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Short communication

Inoculation of newborn mice with non-coding regions of foot-and-mouth disease virus RNA can induce a rapid, solid and wide-range protection against viral infection

Miguel Rodríguez-Pulido [a,b], Francisco Sobrino [b], Belén Borrego [c], Margarita Sáiz [b,*]

[a] Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Bellaterra, 08193 Barcelona, Spain
[b] Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Cantoblanco, 28049 Madrid, Spain
[c] CISA-INIA, Valdeolmos, 28130 Madrid, Spain

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ABSTRACT

We have recently described the ability of in vitro-transcribed RNAs, mimicking structural domains in the 5′ and 3′ non-coding regions (NCRs) of the foot-and-mouth disease virus (FMDV) genome, to trigger the innate immune response in porcine cultured cells and mice. In this work, the antiviral effect exerted in vivo by these small synthetic non-infectious RNA molecules was analyzed extensively. The susceptibility of transfected newborn Swiss mice to FMDV challenge was tested using a wide range of viral doses. The level of protection depended on the specific RNA inoculated and was dose-dependent. The RNA giving the best protection was the internal ribosome entry site (IRES), followed by the transcripts corresponding to the S fragment. The time course of resistance to FMDV of the RNA-transfected mice was studied. Our results show the efficacy of these RNAs to prevent viral infection as well as to contain ongoing FMDV infection in certain time intervals. Protection proved to be independent of the serotype of FMDV used for challenge. These results support the potential use of the FMDV NCR transcripts as both prophylactic and therapeutic molecules for new FMDV control strategies.

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly infectious and economically devastating disease of livestock, recognized as the most important constraint to international animal trade (Kitching, 2005; OIE, 2007; Sáiz et al., 2002). FMDV is a member of the Picornaviridae family, and its genome consists of a single-stranded RNA molecule of about 8.5 Kb in length containing a unique open reading frame flanked by two highly structured 5′ and 3′ non-coding regions (NCRs) (Belsham and Martinez-Salas, 2004). We have recently described the ability of synthetic RNA transcripts corresponding to structural elements present in the FMDV 5′ and 3′ NCRs to trigger the innate immune response and induce an antiviral state in vivo (Rodríguez-Pulido et al., 2011). The 5′-terminal S fragment is a 360-nt long region predicted to fold into a stable and long hairpin structure (Escarmís et al., 1992; Witwer et al., 2001). The internal ribosome entry site (IRES) mediates the cap-independent translation of the viral RNA through its approximately 450-nt-long multi-domain structure (Belsham, 2009; Fernández-Miragall et al., 2009). The 3′NCR is about 90 nt long, including two stem-loop structures, ending by a poly A tail (Serrano et al., 2006). We previously showed the detection of IFN-α/β proteins in sera of newborn mice inoculated intraperitoneally (IP) with 100 μg of in vitro-transcribed RNA corresponding to the S, IRES or 3′NCR sequences. These mice showed a reduced susceptibility to FMDV infection (Rodríguez-Pulido et al., 2011). Thus, 24 h after RNA inoculation, all animals survived FMDV challenge with 10^2 plaque forming units (PFU), whereas only 28% of the control group survived. Upon challenge with 10^4 PFU none of the control mice survived at day 2 postinfection, while survival at day 11 postinfection was 10%, 60% and 100% for 3′NCR-, S- and IRES-inoculated mice, respectively (Rodríguez-Pulido et al., 2011).

In this work, the level of susceptibility to FMDV infection of suckling mice inoculated with each of the FMDV NCR transcripts has been analyzed using a wide range of viral doses. The contribution to the RNA-mediated protection of the RNA dose, transfection reagents, time of inoculation and viral serotype used for challenge has also been studied.

Groups of 3–7 day-old Swiss mice were inoculated IP with 100 μg of the corresponding RNA transcripts as described (Baranowski et al., 2003; Rodríguez-Pulido et al., 2011), and inoculated 24 h later with different doses of type-O FMDV O1K isolate. The double-stranded RNA analogue poly I:C, previously reported to induce an anti-FMDV effect when inoculated IP into suckling mice (Richmond and Hamilton, 1969; Rodríguez-Pulido et al., 2011), was included as a positive control in these assays. The animals were monitored for 11 days. Mice showing severe signs of disease

* Corresponding author. Tel.: +34 91 1964522; fax: +34 91 1964420.
E-mail address: msaiz@cbm.uam.es (M. Sáiz).

