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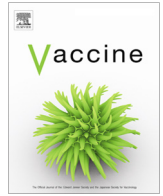
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## A canine adenovirus type 2 vaccine vector confers protection against foot-and-mouth disease in guinea pigs

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### ABSTRACT

Vaccination is a key element in the control of foot-and-mouth disease (FMD). The majority of the antigenic sites that induce protective immune responses are localized on the FMD virus (FMDV) capsid that is formed by four virus-encoded structural proteins, VP1 to VP4. In the present study, recombinant canine adenovirus type 2 (CAV2)-based FMD vaccines, Cav-P1/3C R<sup>o</sup> and Cav-VP1 R<sup>o</sup>, respectively expressing the structural P1 precursor protein along with the non-structural 3C protein or expressing the structural VP1 protein of the FMDV strain O/FRA/1/2001, were evaluated as novel vaccines against FMD. A strong humoral immune response was elicited in guinea pigs (GP) following immunization with Cav-P1/3C R<sup>o</sup>, while administration of Cav-VP1 R<sup>o</sup> did not induce a satisfying antibody response in GP or mice. GP were then used as an experimental model for the determination of the protection afforded by the Cav-P1/3C R<sup>o</sup> vaccine against challenge with the FMDV strain O<sub>1</sub> Manisa/Turkey/1969. The Cav-P1/3C R<sup>o</sup> vaccine protected GP from generalized FMD to a similar extent as a high potency double-oil emulsion O<sub>1</sub> Manisa vaccine. The results of the present study show that CAV2-based vector vaccines can express immunogenic FMDV antigens and offer protection against generalized FMD in GP. This suggests that Cav-P1/3C R<sup>o</sup> FMDV vaccine may protect natural host species from FMD. In combination with an appropriate diagnostic test, the Cav-P1/3C R<sup>o</sup> FMDV vaccine may also serve as a marker vaccine to differentiate vaccinated from infected animals.

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### 1. Introduction

Foot-and-mouth disease (FMD) is one of the most important infectious diseases of cloven-hoofed livestock and wildlife. The FMD virus (FMDV) belongs to the genus Aphthovirus within the Picornaviridae family. To date, seven different serotypes (O, A, C, Asia 1, (South African territories) SAT1, SAT2 and SAT3) and multiple antigenic subtypes have been identified and new subtypes arise frequently [1]. Control and eradication of FMDV by use of inactivated vaccines has been successful in Europe, South America and parts of Africa and Asia [2,3]. However, shortcomings of classical inactivated vaccines including serotype-dependency, limited anti-

genic matching between vaccine and outbreak strains, short-term protection after vaccination, antigen instability particularly after vaccine formulation, and a high production cost resulting from the high-containment facilities required for the production of live virus thrive the search for novel vaccine strategies [4]. An alternative vaccine approach relies on the use of recombinant viruses to express FMDV antigens. Adenovirus-based vectors (AdV) represent a promising antigen delivery system. A single parenteral inoculation of a live but replication-defective human adenovirus engineered to express FMDV capsomers induced protection against FMD in pigs and cattle at 7 days post vaccination (dpv) [5,6]. Because veterinary vaccines based on human AdV may for safety reasons be less suitable for mass-vaccination campaigns in food producing animals, non-human adenovirus-based vector vaccines are proposed as an appropriate alternative [7]. Recombinant canine adenovirus type 2 (CAV2) vectors are well characterized and have significant potential for vaccine purposes [8,9].

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Here, non-replicative CAV2 vectors expressing either the FMDV capsid polyprotein precursor P1 along with the non-structural 3C protein for its cleavage (Cav-P1/3C R<sup>o</sup>) or the structural VP1 protein (Cav-VP1 R<sup>o</sup>) were developed and evaluated *in vivo*.

## 2. Materials and methods

### 2.1. CAV2 recombinant FMDV vaccines and cells

Two CAV2 vectors expressing the FMDV capsid polyprotein precursor P1 and the 3C protein for its cleavage (Cav-P1/3C R<sup>o</sup>) or expressing the VP1 capsid subunit protein (Cav-VP1 R<sup>o</sup>) using coding sequences of FMDV strain O/FRA/1/2001 (GenBank AJ633821) were developed as previously described [9]. In addition, isogenic vectors expressing either the glycoprotein of rabies virus (Cav-G R<sup>o</sup>) [10] or the non-structural protein-1 of bluetongue virus (Cav-NS1 R<sup>o</sup>) [11] were used. All CAV2 vectors were amplified and titrated in dog kidney cells expressing the E1 region of canine adenovirus type 2 (DK-E1) cells [12].

