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# The VP1 G-H loop hypervariable epitope contributes to protective immunity against Foot and Mouth Disease Virus in swine

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ABSTRACT

Foot and Mouth Disease is a highly contagious and economically important disease of livestock. While vaccination is often effective at controlling viral spread, failures can occur due to strain mismatch or viral mutation. Foot and Mouth Disease Virus (FMDV) possesses a hypervariable region within the G-H Loop of VP1, a capsid protein commonly associated with virus neutralization. Here, we investigate the effect of replacement of the G-H loop hypervariable epitope with a xenoepitope from PRRS virus on the immunogenicity and efficacy of an adenovirus vectored FMDV vaccine (Ad5-FMD). Pigs were vaccinated with Ad5-FMD, the modified Ad5-$FMD_{xeno}$, or PBS, followed by intradermal challenge with FDMV strain $O_1$ Manisa at 21 days post-vaccination. While overall serum antibody titers were significantly higher in Ad5-$FMD_{xeno}$ vaccinated animals, neutralizing antibody titers were decreased in pigs that received Ad5-$FMD_{xeno}$, when compared to those vaccinated with Ad5-FMD, prior to viral challenge, indicative of immune redirection away from VP1 towards non-neutralizing epitopes. As expected, animals vaccinated with unmodified Ad5-FMD were protected from lesions, fever, and viremia. In contrast, animals vaccinated with Ad5-$FMD_{xeno}$ developed clinical signs and viremia, but at lower levels than that observed in PBS-treated controls. No significant difference was found in nasal shedding of virions between the two Ad5-FMD vaccinated groups. This data suggests that the hypervariable epitope of the VP1 G-H loop contributes to protective immunity conferred by Ad5 vector-delivered FMD vaccines in swine, and cannot be substituted without a loss of immunogenicity.



## 1. Introduction

Foot-and-Mouth Disease is a highly transmissible viral disease that can result in enormous economic losses to the livestock industry worldwide [13,14]. Although typically effective, prophylactic vaccination against FMDV is complicated in part due to the high rate of viral mutation, which results in year-to-year strain differences rendering some vaccination efforts unsuccessful [13,15]. It has been speculated that viral evolution along with relatively easy adaptation to many susceptible hosts results in heterogeneity within the FMDV VP1 capsid protein which may be responsible for some vaccine failures [42,44].

FMDV is a small RNA virus of the *Aphthovirus* genus in the *Picornaviridae* family [1,21,22,26]. The FMDV genome comprises 8.4 kilobases and codes for 12 proteins, which must be proteolytically cleaved to form mature and functional proteins [21]. The virus forms an icosahedral capsid comprising 60 copies each of the structural viral proteins VP0, VP1 and VP3, and organized in the form of twelve pentamers. A final maturation cleavage of VP0

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occurs in the presence of RNA to produce VP4 (the N-terminal 85 residues of VP0) and VP2. VP1, 2, and 3 are surface exposed, with VP1 surrounding the 5-fold axes of symmetry, and VP2 and VP3 alternating around the icosahedral 3-fold axes [1]. FMDV structural proteins are important facets of immune recognition of the virus [2]. Within VP1, the G-H loop plays a major role in the viral infection cycle, largely due to the highly conserved RGD amino acid motif [10]. This motif binds to αV family of integrin receptors expressed on host epithelial cells to mediate viral entry, and strains lacking this domain have diminished abilities to infect permissive cells [3,6,7,18].

Immune recognition of RNA viruses can be complicated by rapid mutation of progeny virions during the course of infection. Low fidelity 'error-prone' RNA polymerases incorporate mutations that may affect immunologically relevant epitopes, increasing viral variance and thus presenting substantial hurdles for vaccine development. Within the type O VP1 G-H loop, an eight amino acid region has been identified as hypervariable (HV), and its proximity to the highly conserved and immunogenic RGD motif has suggested a potential role of this element in immune evasion by the virus [4,11,31,32,41,43]. Some reports have shown that mutations in this epitope are well tolerated by the virus, but have a detrimental impact on antibody binding affinity and virus neutralization [2,31,43,47,49]. However, in some cases, related mutants induced significant levels of neutralizing antibodies in the natural host, comparable to those elicited by wild type (WT) virus [30,40,47,48]. Additionally, it has been shown that the VP1 G-H loop is a highly immunogenic site of FMDV, with some reports indicating that more than 25% of neutralizing antibodies elicited during FMDV infection are directed towards this domain of VP1 [33,45].