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Table 1
Susceptibility to FMDV infection of suckling mice inoculated IP with 100 μg of the corresponding RNA 24 h before infection.

RNA	Viral dose (PFU/mouse)[a]	Survival/ total (%)	Mean day to death ± SEM[b]	LD_{50} (PFU/ml)[c]
S	7×10^6	5/10 (50)	3.8 ± 0.2	$>7 \times 10^7$
	7×10^5	6/8 (75)	8.5 ± 0.0	
	7×10^4	13/18 (72)	4.0 ± 0.5	
	7×10^3	10/11 (90)	4.5 ± 0.0	
	7×10^2	12/12 (100)	–	
IRES	7×10^6	9/10 (90)	4.0 ± 0.0	$>7 \times 10^7$
	7×10^5	7/8 (87,5)	8.5 ± 0.0	
	7×10^4	16/17 (93)	4.0 ± 0.00	
	7×10^3	10/10 (100)	–	
3′NCR	7×10^5	0/8 (0)	2.5 ± 0.5	6×10^5
	7×10^4	1/10 (10)	4.1 ± 0.6	
	7×10^3	6/11 (54)	3.9 ± 1.3	
	7×10^2	5/5 (100)	–	
Poly I:C	7×10^6	1/10 (10)	4.8 ± 0.22	4×10^6
	7×10^5	0/10 (0)	4.2 ± 0.13	
	7×10^4	7/11 (64)	4.5 ± 0.0	
	7×10^3	7/8 (87,5)	7.0 ± 0.0	
	7×10^2	12/12 (100)	–	
–	7×10^4	0/9 (0)	1.0 ± 0.0	3×10^3
	7×10^3	1/24 (4)	1.0 ± 0.0	
	7×10^2	7/25 (28)	1.9 ± 0.14	
	7×10^1	18/27 (67)	1.9 ± 0.11	
	7	9/9 (100)	–	

[a] Mice were inoculated IP with the indicated FMDV dose in a final vol of 100 μl.
[b] Estimated as the average number of days that the mice in each group survived after viral infection with the assistance of GraphPad Prism version 5.01 (GraphPad software, San Diego, CA). -No death was observed in these groups.
[c] Determined on survival at day 11 postinfection with 10-fold dilutions of FMDV O1K (7×10^7 PFU/ml) recovered after transfection and passage on BHK-21 cells (Baranowski et al., 2003), as described (Reed and Muench, 1938).

were euthanized. Susceptibility of each group to FMDV is shown in Table 1. Inoculation with all RNAs remarkably increased the 50% lethal dose (LD_{50}) of the virus, determined for the control group. In the case of the 3′NCR, the increase was about 2 log, 3 log for the poly I:C-transfected mice and more than 4 log for both the IRES- and S-transfected groups. Therefore, suckling mice inoculated with IRES or S transcripts 24 h before challenge became at least 10,000-fold less susceptible to the virus than PBS-inoculated mice. Interestingly, 90% of the IRES-transfected mice survived after infection with a viral dose of 7×10^6 PFU (undiluted viral stock), showing the outstanding protective effect of these RNA molecules. The average number of days that the mice survived after viral infection increased significantly in the RNA-treated groups (Table 1).

The RNA dose used in our experiments was 100 μg per animal, about 20 mg/kg, in the range of previous reports (Richmond and Hamilton, 1969) and according to our recent results (Rodríguez-Pulido et al., 2011). To analyze the effect of the amount of RNA inoculated on protection, groups of newborn mice were transfected with decreasing amounts of IRES RNA and survival was monitored after challenge (Fig. 1). The level of protection against viral infection was dose-dependent. A slight decrease in protection was observed for the 50 μg dose and a higher decrease (10–40% survival) in animals receiving 1–10 μg. Survival differences shown in Fig. 1 were statistically significant by the log-rank test ($p < 0.01$). These results suggest that the resistance observed is based on specific antiviral mechanisms, and also indicate that a dose of 100 μg per mouse was suitable for optimal protection.

