

1 **TITLE: Delivery of synthetic RNA can enhance the immunogenicity of vaccines**  
2 **against foot-and-mouth disease virus (FMDV) in mice**

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14 **KEYWORDS**

15 FMDV; FMD vaccine; RNA delivery; vaccine adjuvant

16

17 **ABBREVIATIONS**

18 FMDV, foot-and-mouth disease virus; ncRNA, non-coding RNA; WNV, West Nile virus;

19 PAMP, pathogen-associated molecular pattern; PRR, pattern-recognition receptor;

20 IRES, internal ribosome entry site

21

1 **ABSTRACT**

2 We have recently described the antiviral effect in mice of in vitro-transcribed RNAs  
3 mimicking structural domains in the non-coding regions of the foot-and-mouth disease  
4 virus (FMDV) genome RNA. These small, synthetic and non-infectious RNA molecules  
5 (ncRNAs) are potent type-I interferon (IFN) inducers in vivo. In this work, the  
6 immunomodulatory effect of the ncRNA corresponding to the internal ribosome entry site  
7 (IRES) on immunization with two different FMD vaccine formulations, both based on  
8 inactivated virus, including or not a commercial adjuvant, was analyzed in the mice model.  
9 The effect of the time interval between RNA inoculation and immunization was also  
10 studied. RNA delivery consistently increased the titers of specific anti-FMDV antibodies,  
11 including neutralizing antibodies, elicited after vaccination. Moreover, at day 2 after  
12 immunization, significant differences in mean antibody titers could be detected between  
13 the groups of mice receiving either vaccine co-administered with the RNA and the  
14 control group, unlike those immunized with the vaccine alone. When vaccinated mice  
15 were challenged with FMDV, the mean values of viral load were lower in the groups  
16 receiving the RNA together with the vaccine. Our results show the enhancing effect of  
17 the IRES RNA on the immune response elicited after vaccination and suggest the  
18 potential of this molecule as an adjuvant for new FMD vaccine design.

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## 1 **1. INTRODUCTION**

2 Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious  
3 disease of livestock considered as a major animal health problem worldwide [1-3]. In areas  
4 where FMD is enzootic, vaccination is based on chemically-inactivated virus inducing a  
5 short-term immunity and involving periodical re-vaccination [4]. In many FMD-free  
6 countries, only emergency vaccination is accepted in response to an outbreak, taking the  
7 onset of protective immunity 5-7 days. Currently, many efforts are being focussed on the  
8 induction of earlier and long-lasting protective immunity.

9 The vaccine development field is exploiting the innate responses in new vaccine  
10 adjuvant design, such as the use of type-I interferon (IFN). Besides its broad antiviral  
11 activity, IFN can induce higher and earlier protective immune responses [5, 6]. IFN  
12 induction can be triggered by administration of a variety of compounds, recognized as  
13 pathogen-associated molecular patterns (PAMPs) by the cellular pattern-recognition  
14 receptors (PRRs) acting as sentinels in the host innate immune system [7-9].

15 We have recently shown that in vitro-transcribed RNAs, mimicking structural domains  
16 in the 5' and 3' non-coding regions of the FMDV genome (ncRNAs), can act as potent  
17 type-I-IFN inducers in vivo [10, 11]. Consistently, RNA inoculation induced protection  
18 against lethal doses of FMDV or West Nile virus (WNV) in mice [10-12]. Among all  
19 the ncRNAs tested, the transcripts corresponding to the internal ribosome entry site  
20 (IRES) in the 5'NCR of the viral genome conferred the highest levels of protection  
21 against both viruses [11, 12].

22 Here, we aimed to explore the immunomodulatory properties of these synthetic non-  
23 infectious RNA molecules and address whether their ability to induce IFN could be  
24 exploited for their use as immune adjuvants for FMD vaccination in mice. Our results  
25 show that inoculation of the IRES transcripts, together with two different vaccine

1 formulations, both based on inactivated virus, including or not a commercial adjuvant,  
2 may improve the induced immune response in terms of promptness, magnitude and  
3 endurance of the specific antibody titers elicited.

4

## 5 **2. MATERIALS AND METHODS**

### 6 **2.1. Mice**

7 Swiss ICR-CD1 mice from Harlan were used and handled in strict accordance with the  
8 guidelines of the European Community 86/609/CEE. The protocol was approved by the  
9 Committee on the Ethics of Animal Experiments of INIA (Permit Number: CEEA  
10 2011/015).

### 11 **2.2. RNA synthesis**

12 RNA transcripts corresponding to the IRES of FMDV C-S8c1 were generated by in  
13 vitro transcription from a PGEM-derived clone [10, 13]. Then, RNA was extracted and  
14 analyzed as described [10]. Prior to inoculation, IRES transcripts were heated at 92 °C  
15 for 5 min, cooled down to room temperature for 10 min, and then chilled on ice.

### 16 **2.3. Vaccine formulation and vaccination**

17 The inactivated vaccine consisted of a single dose of BEI-inactivated FMDV C-S8c1  
18 [14], equivalent to  $2 \times 10^5$  plaque forming units (PFU). The conventional vaccine was  
19 formulated as above but in a 1/1 (water/oil) emulsion in a commercial adjuvant  
20 (Montanide ISA50 V2, kindly provided by Seppic SA). In all cases, a single dose of the  
21 corresponding vaccine was injected intraperitoneally (IP).

22 Mice were IP inoculated with 200 µg of IRES RNA emulsified with lipofectine  
23 (Invitrogene), as described [10, 12], 24 h prior to, at the same time (co-inoculation) or

1 24 h after vaccine administration; for co-inoculation, RNA and vaccine were injected in  
2 two different points of the abdomen (about 1.5 cm distant).

3 Animals were bled through the maxillary vein. Sera were inactivated and kept at -80°  
4 until use.

#### 5 **2.4. Viral challenge and viremia**

6 Mice were inoculated with 10<sup>3</sup> PFU of infectious FMDV C-S8c1 in the footpad [14].  
7 For viral load analysis, total RNA was extracted using Tri Reagent (Sigma) from serum  
8 samples (10 to 20 µl) collected 2 days post-challenge, and resuspended in 15 µl of  
9 DEPC-treated water. One µl of each RNA was assayed in an RT-PCR reaction targeting  
10 the 3Dpol gene [15]. Viremia levels were expressed as relative values to the mean of  
11 PBS-inoculated controls.