We have previously found that replacing the HV region of the VP1 G-H loop could broaden immunity in mice, wherein antibodies induced by a peptide vaccine were found to be capable of binding to heterologous isolates [43]. However, in other mouse studies, it was demonstrated that complete removal of the G-H loop did not induce sufficient protection against lethal challenge, suggesting a key role for this region in eliciting protective immunity [19]. For this reason, we aimed to identify whether stable replacement of the G-H loop HV region by an unrelated epitope could broaden immunity without affecting the immunogenicity of an Ad5-vectored FMD vaccine in swine, a natural host for FMDV. The 8 amino acid HV region of the VP1 G-H loop was replaced with a xenoepitope from the Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) gp5 protein, in the context of an Ad5-FMD Manisa capsid-based vaccine [16]. Similarly to other Ad5-FMD vaccines directed to serotype A [34,35,38], this construct has been demonstrated to be highly efficacious in swine and cattle [14,16]. The modified vaccine, denoted Ad5-FMD$_{xeno}$, was assessed for protective efficacy in swine, as compared to an unmodified Ad5-FMD which delivers $O_1$ Manisa full capsid antigen.

## 2. Methods

### 2.1. Cells and viruses

HEK 293 cells (ATTC CRL-1573) from the American Type Culture Collection (ATCC; Rockville, MD) were used to generate, grow and titer all recombinant human Ad5 viruses used in this study [16,20] including Ad5-FMD$_{xeno}$ and Ad5-FMD, which contain an FMDV $O_1$ Manisa cassette (FMDV capsid –P1- and non-structural full length 2B and 3C coding regions) under the control of the cytomegalovirus (CMV) promoter [16]. All Ad5 vectors were purified by CsCl gradient centrifugation and viral titers were determined by standard protocols [12,35]. Swine kidney cells, (IBRS-2 cells), and/or HEK 293 cells were utilized to measure FMDV transgene expression following infection by the Ad5 vectors. Serum neutralization assays and FMDV titration from blood and nasal swabs samples were performed on LFPK αVβ6 cells as previously described [16,27,28]. FMDV $O_1$ Manisa used as a challenge virus in this study was collected from the vesicular fluid of an infected pig and titrated in LFPK αVβ6 cells, and in pigs [8]. All FMDV $O_1$ strains used for the cross-neutralization assays were obtained from the Animal and Plant Health Inspection Service (APHIS) of the USDA at PIADC. Sequences were extracted from GeneBank and ID indicated in parentheses: O1-Campos/94 (AY593819), O1-Caseros/67 (AY593821), O1-Manisa/69 (AY593823), O1-UK-2001 (AJ539141), O1-SKR-2010 (KF112887). Sequences for O1-Pak/KCH19/12 and O1-Pak/ICT254 were provided by Dr. Zaheer Ahmed (PIADC).

### 2.2. Construction of recombinant Ad5 viruses expressing xenoepitopes within the FMDV VP1 capsid protein

Replication-incompetent human Ad5 vector encoding FMDV $O_1$ Manisa P1-2A-2B and FMDV A12 3B3C regions (Ad5-FMD) has been previously described [16]. Ad5-FMD$_{xeno}$ was constructed by substituting a 786 nucleotide *Bsrg*I-*Nhe*I fragment containing the PRRSV xenoepitope (VP1 amino acids 135-142: HFQSIYNL) coding sequences instead of corresponding $O_1$ Manisa wild-type VP1 sequences (KYGDGTVA) in the plasmid Ad5-FMD infectious clone [16]. The substitution was positioned to begin 10 amino acids upstream of the conserved RGD motif within the G-H loop, and end 2 amino acids upstream of the RGD motif. The plasmid constructs were confirmed by restriction enzyme analysis and sequencing. The resulting vectors were linearized with *Pac*I and transfected into HEK-293 cells using Lipofectamine® and following manufacturer's protocols (Life Technologies, Grand Island, NY). Recombinant viruses were harvested 10 days post-transfection, amplified in HEK293 cells and purified twice by CsCl isopycnic centrifugation as previously described [35]. Assessment of recombinant FMDV transgene expression was performed in IBRS-2 cells infected at a MOI of 10. After 24 h of incubation, cells were lysed and protein extracts were analyzed by SDS-PAGE, followed by Western blot using rabbit polyclonal antibodies against FMDV structural and non-structural viral proteins made in our lab. Lysate of BHK-21 cells infected with wild type FMDV $O_1$ Manisa was used as a positive control for expression of viral protein products.