Next, we analyzed the contribution of Lipofectine to protection, as all the RNA transcripts were delivered in a mixture containing 20 μg/ml of this transfection reagent (Baranowski et al., 2003; Rodríguez-Pulido et al., 2011). The level of protection against FMDV induced by inoculation with the IRES transcripts in the

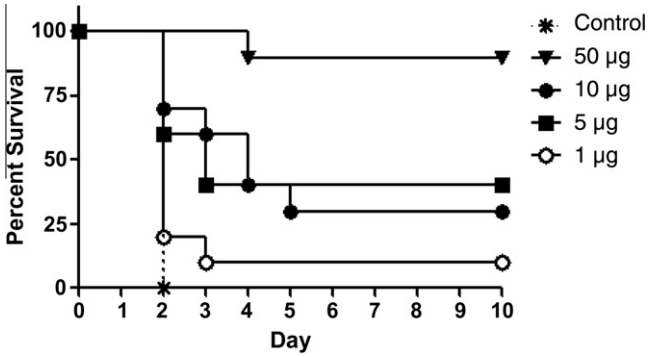

Fig. 1. The antiviral effect of the IRES RNA is dose-dependent. Groups of mice were inoculated IP with 1, 5, 10 or 50 μg of IRES transcripts or PBS (control) and 24 h later were infected IP with 7×10^4 PFU of FMDV-O1K. Survival was recorded for up to 10 days post-challenge; $n = 10$ mice per group. Kaplan–Meier survival curves were analyzed by the log rank test using GraphPad Prism version 5.01 (GraphPad software, San Diego, CA).

presence or absence of Lipofectine was assayed (Fig. 2). Survival differences between IRES inoculation with or without LP were statistically non-significant by the log rank test ($p > 0.05$). However, survival against viral challenge in animals receiving IRES without Lipofectine decreased by 30% compared to the group of mice inoculated with IRES transcripts emulsified in that transfection reagent (70% survival versus 100%). All mice inoculated with PBS with or without Lipofectine died and survival differences between these groups were statistically non-significant by the log rank test ($p > 0.05$), showing that Lipofectine *per se* does not confer protection, although it may enhance to some extent the protective effect of the RNAs by increasing their stability and/or their access to the target cells.

In order to assess the time window of the RNA-induced protection against FMDV infection, IRES and S transcripts, proven to give the best results in protection (Table 1), but showing differences in the kinetics of IFN-I induction (Rodríguez-Pulido et al., 2011), were inoculated at different times before and after viral challenge (Fig. 3). Complete or very high protection was achieved when IRES RNA was inoculated 8 or 24 h prior to FMDV infection (Fig. 3A), with 100% and 86% survival, respectively. However, the susceptibility to the virus increased when the IRES transcripts were inoculated 4 h before infection (60% survival). Inoculation of the transcripts at longer times pre-infection strongly decreased their protective effect against viral infection, with 50% and 0% survival for 48 and 72 h before challenge, respectively. A similar effect was observed for S RNA transcripts (Fig. 3B). Inoculation at 8 h before infection dramatically reduced protection, with 36% survival, compared to 100% when the S RNA was inoculated 24 h before infection. A smaller increase in susceptibility (70% survival) was also observed for inoculation at 4 h pre-infection. Inoculation at 48 h before infection reduced survival to 56%. Remarkably, even when the S RNA was inoculated 3 days before challenge, 36% of the animals survived, unlike 0% survival with the IRES. This is in agreement with the detection of IFN-α/β during longer periods of time in mice serum after inoculation with the S transcripts compared to the IRES-inoculated group (Rodríguez-Pulido et al., 2011). IFN levels reached highest values at 8 h after inoculation of both S and IRES RNAs and decayed 24 h after for the IRES-inoculated animals, while still remained high for the S-inoculated group. At 48 h after inoculation, IFN levels were low for both groups. This difference could account for the higher survival rates observed for the S-inoculated groups compared to IRES-inoculated groups after challenge at longer times with RNA inoculation. Then, the window of protection induced by the S transcripts would be