### 2.2. Immunization of mice

In the first experiment, six-week-old CD1 female mice were randomly assigned to 3 groups. Two groups (n = 3) were immunized with either Cav-P1/3C R<sup>o</sup> or Cav-NS1 R<sup>o</sup>. Animals received intramuscularly (IM) a single dose of  $2 \times 10^8$  TCID<sub>50</sub> of CAV2-based vaccines in 50 µl of PBS. One unvaccinated mouse served as control. Blood was collected via the retro-orbital sinus at 2 and 4 weeks after vaccination (wvpv).

In the second experiment, seven-week-old C57BL/6 female mice were randomly assigned to 5 groups. Groups 1 to 3 (n = 4) received IM  $2 \times 10^8$  TCID<sub>50</sub> of Cav-P1/3C R<sup>o</sup>, Cav-VP1 R<sup>o</sup> or Cav-NS1 R<sup>o</sup> vectors, respectively. Four mice in group 4 were inoculated, by the intraperitoneal route, with 2 µg of inactivated O<sub>1</sub> Manisa FMDV antigen (Merial) formulated with an oil adjuvant (Montanide ISA 50 V2, Seppic). All inoculated animals were booster-vaccinated with the same dose 3 weeks later. Two unimmunized mice served as controls (group 5). Blood was collected before each vaccination and 3 weeks after the booster vaccination.

### 2.3. Antibody responses in mice

Antibody responses against FMDV were assessed by a validated in-house indirect ELISA performed on PrioCHECK FMDV type O kit plates (Prionics AG) with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Millipore) as secondary antibodies.

Antibodies against the CAV2 vectors were detected by ELISA as described previously [8]. Western Blot analysis was performed by resolving lysates from Cav-FMDV construct-transduced CHO-CAR cells in SDS-PAGE 4–12% Bis-Tris gels (Invitrogen). Proteins were transferred from the gel onto 0.45 µm nitrocellulose membranes. Proteins were immunoblotted by sequentially applying the serum samples from immunized animals and alkaline phosphatase-conjugated goat anti-mouse IgG (Promega).

### 2.4. Vaccination of guinea pigs

Outbred male Dunkin Hartley guinea pigs (GP) (350–450 g) were used. In the first experiment, 9 GP were randomly assigned to 3 groups (n = 3). Groups 1 and 2 were individually IM inoculated with  $1 \times 10^9$  TCID<sub>50</sub> of Cav-P1/3C R<sup>o</sup> or Cav-VP1 R<sup>o</sup> constructs. Group 3 was inoculated IM with 2 µg of inactivated O<sub>1</sub> Manisa FMDV antigen (Merial) formulated with an oil adjuvant (Montanide ISA 50 V2, Seppic). All animals were immunized with the same dose 3 weeks later. GP were sedated with Xylazine (20 mg/

kg) and blood was collected from the jugular vein before each injection and 3 weeks after the second injection. Sera were examined in the in-house indirect ELISA performed on PrioCHECK FMDV type O kit plates (Prionics AG) as described above and in a virus neutralization (VN) assay. In brief, serial dilutions of serum were incubated with 100 TCID<sub>50</sub> of FMDV O at 37 °C for 1 h in 96-well plate. Then,  $4 \times 10^4$  IBRS-2 cells/well were added and incubated at 37 °C for 3–4 days. The anti-VP1 MAb D9 and FMDV negative sera were used as positive and negative controls, respectively. Appearance of CPE was used to determine the end-point titers, expressed as the reciprocal of the highest dilution that neutralized 100 TCID<sub>50</sub> of FMDV type O.