#### 12 **2.5. Antibody detection**

##### 13 **2.5.1. Total anti-FMDV antibodies**

14 Mice sera in 3-fold serial dilutions were analyzed by a trapping ELISA against unpurified  
15 C-S8c1 virus captured using a rabbit anti-serotype C FMDV serum (IAH, Pirbright, UK).  
16 Antibodies were detected using an anti-mouse IgG (H+L)-HRP conjugate (BioRad)  
17 antibody or a goat anti-mouse µ-chain-specific-HRP conjugate antibody (Sigma).

##### 18 **2.5.2. Anti-FMDV antibodies isotyping**

19 For isotype determination, sera at a 1/25 dilution were tested in an anti-virus ELISA, like  
20 above, but using a mouse immunoglobulin isotyping kit (BioRad) [14].

##### 21 **2.5.3. Neutralizing antibodies**

1 Sera were analyzed by a plaque-reduction neutralization assay [16] in 3-fold serial  
2 dilutions from 1/20, and titers were expressed as PRN70, except for sera with neutralizing  
3 titers too low for PRN70 calculation; in this case, titers were expressed as plaque reduction  
4 percentage PRP [17] at the lowest serum dilution analyzed (1/20).

## 5 **2.6. Statistical analysis**

6 ANOVA and Bonferroni's post hoc test and the Student's *t*-test were used to compare  
7 data among groups.

## 8 **3. RESULTS**

### 9 **3.1. Effect of RNA inoculation on antibodies elicited after conventional FMD** 10 **vaccination**

11 In a first vaccination experiment, groups of five mice (5 to 7-month-old) were  
12 inoculated with the IRES transcripts either 24 h before, at the same time of, or 24 h after  
13 immunization with a conventional vaccine. A fourth group (n=3) was given the vaccine  
14 alone. Serum samples were collected at early (days 5 and 12) or late (day 56) times  
15 post-vaccination, and the humoral immune response was analyzed by ELISA (Fig. 1)  
16 and seroneutralization (Fig. 2).

17 The mean anti-FMDV antibody titers reached were in all cases higher in the RNA-  
18 inoculated groups, compared to the control group receiving the vaccine alone. This  
19 trend was observed both at early and late times after vaccination, though only  
20 statistically significant differences were found at 56 days after vaccination between the  
21 group co-inoculated with the RNA and the vaccine at the same time and the control  
22 group (Fig. 1).

23 At day 5 after vaccination, high neutralization titers were detected in all the groups, with  
24 statistically significant differences between mice immunized only with the vaccine and

1 those inoculated with the RNA at the same time of vaccination or 24 h later (Fig. 2). At  
2 later times, a decrease in neutralization titers was observed, particularly stronger in the  
3 group receiving the vaccine alone, with undetectable levels of neutralizing antibodies at  
4 day 56 in any of the three vaccinated animals. In the RNA-inoculated groups, a decrease  
5 was also observed at this time, but most of the animals still maintained high neutralizing  
6 antibody titers above the detection limit of the assay; the group co-inoculated with the  
7 RNA and the vaccine showed the highest average values at all the time points assayed.  
8 The results shown in Figs. 1 and 2, revealed a quantitative difference between mice  
9 immunized with the vaccine alone or with the IRES RNA, respectively. With the aim of  
10 investigating qualitative differences in the antibody response associated to RNA  
11 delivery, immunoglobulin isotype profiles were analyzed by ELISA five weeks after  
12 vaccination (Fig. 3). Interestingly, in mice receiving the vaccine alone, IgM was the  
13 predominant isotype, while in the RNA-inoculated groups there was a switch to IgG,  
14 mainly IgG2a. In the group receiving the RNA 24 h before vaccination, high titers of  
15 IgG2a and IgG2b were detected though these animals maintained high titers of IgM.  
16 The mean values of the vaccine control group were always lower for IgG1, IgG2a,  
17 IgG2b and IgG3 than those for the three groups of RNA-inoculated and vaccinated mice  
18 (Fig. 3). Although the differences observed between groups did not reach statistically  
19 significant levels, this qualitative difference in the isotype profile was maintained at  
20 week nine after vaccination (not shown).

### 21 **3.2. Effect of RNA co-inoculation with conventional or inactivated FMD vaccines** 22 **on immunization**

23 A second vaccination experiment was performed including two different vaccine  
24 formulations: the conventional vaccine, formulated as above with an oil adjuvant, and  
25 the inactivated vaccine, used directly with no adjuvant added. In both cases, the IRES

1 RNA was co-inoculated at the same time of vaccination. This approach was based on  
2 the above results showing overall higher antibody titers in the co-inoculated groups  
3 (Figs. 1 and 2), and the obvious inherent technical advantages of co-administration.  
4 Groups of 4-5 mice (8-week-old) were immunized with each vaccine, including or not  
5 RNA inoculation. A group of naive mice was included as a control. Serum samples  
6 were collected 2, 5 and 56 days after vaccination, and serological analyses were carried  
7 out as above. First, the levels of anti-FMDV antibodies were analyzed by ELISA (Fig.  
8 4). Since the induction of very rapid antibody responses was pursued, an ELISA  
9 specific for IgM isotype detection was used for the analysis of sera collected at early  
10 times post-vaccination (Fig. 4A and B), while detection of specific IgGs was tested for  
11 the latest time assayed (Fig. 4C). Total anti-FMDV antibodies at day 2 had already been  
12 detected, with OD readings close to the sensitivity limit of the assay (not shown).  
13 Interestingly, despite of the low titers detected, statistically significant differences could  
14 be found between the mean titers of the RNA-inoculated vaccinated groups and control  
15 mice as early as 2 days post-vaccination with both formulations (Fig. 4A). At day 5  
16 post-vaccination, the titers were higher and more similar among all the groups (Fig.  
17 4B), while 12 days after immunization the specific IgMs had already decreased in all  
18 the groups (not shown). In all cases, the mean anti-FMDV antibody titers were higher  
19 for mice receiving the RNA compared to those immunized with the corresponding  
20 vaccine alone, being these differences higher at day 56 post-vaccination (Fig. 4C).  
21 The neutralizing antibodies profiles in the different groups were similar to those  
22 observed in the first experiment, with high titers at day 5 post-vaccination in all the  
23 groups (Fig. 5B). However, in this case all the vaccinated animals conserved detectable  
24 levels of neutralizing antibodies 56 days after vaccination, though mean titers of the



1 groups that received only the vaccines were significantly lower than those of their  
2 RNA-inoculated counterparts (Fig. 5B).