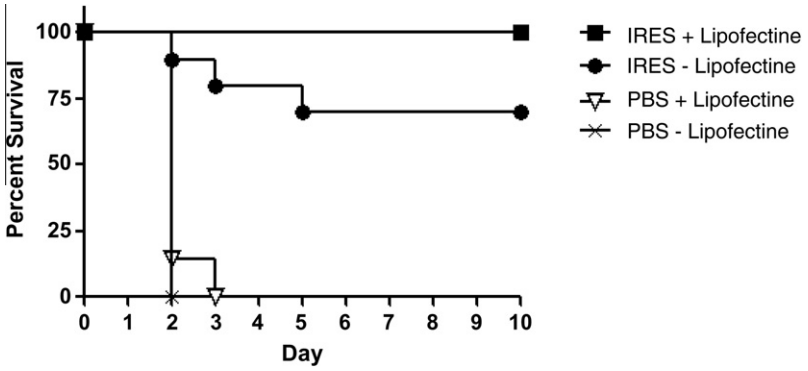

Fig. 2. Effect of Lipofectine (Invitrogen) in IRES-mediated protection against FMDV infection. Mice were inoculated IP with 100 μg of IRES transcripts or PBS in the presence or absence of 0.2 μg/μl of Lipofectine; mice were infected IP 24 h later with 7×10^4 PFU of FMDV-O1K. Survival was recorded up to 10 days post-challenge; $n = 7–10$ mice per group. Kaplan–Meier survival curves were analyzed by the log rank test as in Fig. 1.

