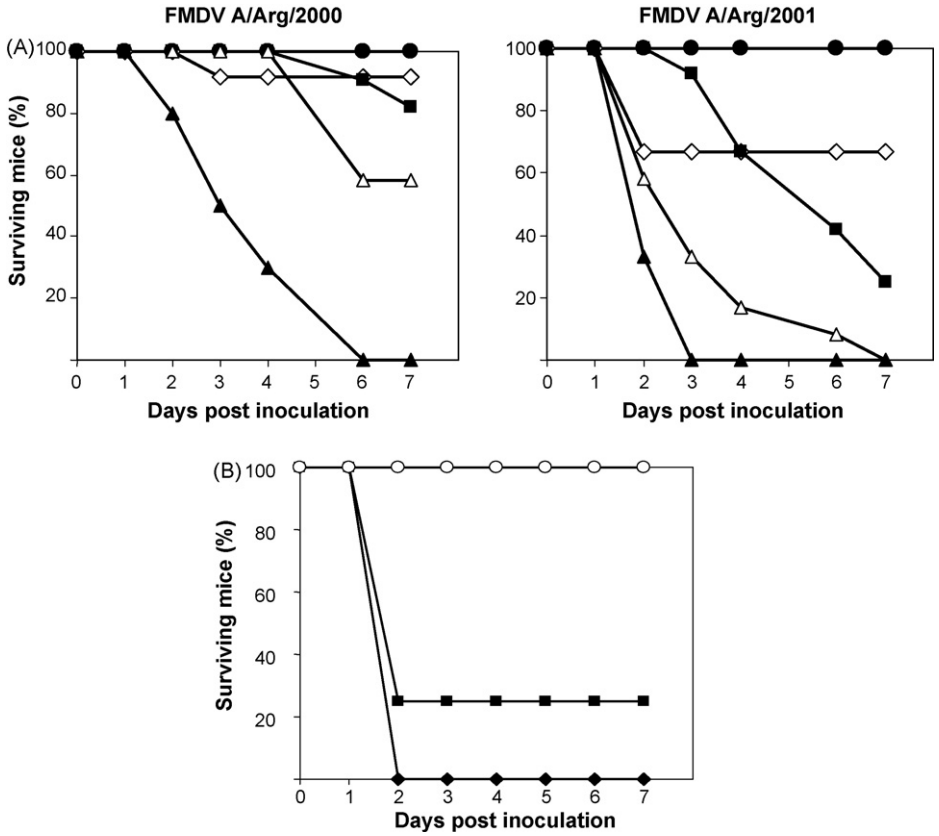

Fig. 2. Survival curves of mice infected with FMDV A/Arg/00 and FMDV A/Arg/01. (A) Seven-day-old CF-1 mice were inoculated with the indicated doses of each virus (PFU/mice) and observed for seven days. FMDV A/Arg/00: (▲) 585 PFU, (△) 59 PFU, (■) 6 PFU, (◊) 0.6 PFU, (●) 0.06 PFU. FMDV A/Arg/01: (▲) 333 PFU, (△) 33 PFU, (■) 3 PFU, (◊) 0.3 PFU, (●) 0.03 PFU. (B) Adult C57Bl/6 mice were infected with 10^7 PFU of FMDV A/Arg/00 (○), with 10^2 PFU of FMDV A/Arg/01 (■) and with 10^3–10^6 PFU of FMDV A/Arg/01 (♦) and observed for 7 days.

that FMDV serotypes O and C, after several passages in cell culture, may acquire the ability to use alternative pathways of entry into cells, such as binding to heparan sulphate or usage of an as-yet-unidentified alternative receptor (Baranowski et al., 2000). This adaptive process generates a repertoire of viral variants, including some with substitutions at the RGD viral receptor site (Rieder et al., 2005), and may cause alterations on viral virulence. In the present study, bovine FMDVs were amplified by two passages in cell cultures. Although neither FMDV A/Arg/01 nor FMDV A/Arg/00 presented any amino acid change in the 1D region after two cell passages (data not shown), further experiments are required to rule out the possibility of different cell receptor usage by FMDVs replicating either in the natural host, cell culture or mice.

Since studies involving natural hosts of FMDV are limited by the complexity, the economical cost, and the animal care regulations, the adult mice model along with the other assays reported here will be useful to provide data that contribute to guide further experiments in cattle.

Complete genomic sequences of both prototype viruses (FMDV A/Arg/00 and FMDV A/Arg/01) are available in GenBank (Carrillo et al., 2005). Comparative genomic analysis shows insertions/deletions in the non-coding regions and several nucleotide substitutions along the genome. Nevertheless, none of these sequence alterations has been associated with FMDV virulence so far, as they do not seem to be located in highly conserved positions. On the other hand, a pairwise comparison of the sequences of the 1D protein of FMDV A/Arg/00 and FMDV A/Arg/01 has been recently reported (Konig et al., 2007). The structural protein 1D is the most studied FMDV protein; it is related to viral cell entry, protective immunity induction and serotype specificity (Domingo et al., 2003; Jackson et al., 2003; Mason et al., 2003). Konig et al. (2007) showed that 1D nucleotide sequences of FMDV A/Arg/00 and FMDV A/Arg/01 differed by 12.8% and the authors also found twenty-two amino acid changes, most of them in the hypervariable region of the studied protein. All together, these facts could explain the antigenic relationship of these two viruses. Nevertheless, further experiments are necessary to define the involvement of the nucleotides and/or amino acid substitutions in viral virulence or pathogenicity. Now, we are performing experiments to construct chimeras based on FMDV A/Arg/01 genome-length clone containing different genome regions of FMDV A/Arg/00. Hereafter, we will evaluate the virulence and pathogenicity of viral chimeras to map virulence determinants on FMDV genome.

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