### 2.5. Vaccination and challenge of guinea pigs

Four groups (n = 4) were vaccinated IM twice with a three-week interval. Per vaccination, group 4 received  $1 \times 10^9$  TCID<sub>50</sub> of Cav-P1/3C R<sup>o</sup>, group 5 received  $1 \times 10^9$  TCID<sub>50</sub> of Cav-G R<sup>o</sup>, group 6 received a full cattle dose (2 ml) of a high potency (>6 PD<sub>50</sub>) double-oil-emulsion (DOE) vaccine containing the inactivated FMDV strain O<sub>1</sub> Manisa/Turkey/1969 (O<sub>1</sub> Manisa) (MSD Animal Health – Intervet, Köln, Germany) and group 7 was left unvaccinated. Six weeks after the second vaccination, all GP were inoculated in the right hind footpad with 100 50% GP infectious doses of the GP-adapted O<sub>1</sub> Manisa strain and monitored daily as described previously [13]. Samples for analysis of the viral RNA load were collected at 2 days after virus inoculation (dpi) (serum) and at 4 dpi when all animals were euthanized (serum and internal organs). The nucleic acids were extracted with the Nucleospin RNA virus columns (Macherey–Nagel, Duren, Germany) according to the manufacturer's instructions. One-step real-time RT-PCR for the FMDV RNA-dependent RNA-polymerase (FMDV 3D gene) was performed as adapted from the Ref. [14]. The crossing-point (Cp) value refers to the cycle that is used to estimate the quantitative value of the RT-qPCR. Cp-values <40 were considered positive. Blood for serological examination was collected at the time of viral challenge. Sera were examined in an indirect ELISA [13].

Mice originated from Charles River Laboratories and GP from Harlan laboratories. The animal experiments were approved and supervised by the Ethical Committees of the CODA-CERVA (reference number 20130628-01) and ANSES/ENVA/UPEC (reference number 20/12/12-25B).

### 2.6. Antibody responses in serum of guinea pigs

Antibodies against FMDV were also quantified by a Luminex-based immunoassay by using a Bio-Plex 200<sup>®</sup> system (Bio-Rad). Briefly, a 1/50 dilution of serum was incubated in 96-well plates with VP1-coupled microspheres (2500 beads/well) at room temperature for 90 min under agitation. After 3 washes with 0.02% PBS using a vacuum manifold, 50 µl volumes of a 1/200 dilution of a rabbit biotinylated anti-GP IgG (Abcam) were dispensed per well and incubated for 45 min at room temperature with agitation. Beads were then washed and incubated for 15 min with 50 µl of a 1/100 dilution of streptavidin-conjugated phycoerythrin (S-PE, Qiagen). After washing to remove the unbound S-PE, beads were analyzed in the Bio-Plex 200<sup>®</sup> system, which monitored the spectral properties of the beads while simultaneously measuring the amount of fluorescence associated with PE. Data were analyzed using Bio-Plex Manager software version 5.0.

Antibodies against the CAV2 vector were detected by ELISA [8]. Briefly, 96-well Maxisorp plates (Nunc) were coated with 10<sup>5</sup> CAV2 particles in PBS overnight at 4 °C. After a blocking step in PBS-3% Skim Milk, 100 µl of 1/100 dilutions of GP sera, prepared in PBS with 3% Skim Milk, were used with 100 µl of a 1/2500 dilution of peroxidase-conjugated rabbit anti-sheep IgG (DakoCytomation).

Wash buffer was PBS-0.1% Tween 20. Assays were performed in duplicate for individual sera at days 0 and 21, and results expressed as means.

### 2.7. Statistical analysis

Antibody titers and viral RNA levels were compared by analysis of variance. A value of  $P \leq 0.05$  was considered as the level of significance.

## 3. Results

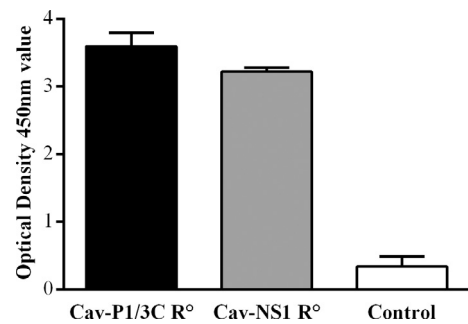
### 3.1. Serological responses after immunization of mice

In the first experiment, at 2 and 4 wpv none of the CD1 mice had antibodies against FMDV at levels detectable by ELISA. However, antibodies in sera of the mice immunized with the Cav-P1/3C R<sup>o</sup> construct reacted with an FMDV protein resolved by SDS-PAGE. Based on its molecular weight, the protein could be identified as VP1. As expected, the VP1-like band was not observed using sera from Cav-NS1 R<sup>o</sup>-immunized mice (Fig. 1). At 4 wpv Cav-P1/3C R<sup>o</sup> and Cav-NS1 R<sup>o</sup> elicited comparable antibody response against the CAV2 vector (Fig. 2).