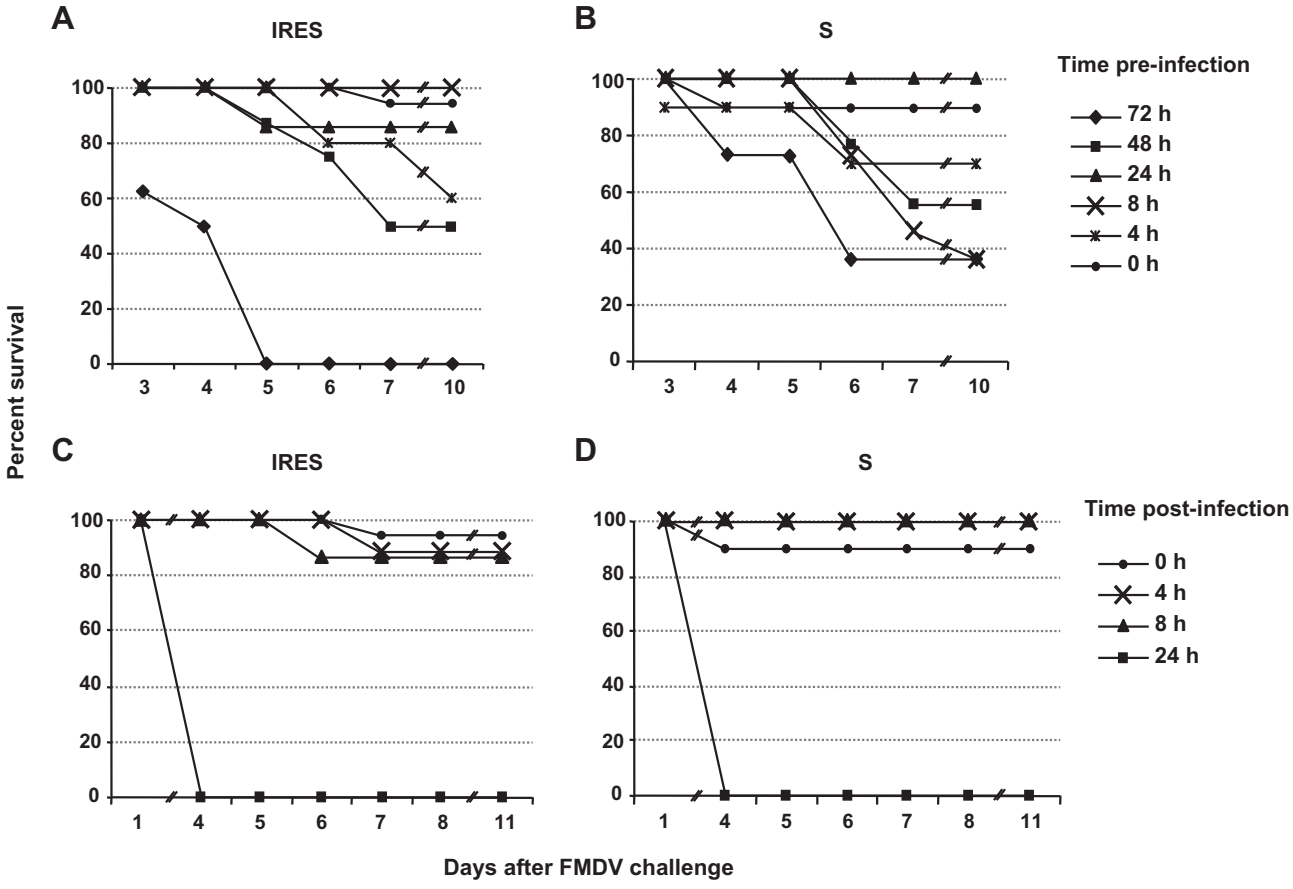

Fig. 3. Effect of the time of inoculation with IRES or S RNA against FMDV infection. Mice were inoculated IP with 100 μg of IRES (A and C) or S (B and D) RNA at the indicated times before (A and B) or after (C and D) intraperitoneal infection with 7×10^4 PFU of FMDV-O1K. Mice survival was recorded at different days after viral challenge; $n = 8–11$ mice per group.

slightly wider than that induced by the IRES and that might correlate with the duration of the IFN response.

The decrease in protection observed for IRES- and S-transfected mice when RNA inoculation was performed at short times before challenge was consistent with previous studies describing the increased mortality of suckling mice associated with near simultaneous injection of poly I:C and FMDV (Richmond and Hamilton, 1969). This interval might be necessary for development of maximal resistance; however, co-inoculation of S or IRES transcripts and the virus induced high levels of protection (Fig. 3A–D), and the IRES RNA had a higher protective effect inoculated at 8 h than at 4 h before infection, suggesting that a fine balance between the routes activating the innate immune response by the RNAs and the viral replication kinetics or antagonistic mechanisms triggered by the virus, might determine either the outcome of disease or the viral clearance.

When the RNAs were inoculated at different times after infection, susceptibility to FMDV was similar for the IRES- and S-inoculated mice (Fig. 3C and D). Remarkably, high survival percentages were observed for those groups inoculated with the RNAs at short times