3 Since the development of a rapid neutralizing response is crucial for FMDV control,  
4 neutralization assays were also performed at day 2 after vaccination; the detected levels  
5 of neutralization at this time point were still too low for PRN70 calculation, most  
6 yielding reduction of infectivity below 70 % at the lowest dilution assayed. Still, in sera  
7 from 3 out of the 5 mice immunized with the inactivated vaccine plus RNA, specific  
8 neutralization was already detected, with a reduction of infectivity above 50% (Fig.  
9 5A).

10 In this second vaccination experiment, no remarkable differences were found in the  
11 average antibody isotype profile between groups receiving or not the RNA with the  
12 vaccines. We failed to detect IgM or IgG3 in any of the groups 8 weeks after  
13 vaccination; however, groups inoculated with the RNA had higher levels of IgG1,  
14 IgG2a and IgG2b (not shown).

15 Together, the above results show the adjuvatory effect of the IRES RNA transcripts  
16 when inoculated in combination with an FMD vaccine in mice.

### 17 **3.3. Effect of RNA co-inoculation with conventional FMD vaccine on viral load** 18 **after FMDV challenge**

19 To determine whether the improvement in the immune response observed in animals  
20 receiving the FMD vaccine plus the RNA correlated with a higher resistance to viral  
21 infection, mice vaccinated with a conventional vaccine and co-inoculated with RNA,  
22 were challenged with  $10^3$  PFU of the homologous virus at different times after  
23 immunization. Groups of mice (n=4; 8-week-old) were challenged 2 days, 5 days, 12  
24 days or 2 months after vaccination. Two control groups were also challenged: one  
25 including RNA-inoculated non-vaccinated mice, and a second group of PBS-inoculated

1 mice. The levels of viremia in serum were analyzed 2 days after viral challenge by a  
2 semi-quantitative RT-PCR assay; at that time point, maximal viral load is consistently  
3 reached under our challenge conditions [14].

4 The viremia in animals vaccinated 2 months prior to viral challenge was also analyzed  
5 as above. In this case, no viral RNA could be detected by RT-PCR in vaccinated mice,  
6 with or without RNA, unlike the RNA- and PBS-inoculated controls (not shown). This  
7 suggests that the induced immunity at this time after a single vaccination, although  
8 lower in terms of neutralizing antibodies in mice receiving the vaccine alone (Fig. 5),  
9 was still good enough to provide protection against late FMDV infection. Fig. 6 shows  
10 the viremia levels corresponding to the groups challenged at early time points post-  
11 vaccination (2, 5 and 12 days). The groups immunized with the conventional vaccine,  
12 with or without RNA, 5 or 12 days before challenge showed a clear reduction in viral  
13 load, when compared to the RNA- or PBS-inoculated groups, the last one rendering the  
14 highest viremia levels (Fig. 6). RNA delivery seemed to improve the results of the  
15 vaccine alone when given 5 and 12 days prior to viral challenge, with lower mean  
16 viremia values.

17 Interestingly, in mice immunized only 2 days before challenge, a clear reduction in viral  
18 load was observed in all the groups, compared to the PBS-inoculated group. This  
19 reduced viremia was also observed in mice inoculated only with RNA (Fig. 6). This  
20 result is in agreement with our previous findings showing the antiviral effect of the  
21 IRES RNA in mice [11, 12]. However, no statistically significant differences were  
22 detected between viral load in the group receiving the vaccine and the RNA at day 2  
23 pre-challenge, compared to the groups receiving the vaccine or the RNA alone,  
24 respectively. This correlates with the levels of neutralizing antibodies detected in the

1 corresponding groups at day 2 after immunization with the conventional vaccine (Fig.  
2 5A).

3

#### 4 **4. DISCUSSION**

5 During infection, FMDV is able to overcome the initial host innate response at different  
6 levels [18-21]. However, FMDV is very sensitive to IFN- $\alpha/\beta/\gamma$  [22, 23], and treatment  
7 with IFN has proved to be an efficient biotherapeutic approach against FMDV,  
8 contributing to shorten the susceptibility window after FMD vaccination and inducing a  
9 more robust and long-lasting adaptive immune response [24-26]. Expression of type-I, -  
10 II and -III IFNs with replication-defective human adenovirus vectors has been  
11 extensively used with good but differential results depending on the host species  
12 assayed [27-30]. The use of baculovirus as an immunopotentiator adjuvant in  
13 combination with an FMDV vaccine has been recently reported to elicit very early  
14 protection against the virus without affecting long lasting immunity in mice [31].  
15 Endogenous or therapeutically induced early type-I IFN responses may confer  
16 protection until adaptive immunity is activated to an extent that the pathogen can be  
17 eliminated [6]. In that context, PRRs come into sight as targets of new vaccine  
18 adjuvants beside their role as sentinels in innate immunity [5, 7, 32].

19 In previous work, we have shown the ability of synthetic RNA transcripts mimicking  
20 the FMDV IRES to induce the highest levels of resistance to FMDV and WNV  
21 infections in mice among all the different RNAs tested, including polyI:C [11, 12]. The  
22 strong innate responses induced by the IRES transcripts led us to test its adjuvatory  
23 effect in the adaptive immune response elicited upon FMD vaccination.