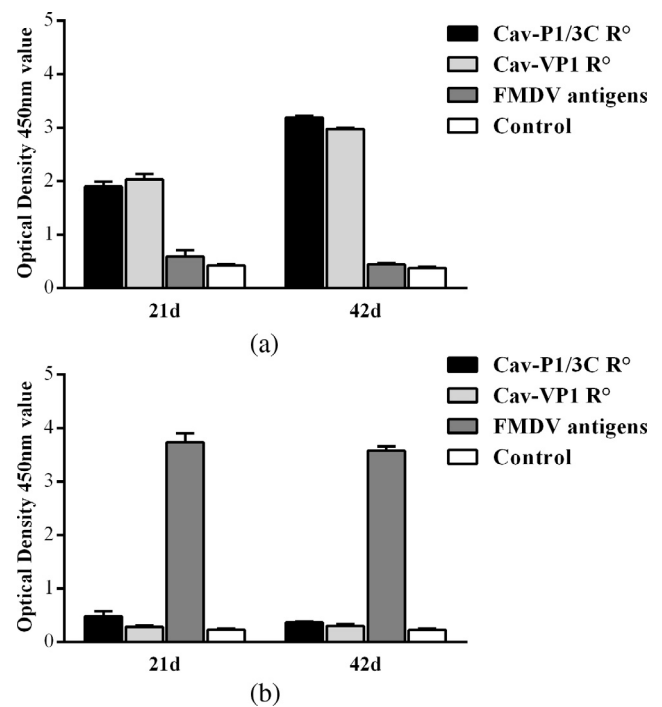
In the second experiment, a strong antibody response against FMDV was observed in C57BL/6 mice immunized with inactivated FMDV antigens from 14 dpv onwards, without apparent booster effect. Despite two immunizations, antibodies against FMDV could not be detected by ELISA in mice immunized with Cav-P1/3C R<sup>o</sup> or Cav-VP1 R<sup>o</sup> (Fig. 3A), whereas antibodies against the CAV2 vectors were detected at 21 dpv with a booster effect observed at day 42 pi. Antibodies against CAV2 were not detected in sera from unimmunized mice or mice immunized with inactivated FMDV antigens (Fig. 3B).

### 3.2. Vaccination of guinea pigs

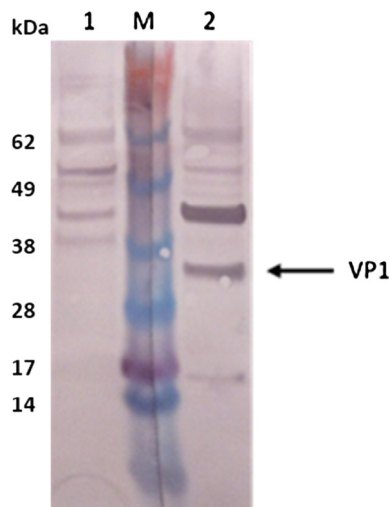
Both CAV2-vectored vaccines induced a strong antibody response against CAV2 (>2 OD values) (Fig. 4A), whereas expectedly inactivated FMDV antigens (group 3) did not. Detectable



**Fig. 2.** CAV2-specific humoral response in immunized CD1 mice. Antibodies against CAV2 were detected by ELISA using 1/100 dilutions of sera from CD1 mice ( $n = 3$ ) at 4 weeks after immunization. Data were presented as mean  $\pm$  standard deviation.