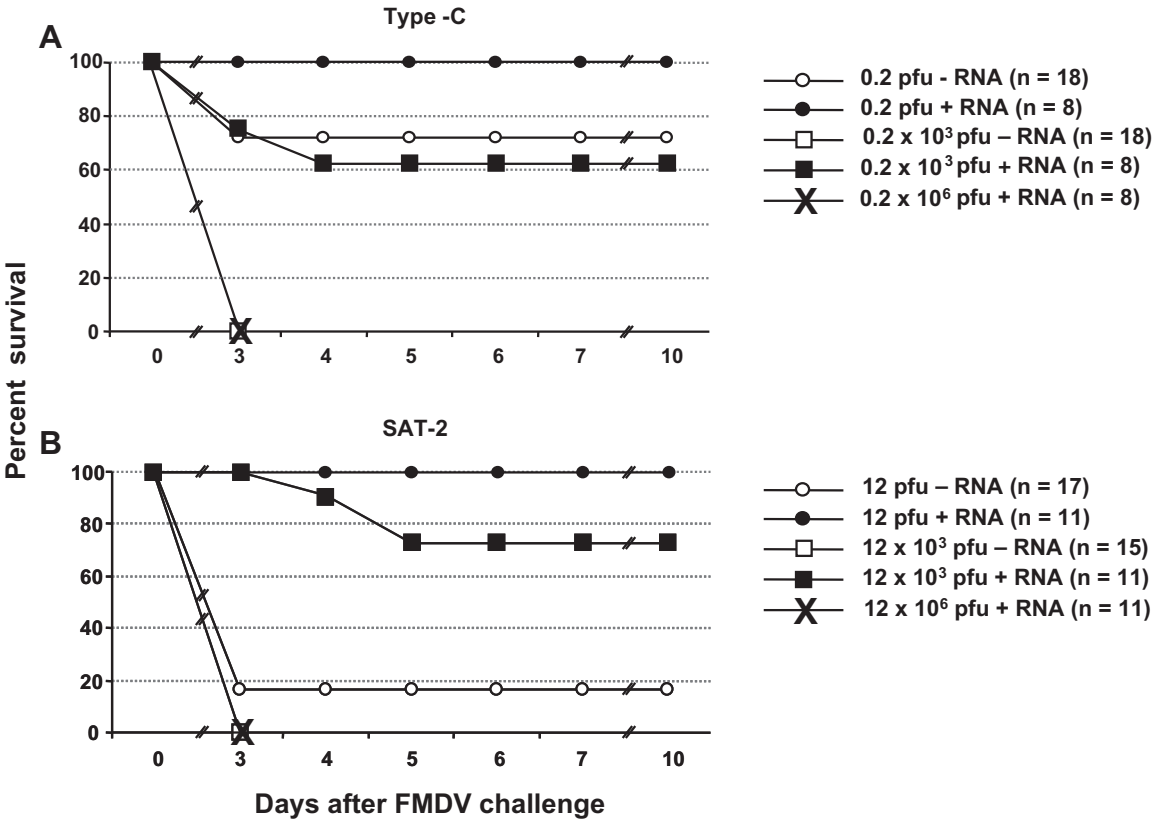

Fig. 4. Inoculation of IRES RNA in mice induces heterotypic protection against FMDV. Groups of mice were inoculated IP with 100 μg of IRES RNA or PBS and 24 h later were infected IP with three different doses of type-C C-S8 c1 (A) or SAT-2 RHO 1/48 (B) FMDV isolates. The relative infectivity of the viral stocks used was 0.3 PFU/LD_{50} for C-S8 and 3 PFU/LD_{50} for SAT-2. Mice survival was recorded at different days after challenge.

after infection (89% and 87% of mice inoculated with the IRES at 4 and 8 h postinfection survived, respectively), and complete protection (100% survival) was achieved when mice were inoculated with the S transcripts at 4 or 8 h postinfection. No protective effect was observed for mice inoculated with the RNAs 24 h after viral infection. These results suggest that the antiviral response induced by the RNAs is rapidly established and effective to counteract the viral replication if administered shortly after infection, while 24 h later it was too late to contain the progress of the infection. Our data support the potential use of this RNAs as both prophylactic as well as therapeutic molecules in a certain time window.

One of the main reasons hampering foot-and-mouth disease control by zoo-sanitary measures and vaccination is the occurrence of multiple serotypes of the virus. In all the above experiments a type-O virus derived from a full-length clone after transfection and passage on cultured cells was used. To test the protective effect induced by the IRES transcripts in mice against FMDV isolates of different serotypes, mice were inoculated with 100 μg of IRES and challenged 24 h later with different doses of C-S8c1 and RHO 1/48 isolates, grouped into the C and SAT-2 serotypes, respectively (Fig. 4). Type-O, -C and -SAT-2 isolates are genetically and serologically divergent, and display different prevalence and geographical distribution (Tully and Fares, 2006; Yoon et al., 2011). Complete protection was observed in RNA-inoculated mice after infection with viral doses corresponding approximately to their LD_{50}, whereas survival was 72% and 17% in the control groups inoculated with type-C and SAT-2 isolates, respectively. The difference in the relative infectious dose inoculated, 0.6 LD_{50} for type-C and 4 LD_{50} for type-SAT-2 viruses, as well as the variability among assays, might account for the different survival rates observed in both control groups. At a higher challenge dose (about 10^3 LD_{50}), none of the mice in the control groups survived at day 3 postinfection, while survival was 63% and 73% for the IRES-inoculated mice infected with type-C and SAT-2 viruses, respectively. Inoculation with 10^6 LD_{50} of each virus was lethal for IRES-inoculated, as well as control animals. Although isolates from other types remain to be tested, these results suggest the wide-range nature of the antiviral effect triggered by inoculation of the IRES RNA in mice.