24 It is well documented the correlation between neutralizing antibodies and protection  
25 against FMDV infection in swine, ruminants and mice [33, 34]. In the groups receiving

1 the vaccine plus the RNA, higher levels of specific neutralizing antibodies were  
2 maintained up to 56 days, unlike the control group immunized with the corresponding  
3 vaccine alone. Taken together, our results suggest that RNA inoculation can enhance the  
4 level and duration of specific antibodies in mice and therefore, promote long-term  
5 protection after a single vaccination. The use of the IRES RNA in combination with the  
6 inactivated virus yield similar or better results in terms of antibody levels, specially at  
7 early times post-vaccination, than the conventionally adjuvated vaccine, thus potentially  
8 reducing the susceptibility window after FMD vaccination.

9 In addition to the quantitative effect observed on antibody response when the FMD  
10 vaccination was supplemented with the IRES RNA, a switch from IgM to IgG isotype  
11 was observed five weeks after vaccination in the first experiment. However, in the  
12 second experiment no detectable levels of IgM could be found in any of the groups  
13 eight weeks after vaccination with or without RNA (data not shown). The difference in  
14 age of the mice used for both experiments might have some influence on that result,  
15 though additional experiments would be necessary to confirm that hypothesis. Previous  
16 reports suggest that the isotype profile elicited, besides the level of neutralizing  
17 antibodies, could be important in terms of the efficacy of vaccine formulation. A  
18 relevant role for the IgG isotypes profile elicited in response to viral infection or  
19 immunization has been proposed [35]. In mice vaccinated with formulations including  
20 immunomodulators, persisting high levels of IgG2b, similar to infected animals, were  
21 detected for longer times post-vaccination, compared to conventional vaccines. Elevated  
22 levels of IgG2a and IgG2b have also been reported using a microparticle adjuvant with  
23 an inactivated vaccine in mice; the different isotype profile induced by this vaccine  
24 indicated a Th1/Th2 response [36]. Though further studies are required to clarify the  
25 role of the IRES RNA on modulation of the immune profile induced after vaccination,

1 the use of these molecules as adjuvants might provide an additional value for new FMD  
2 vaccine design.

3 When conventionally vaccinated mice, plus RNA or not, were challenged with FMDV,  
4 a reduced viremia was detected in all groups. A similar result was observed for animals  
5 inoculated only with RNA. However, higher mean viremia levels were found in the  
6 groups immunized with the vaccine alone 5 and 12 days prior to challenge, though with  
7 non-statistically significant compared to groups receiving the RNA as well. A putative  
8 antiviral effect of the adjuvant included in the conventional vaccine can not be ruled  
9 out; this would account for the low viremia levels detected in the group challenged two  
10 days after receiving the vaccine alone.

11 Together, our results show the enhancer effect of the IRES RNA on the immune  
12 response elicited after FMD vaccination in mice. Despite the differences in FMDV  
13 infection and disease between mice and natural hosts, relevant similarities in their  
14 immune responses have been shown [34, 37, 38]. The use of PAMP agonists as  
15 adjuvants in farm animals, besides increasing the magnitude of the immune response,  
16 might reduce the required time lapse between vaccination and protection; this would be  
17 particularly useful in reactive vaccination in case of FMD outbreaks [32]. Our results in  
18 the mice model suggest the potential use of these small synthetic and non-infectious  
19 RNA molecules as adjuvants for new FMD vaccines strategies.

20

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16

## 17 **FIGURE LEGENDS**

18 **Figure 1.** Total anti-FMDV antibodies (in 5 to 7-month-old mice) elicited after  
19 vaccination with a conventional vaccine alone or with RNA delivery 24 h prior to  
20 vaccination (-24 h), co-inoculated at the same time (0 h) or 24 h after vaccination (+24  
21 h). Sera were collected at the indicated time points after vaccination. Data correspond to  
22 the ELISA titers of individual mice in each group. Mean titers are indicated with bars.  
23 Significant differences between groups using the Student's *t*-test are indicated.

24

1 **Figure 2.** Neutralizing antibody titers elicited after immunization with a conventional  
2 vaccine alone or with RNA delivery 24 h prior to vaccination (-24 h), co-inoculated at  
3 the same time (0 h) or 24 h after vaccination (+24 h). Sera were collected at the  
4 indicated time points after vaccination. Data correspond to PRN70 ( $\log_{10}$ ) titers of  
5 individual mice in each group. Dotted line indicates assay limit of detection; sera below  
6 that limit were arbitrarily assigned a titer of 0.5 in the graph. Mean titers of groups with  
7 all animals giving values above the detection limit are indicated with bars. Significant  
8 differences between groups after statistical analysis using ANOVA and Bonferroni's  
9 post hoc test are indicated.

10

11 **Figure 3.** Isotype profiles of anti-FMDV antibodies in mice after immunization with a  
12 conventional vaccine alone or with RNA delivery 24 h prior to vaccination (-24 h), co-  
13 inoculated at the same time (0 h) or 24 h after vaccination (+24 h). Sera were collected  
14 35 days after vaccination, and 1/25 dilutions were assayed for IgM (A), IgG1 (B),  
15 IgG2a (C), IgG2b (D) or IgG3 (E) detection, respectively. OD readings were corrected  
16 by blank wells. Data correspond to the ELISA titers of individual mice in each group.  
17 Mean titers are indicated with bars.

18

19 **Figure 4.** Anti-FMDV antibody levels (in 2-month-old mice) after immunization with a  
20 conventional or an inactivated vaccine either alone or with RNA co-inoculation. Data  
21 correspond to the antibody titers of sera from individual mice in each group. Mean titers  
22 are indicated with bars. A and B show the titers expressed as OD readings at 450 nm in  
23 a specific IgM-ELISA of sera samples collected at day 2 after vaccination and assayed  
24 at a 1/20 dilution (A) or at day 5 after vaccination and assayed at a 1/50 dilution (B).  
25 OD readings were corrected by blank wells. Sera from naive animals were included as

1 negative controls. Panel C shows the total anti-FMDV antibody titers by ELISA ( $\log_{10}$ )  
2 of sera collected at day 56 after vaccination. Significant differences between groups  
3 after statistical analysis using ANOVA and Bonferroni's post hoc test are indicated.  
4 Asterisk denotes p values using the Student's *t*-test.