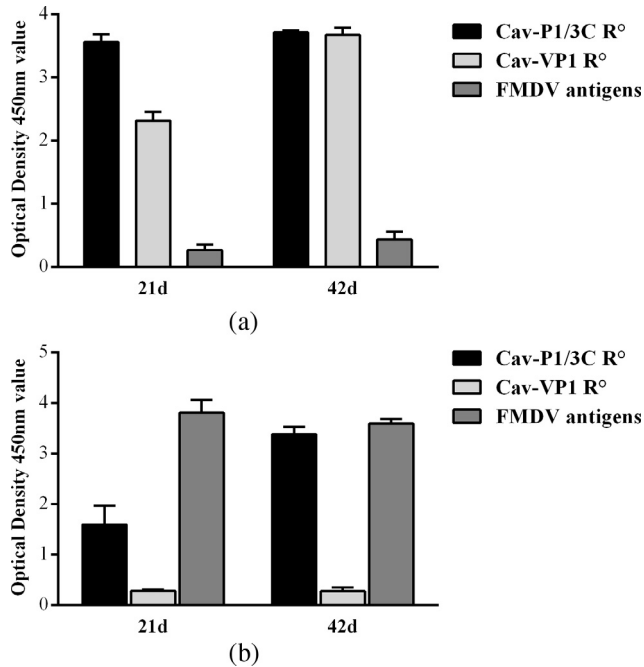
**Fig. 3.** Immunogenicity of Cav-P1/3C R<sup>o</sup> and Cav-VP1 R<sup>o</sup> in C57BL/6 mice. Humoral immune responses against FMDV were assessed by ELISA on immobilized FMDV antigens (PrioCHECK FMDV type O kit plate), 21 and 42 days after initial immunization. Results obtained for 1/50 dilutions of sera are shown (A). Humoral responses elicited against the CAV2 vector were determined by ELISA on immobilized CAV2 particles. Results obtained for 1/50 dilutions of sera are presented (B). Mice immunized with FMDV proteins and naive mice were used as positive and negative controls, respectively. A booster immunization was administered 2 weeks after the initial administration.



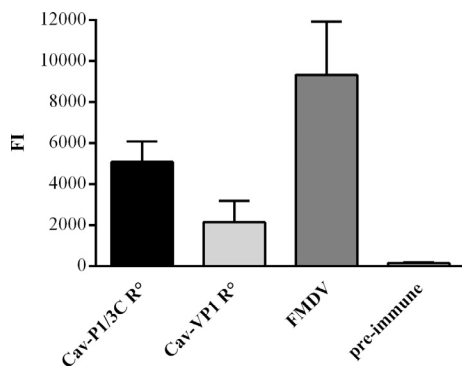
**Fig. 1.** Immunoblotting of FMDV proteins using sera from CD1 mice immunized with CAV2 vectors. CD1 mice were immunized with either Cav-P1/3C R<sup>o</sup> or Cav-NS1 R<sup>o</sup> by the intramuscular route. Four weeks after immunization, blood samples were collected and sera were prepared. Sera from mice immunized with Cav-NS1 R<sup>o</sup> (1) or Cav-P1/3C R<sup>o</sup> (2) were used to stain FMDV proteins on a nitrocellulose membrane. Lane M is the SeeBlue™ Plus2 pre-stained protein standard (Invitrogen). Molecular weight markers (kDa) are indicated in the left margin.

levels of antibodies against FMDV were induced after the first vaccination with Cav-P1/3C R<sup>o</sup> (group 1) or inactivated FMDV antigens (group 3). The antibody response elicited by Cav-P1/3C R<sup>o</sup> after the first vaccination was lower compared to the inactivated FMDV antigens, but this response increased following booster vaccination at day 21. At day 42, the antibody levels induced by Cav-P1/3C R<sup>o</sup> and inactivated FMDV antigens were comparable. In contrast, the Cav-VP1 R<sup>o</sup> vaccine (group 2) failed to induce detectable levels of antibodies, even after two vaccinations (Fig. 4B).

A humoral immune response specific for the FMDV VP1 protein was demonstrated in a Luminex-based immunoassay, using serotype O VP1 protein-conjugated beads. All vaccinated animals produced detectable levels of antibodies against the VP1 protein (Fig. 5). VN antibodies were not detectable.