In summary, we have studied the host resistance induced in suckling mice by inoculation of FMDV NCR transcripts. The IRES RNA was the most effective transcript among those assayed, followed by the S RNA. High levels of protection were achieved using these RNAs against increasing FMDV doses, and time course experiments proved their prophylactic as well as therapeutic ability to control FMDV infection in several time intervals. Our results suggest the potential use of these small, non-infective synthetic molecules in the development of new broad-spectrum anti-FMDV strategies.

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References

Baranowski, E., Molina, N., Nunez, J.I., Sobrino, F., Sáiz, M., 2003. Recovery of infectious foot-and-mouth disease virus from suckling mice after direct inoculation with in vitro-transcribed RNA. J. Virol. 77, 11290–11295.
Belsham, G.J., 2009. Divergent picornavirus IRES elements. Virus Res. 139, 183–192.

Belsham, G.J., Martinez-Salas, E., 2004. Genome organisation, translation and replication of FMDV RNA. In: Sobrino, F., Domingo, E. (Eds.), Foot-and-Mouth Disease: Current Perspectives. Horizon Bioscience, Norfolk, UK, pp. 19–52.

Escarmís, C., Toja, M., Medina, M., Domingo, E., 1992. Modifications of the 5′ untranslated region of foot-and-mouth disease virus after prolonged persistence in cell culture. Virus Res. 26, 113–125.

Fernández-Miragall, O., López de Quinto, S., Martinez-Salas, E., 2009. Relevance of RNA structure for the activity of picornavirus IRES elements. Virus Res. 139, 172–182.

Kitching, R.P., 2005. Global epidemiology and prospects for control of foot-and-mouth disease. Curr. Top. Microbiol. Immunol. 288, 133–148.

OIE, 2007. Terrestrial animal health code. Chapter 2.2.10. FMD article.

Reed, L.J., Muench, H., 1938. A simple method of estimating fifty per cent endpoints. Am. J. Hyg. 27, 493–497.

Richmond, J.Y., Hamilton, L.D., 1969. Foot-and-mouth disease virus inhibition induced in mice by synthetic double-stranded RNA (polyriboinosinic and polyribocytidylic acids). Proc. Natl. Acad. Sci. USA 64, 81–86.

Rodríguez-Pulido, M., Borrego, B., Sobrino, F., Sáiz, M., 2011. RNA structural domains in non-coding regions of foot-and-mouth disease virus genome trigger innate immunity in porcine cells and mice. J. Virol. 85, 6492–6501.

Sáiz, M., Nunez, J.I., Jimenez-Clavero, M.A., Baranowski, E., Sobrino, F., 2002. Foot-and-mouth disease virus: biology and prospects for disease control. Microbes Infect. 4, 1183–1192.

Serrano, P., Pulido, M.R., Sáiz, M., Martínez-Salas, E., 2006. The 3′ end of the foot-and-mouth disease virus genome establishes two distinct long-range RNA–RNA interactions with the 5′ end region. J. Gen. Virol. 87, 3013–3022.

Tully, D.C., Fares, M.A., 2006. Unravelling selection shifts among foot-and-mouth disease virus (FMDV) serotypes. Evol. Bioinform. Online 2, 211–225.

Witwer, C., Rauscher, S., Hofacker, I.L., Stadler, P.F., 2001. Conserved RNA secondary structures in Picornaviridae genomes. Nucleic Acids Res. 29, 5079–5089.

Yoon, S.H., Park, W., King, D.P., Kim, H., 2011. Phylogenomics and molecular evolution of foot-and-mouth disease virus. Mol. Cells 31, 413–421.