5

6 **Figure 5.** Induction of neutralizing antibodies in mice after immunization with a  
7 conventional vaccine or an inactivated vaccine either alone or with RNA co-inoculation.  
8 Data correspond to the antibody titers of sera from individual mice in each group. Mean  
9 titers are indicated with bars. A, sera collected at day 2 postvaccination were assayed at  
10 a dilution of 1/20. Titers are expressed as plaque reduction percentage (PRP). In B,  
11 neutralization titers of sera collected at days 5 or 56 days after vaccination were  
12 expressed as PRN 70 ( $\log_{10}$ ). Dotted line indicates assay limit of detection. Sera from  
13 naive animals were included as negative controls and were arbitrarily assigned a titer of  
14 0.5 in the graph. Significant differences between groups after statistical analysis using  
15 the Student's *t*-test are indicated. Asterisk denotes significant differences between that  
16 group and the negative control group ( $p < 0.0001$ ).

17

18 **Figure 6.** Viral load after vaccination and viral challenge. Groups of mice were  
19 vaccinated with a conventional vaccine either alone or with RNA co-inoculation,  
20 inoculated with RNA or with PBS. Then, mice were challenged with  $10^3$  PFU of FMDV  
21 at 2, 5 or 12 days after vaccination. Sera were collected from mice 2 days after viral  
22 challenge and viremia levels were determined by RT-PCR and expressed as relative  
23 values to PBS-inoculated animals after gel densitometry. Data correspond to the viremia  
24 titers of sera from individual mice in each group. Mean titers are indicated with bars.

- 1 Significant differences between groups after statistical analysis using ANOVA and
- 2 Bonferroni's post hoc test are indicated.