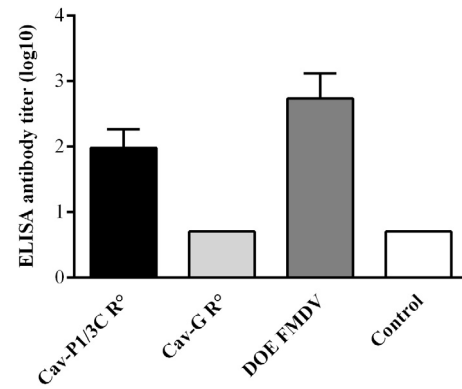
**Fig. 4.** Immunogenicity of Cav-P1/3C R° and Cav-VP1 R° in guinea pigs. Humoral responses elicited against the CAV2 vector were determined by ELISA on immobilized CAV2 particles. Results obtained for 1/100 dilutions of sera are presented (A). Humoral responses against FMDV were assessed by ELISA 21 and 42 days after initial immunization. Results obtained for 1/100 dilutions of sera are shown (B). Animals immunized with inactivated FMDV antigens were used as positive controls. A booster immunization was administered 3 weeks after the initial vaccination.



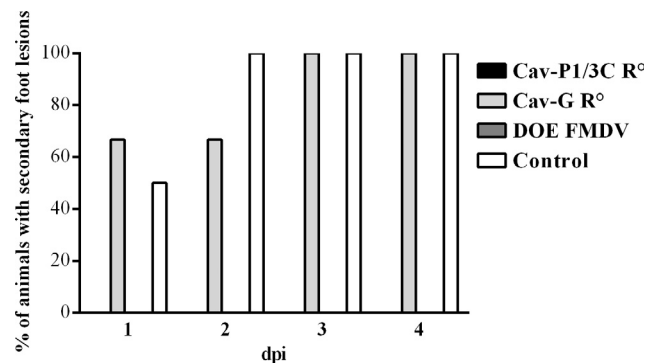
**Fig. 5.** Luminex-based immunoassay to detect immune response against FMDV VP1 protein. Mean fluorescent intensities of diluted guinea pig sera (1/50) were recorded. Error bars indicate the standard deviation from the mean.

### 3.3. Vaccination of guinea pigs and protection against viral challenge

At the time of viral challenge all Cav-P1/3C R° vaccinated animals (group 4) and all O<sub>1</sub> Manisa DOE vaccinated animals (group 6) had antibodies against O<sub>1</sub> Manisa with group mean ELISA titers of  $2.0 \pm 0.3 \log_{10}$  and  $2.7 \pm 0.4 \log_{10}$ , respectively (Fig. 6), whereas all Cav-G R° vaccinated animals (group 5) and all unvaccinated animals (group 7) remained seronegative. All unvaccinated animals and all Cav-G R° vaccinated animals developed a vesicular lesion at the inoculation site in the right hind footpad from 1 dpi onwards (mean score of primary lesion at 4 dpi  $\pm$  standard deviation  $2.7 \pm 0.5$ ) and at 3 dpi all of these animals showed secondary vesicular lesions at the left hind footpad and redness, swelling or scaling at one or both front feet (mean score of secondary lesions



**Fig. 6.** Antibody titers in ELISA in vaccinated and unvaccinated guinea pigs before virus inoculation.



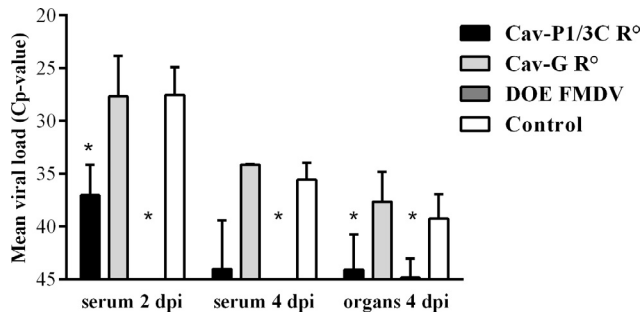
**Fig. 7.** Percentage of GP with secondary foot lesions after inoculation with O<sub>1</sub> Manisa.