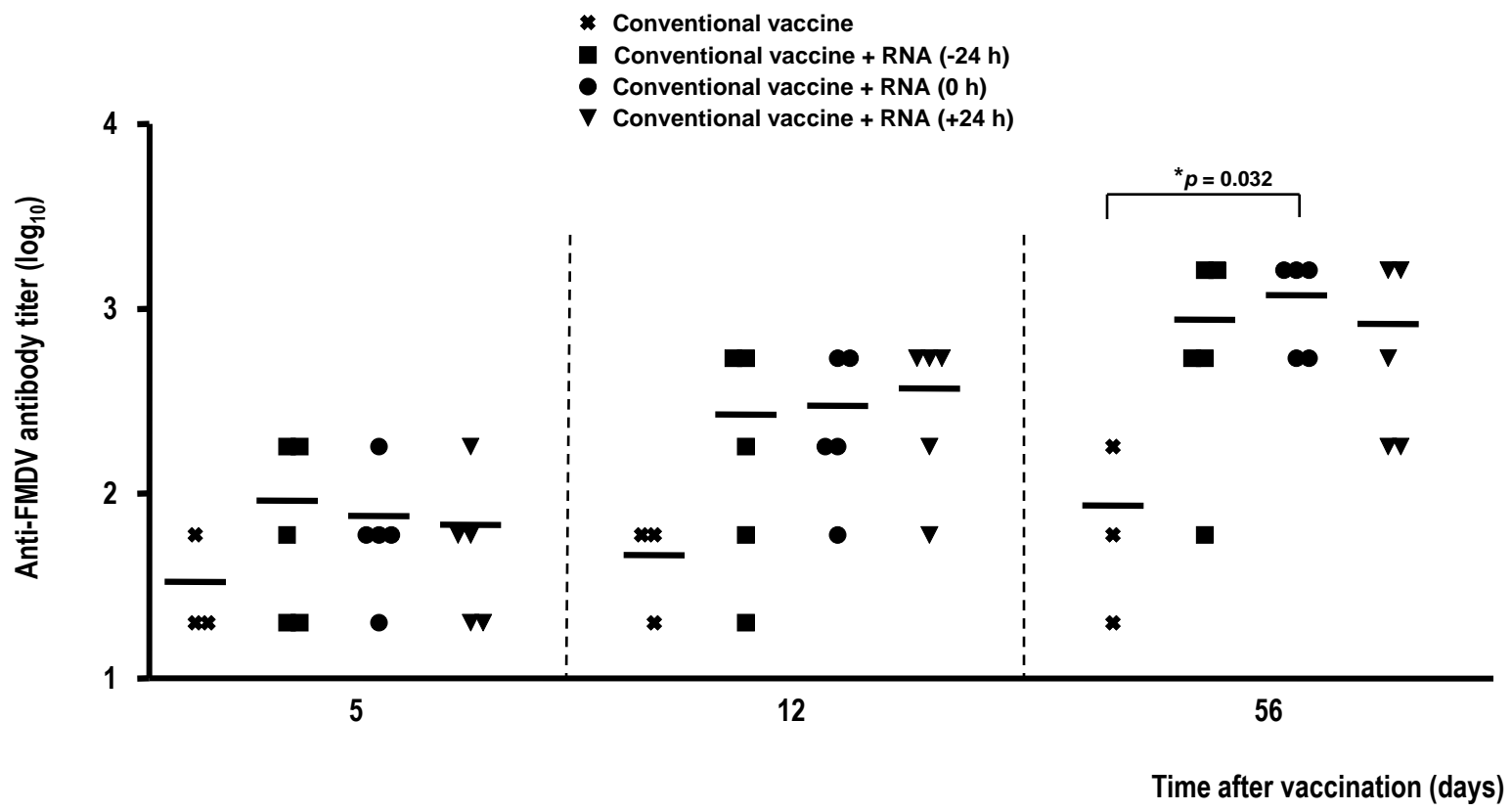


Figure 1

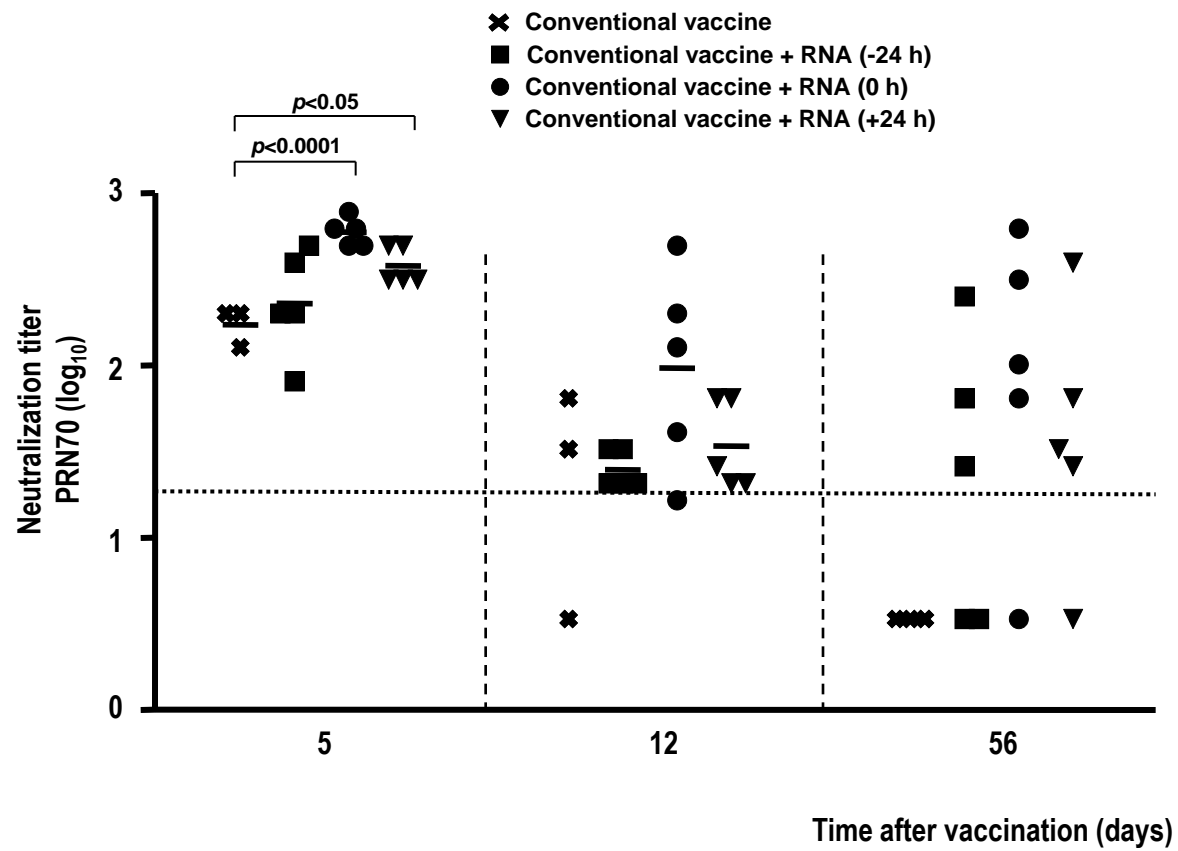


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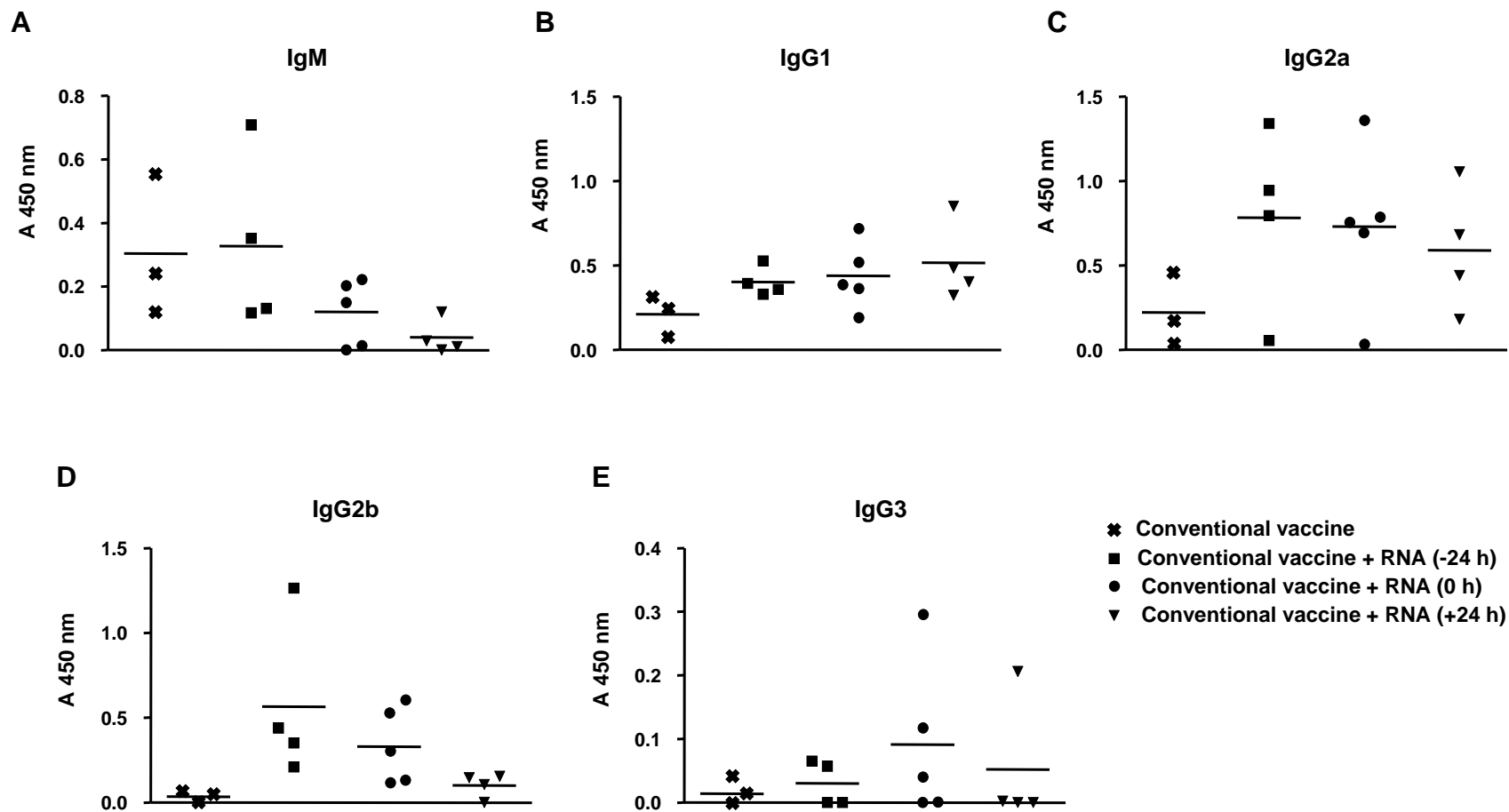
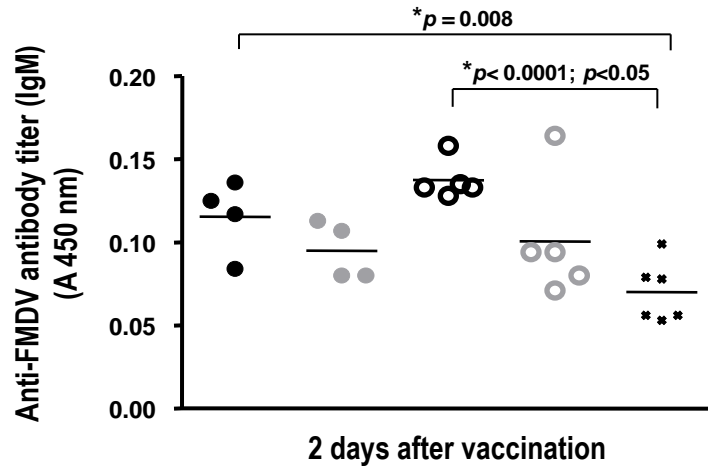