4 dpi  $\pm$  standard deviation  $1.4 \pm 0.7$ ). All Cav-P1/3C R° vaccinated animals developed a lesion at the inoculation site (mean score of primary lesion 4 dpi  $\pm$  standard deviation  $1.6 \pm 0.7$ ), but these lesions were less severe than in the unvaccinated and the Cav-G R°-vaccinated groups and none of the Cav-P1/3C R° vaccinated animals showed secondary lesions. Similarly, one O<sub>1</sub> Manisa DOE vaccinated animal developed a lesion at the inoculation site (mean score of primary lesion 4 dpi  $\pm$  standard deviation  $0.25 \pm 0.5$ ) but none of these animals developed secondary lesions (Fig. 7). Mouth lesions, weight loss or depression was not observed in any of the 4 experimental groups.

All unvaccinated animals and all Cav-G R° vaccinated animals had high levels of viral RNA in their serum at 2 dpi and moderate to low levels in their serum and internal organs at 4 dpi. FMD viral RNA was detected in all serum samples collected at 2 dpi from the Cav-P1/3C R° vaccinated animals but the mean viral RNA levels were significantly reduced compared to unvaccinated and Cav-G R° vaccinated animals ( $P < 0.01$ ). FMD viral RNA was not detected in the serum of O<sub>1</sub> Manisa DOE vaccinated animals at 2 dpi (Fig. 8).

At 4 dpi, a low level of viral RNA was detected in the serum and internal organs of one of the Cav-P1/3C R° vaccinated animals, with significantly reduced group mean viral RNA levels compared to unvaccinated animals or Cav-G R° vaccinated animals ( $P < 0.01$ ). Similarly, a low level of viral RNA was detected in the lungs of one of the O<sub>1</sub> Manisa DOE vaccinated animals (the same animal that developed a lesion at the inoculation site) with group mean viral RNA levels comparable to the Cav-P1/3C R° vaccinated animals ( $P > 0.05$ ) (Fig. 8).





**Fig. 8.** Viral RNA detection in serum and internal organs of vaccinated and unvaccinated guinea pigs after inoculation with O1 Manisa. \* Significant difference compared to O1 Manisa control group ( $P \leq 0.05$ ); dpi: days after virus inoculation.

#### 4. Discussion

Vaccination is an indispensable tool in the fight against FMD. Due to limitations of inactivated vaccines, a new generation of engineered vaccines is under evaluation. Among these, viral vector delivery systems have been tested against FMDV. Several studies have reported that the FMDV P1 precursor protein, in association with the 3C or 3CD protein, could generate virus-like particles (VLP) when expressed by different systems such as baculovirus [15], vaccinia virus [16], fowlpox virus [17] or adenovirus [18,19]. Among these gene-delivery systems, AdV were reported to express high levels of the gene of interest for vaccine applications. Recombinant AdV5-FMDV protected both cattle and swine from clinical disease, as early as 7 dpv [5,20]. To circumvent possible safety issues, recombinant vectors based on non-human adenoviruses are preferable for developing veterinary vaccines. Canine adenovirus has been widely used to derive vaccine vectors for use in many species for different infectious diseases [10]. In this study, two recombinant CAV2 viruses expressing the FMDV structural precursor P1 along with the 3C nonstructural protein (Cav-P1/3C R°) or expressing VP1 (Cav-VP1 R°) were evaluated *in vivo* as potential vaccines against FMD.

Mouse is a valuable laboratory animal model to investigate new FMDV vaccines [21]. CD1 or C57BL/6 mice were immunized with either Cav-P1/3C R° or Cav-VP1 R°. It was shown by SDS-PAGE that mice immunized with Cav-P1/3C R° mounted antibodies towards the VP1 protein but these were undetectable by ELISA. The reason for this is not clear, as both vaccines elicited an immune response against the CAV2 vector. The lack of anti-FMDV antibodies detectable with ELISA might be sought in differences in the epitope structure as expressed by the CAV2 recombinant vaccines (based on O/FRA/1/2001 and used in the Western Blot) and as present on the ELISA plates (based on O1Manisa) as well as in the serum dilution used. The mice sera were diluted 1/50 whereas the manufacturer's instructions of the PrioCHECK FMDV type O kit plates indicate that the serum should be diluted 1/10. It is however not easy to sequentially collect large volumes of blood in mice without compromising animal welfare. Therefore, other authors have optimized the detection methods to analyze immune responses following adenovirus-based vaccination in mice [22,23]. In contrast, a strong anti-FMDV antibody response was observed in mice immunized with inactivated FMDV vaccines; as observed by other authors [24].