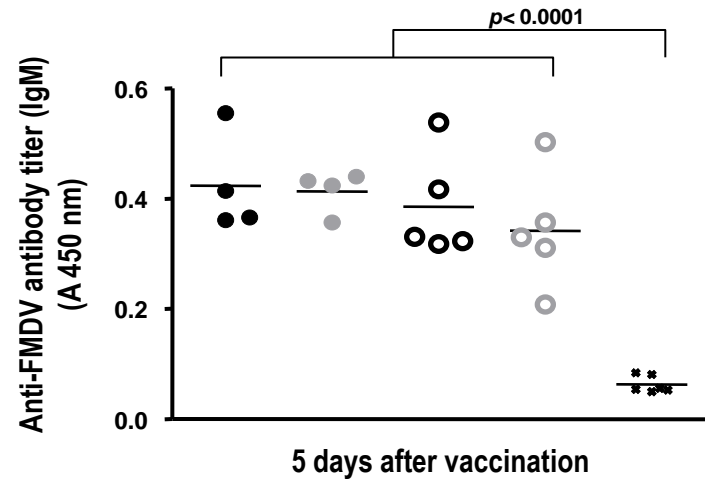
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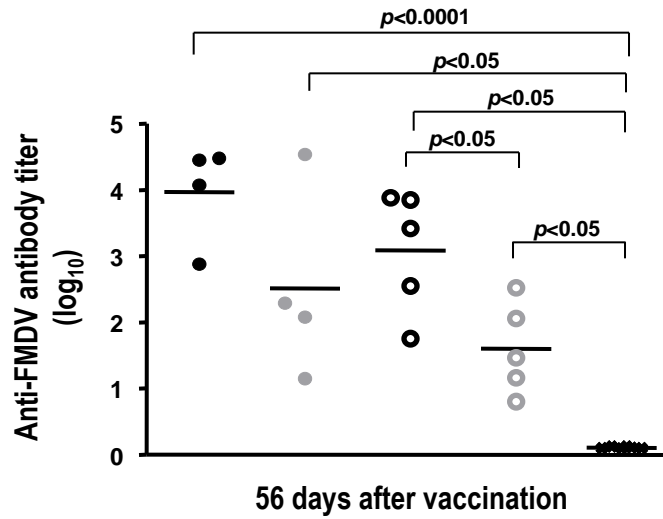
A



B



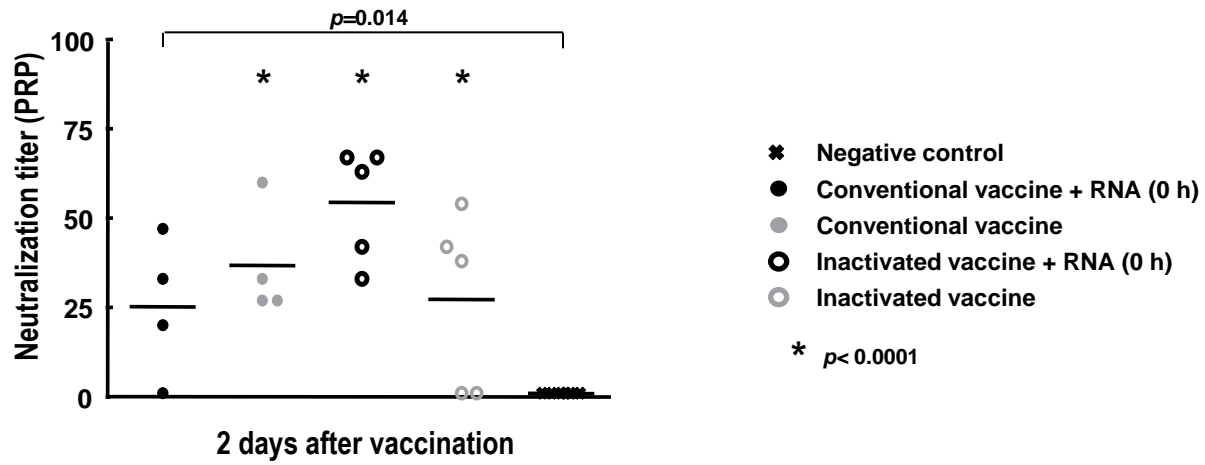
C



- \* Negative control
- Conventional vaccine + RNA (0 h)
- Conventional vaccine
- Inactivated vaccine + RNA (0 h)
- Inactivated vaccine

Figure 4

**A**



**B**

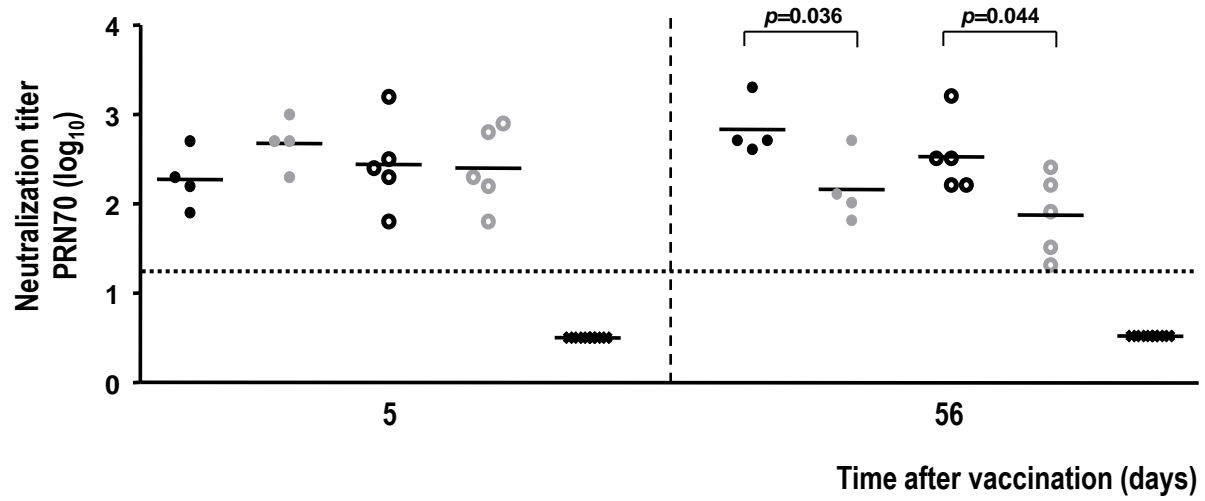


Figure 5

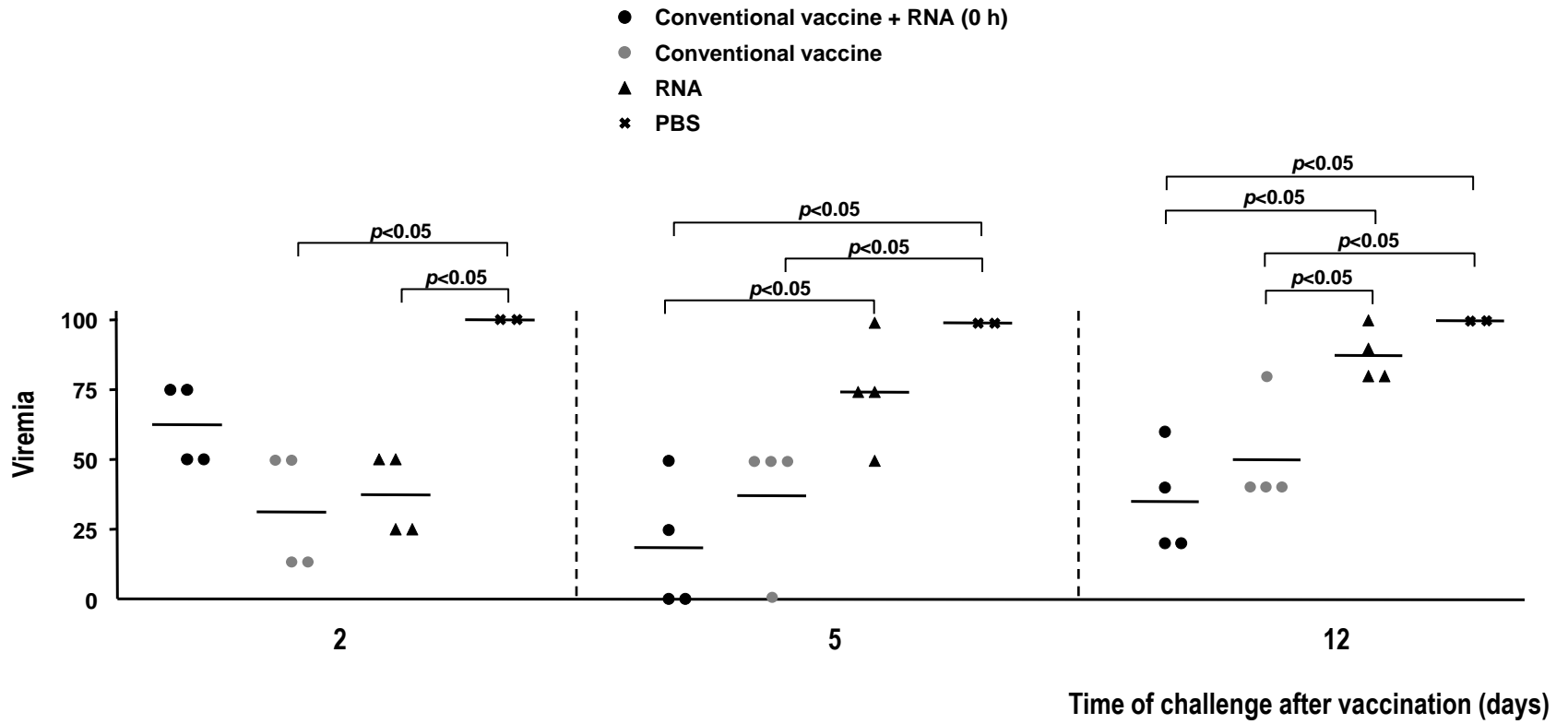


Figure 6