For the subsequent experiments in GP, a novel method was chosen to evaluate the humoral immune responses in GP immunized with Cav-P1/3C R° or Cav-VP1 R°. Using a bead-based immunoassay, antibodies directed against the FMDV VP1 were detected in all GP immunized against FMDV. While VN antibodies were not detected in GP immunized with the CAV2 vectors, it is possible that the detection limit of our VN test (1/32) was too high. In this regard, the mean VN antibody titer was found to be 1/24 at the

time of challenge (6 weeks after booster vaccination) in a fully protective vaccine trial with a modified live pseudorabies virus expressing the FMDV P1-2A-3C [25]. In another study, in which GP received a prime-boost vaccination with purified VP1 protein, GP were fully protected against challenge with VN antibody titers as low as 16 [26].

Our data obtained from the vaccination of GP show that the Cav-P1/3C R° construct elicits antibodies against FMDV, whereas the immunogenicity of Cav-VP1 R° is less clear, as an antibody response was detected in a Luminex® assay but not by ELISA. As suggested above, the nature of the antigen involved in these serological assays might explain the discrepancy observed. The VP1 protein that was coated on beads for the immunoassay is a recombinant protein produced in *E. coli*, whereas the antigen for the ELISA was prepared from inactivated virus. The FMDV VP1 protein is a major immunogenic antigen that harbors B and T cell epitopes [27,28] which have been extensively used in the development of vaccines against FMDV [29,30]. Nevertheless, the VP1 protein on its own appears to be less immunogenic than when associated with the viral capsid [31]. This might – at least to some extent – explain the negative results for Cav-VP1 R° in the present study.

Subsequently, GP were immunized with Cav-P1/3C R° in order to evaluate the vaccine's efficacy to afford clinical and virological protection after viral challenge in a validated infection model [13]. The Cav-P1/3C R° recombinant vaccine protected GP from generalized FMD. At the time of challenge, antibodies were detected by ELISA in all Cav-P1/3C R° vaccinated animals, with titers only slightly lower than the titers of the O<sub>1</sub> Manisa DOE-vaccinated animals. It should however be taken into account that the O<sub>1</sub> Manisa DOE vaccine is homologous to the O<sub>1</sub> Manisa virus strain that is used in the ELISA whereas the O/FRA/1/2001 strain of the Cav-P1/3C R° vaccine is heterologous. Secondly, a full cattle dose of a high potency O<sub>1</sub> Manisa DOE vaccine was used for prime and boost administration whereas a GP-adapted vaccine dose was used for the Cav-P1/3C R° vaccine. Nonetheless, within the four-day experimental time frame the Cav-P1/3C R° recombinant vaccine protected GP from generalized FMD to a similar extent as the high potency O<sub>1</sub> Manisa DOE vaccine. Protection against FMDV has also been reported with intermediate antibody titers in vaccinated pigs [32].

Beyond the scope of the present study, it should be noted that the Cav-P1/3C R° vaccine has the potential to be used as a marker vaccine to differentiate infected from vaccinated animals (DIVA concept). We expect that animals vaccinated with Cav-P1/3C R° will develop antibodies against the non-structural 3C protein, but not against the other non-structural proteins (NSP) of FMDV. At present, most commercially available NSP ELISA kits are 3ABC-based, suggesting that Cav-P1/3C R° vaccinated animals may react positive in these commercial tests. However, there are a number of assays that detect NSP-specific antibody responses including 2B [33], 2C [34,35], 3A [36], 3B [37], 3AB [38], 3AB1 [39], 3AB3 [34,36] and/or 3D [35]. In theory, Cav-P1/3C R° vaccinated animals should not react in these assays but further research is needed on this subject.

In conclusion, the Cav-P1/3C R° recombinant vaccine conferred substantial clinical and virological protection against FMD in GP. Moreover, adenovirus-based vaccine efficacy could be enhanced with adjuvants [40,41]. In addition, the Cav-P1/3C R° vaccine has the potential to be exploited as a marker vaccine against FMD. Further investigations on sensitive serological assays for DIVA testing and on the correlation between serological response and protection in target species are needed.

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