### 2.3. Vaccine trial in swine

A swine experiment was performed in the BSL3 Ag lab at PIADC. All experimental procedures were carried out in compliance with the Animal Welfare Act (AWA), the 2011 Guide for Care and Use of Laboratory Animals, the 2002 PHS Policy for the Humane Care and Use of Laboratory Animals, and U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training (IRAC 1985), as well as specific animal protocols reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of PIADC, protocol 151-04.16R. Twelve castrated male Yorkshire swine (five-six weeks old and weighing approximately 18–23 kg each) were divided into three groups according to the vaccination regimen and housed in a common room. All animals were vaccinated subcutaneously (SC) by injecting 1 mL of the Ad5 vaccine preparation in each side of the neck at a total dose of $1x10^9$ pfu of Ad5-FMD or Ad5-FMD$_{xeno}$ and boosted at 21 days post-prime with the same dose. A control group was inoculated with phosphate-buffered saline solution (PBS) on the same schedule. All animals were challenged with $10^4$ $TCID_{50}$/animal at 14 days post boost, by intradermal inoculation in the heel bulb (IDHB) of the rear foot (4 inoculation sites, 100 μL/site). Serum samples were collected weekly from all animals until the

termination of the experiment (14 days post challenge [dpc]), when the animals were humanely euthanized. Serum aliquots were inactivated at 56 °C for 30 min and stored at −70 °C. After challenge, animals were examined daily for 7 days and a clinical score was recorded. A final clinical examination was performed at 14 dpc. Rectal temperature was monitored daily throughout the experimental period. Clinical scores were determined by counting the number of toes presenting FMD lesions plus the presence of lesions in the snout and/or mouth. The maximum score was set at 17, and lesions restricted to the site of challenge were not counted. Nasal swabs, collected in 2.5 mL MEM, containing 2% antibiotics, 0.2% bovine serum albumin (BSA), and 25 mM Hepes buffer, and serum samples were taken prior and after challenge to assess virus shedding and viremia, and virus neutralizing antibody titers.

### 2.4. Determination of virus neutralizing antibody titers (VNTs)

Neutralizing antibodies against FMDV $O_1$-Manisa in serum samples were measured by virus neutralization test (VNT) in 96-well tissue culture plates, according to the method described in the OIE Manual [36]. Antibody titers were calculated according to the method of Kärber [23] and expressed as the log10 of the reciprocal of the final serum dilution that neutralized 100 TCID50 of virus in 50% of the wells. The limit of detection was log10 $\geq$ 0.3.

### 2.5. Determination of cross neutralizing antibody titers

Serum samples collected at 21 days post-vaccination from vaccinated pigs in the first animal trial were also tested for FMDV neutralizing antibodies against different subtypes within the O serotype. The viral strains used to determine cross-neutralization were O1-UK-2001 and O1-SKR-2010 of the O/SEA topotype, O1-Pak/KCH19/12 andO1-Pak/ICT254 of the O/ME-SA topotype Pan–Asia 2 lineage (Zaheer Ahmed, personal communication), O1-Caseros/67 and O1-Campos/94 of Euro- SA topotypes [24], and the homologous O1-Manisa/69, O/ME-SA topotype [25]. Antibody titers were calculated using end point titrations [23].

### 2.6. Determination of total antibody titers by liquid-phase binding enzyme-linked immunosorbent assay

Liquid-Phase Binding ELISA (LPBE) was performed per the protocol published in the 2012 OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals [36]. The reagents used for LPBE, including rabbit and guinea pig antisera against FMDV O1 Manisa, BEI inactivated FMDV $O_1$ Manisa antigen and FMDV $O_1$ Manisa positive antiserum, were produced at the Reagent and Vaccine Services Section of the Foreign Animal Disease Diagnostic Laboratory, National Veterinary Diagnostic Laboratories, Animal and Plant Health Inspection Service, US Department of Agriculture. The test samples were diluted 2-fold with diluent buffer (1X-PBST/10% normal calf serum/5% normal rabbit serum) from 1:4 to 1:512. The blocking reaction was performed by mixing 50 μL BEI inactivated FMDV $O_1$ Manisa antigen (1:7.5 dilution) and 50 μL of diluted sample (pig serum), and incubated at 37 °C for 60 min. Percentage inhibition (PI) titer was reported as the highest serum dilution where the PI value was $\geq$50. The titer was calculated based on 50 μL of the serum sample.

### 2.7. Detection of FMDV RNA by real-time RT-PCR (rRT-PCR)

Serum samples and nasal swabs were processed for RNA extraction and rRT-PCR as previously described [16] Samples were considered positive when Ct values were <40.

### 2.8. Virus titration in swine blood and nasal secretions

Serum samples and nasal swabs were assayed for the presence of virus by a standard plaque assay on LFPK αVβ6 cells [15]. Virus titers were expressed as $\log_{10}$ plaque-forming units (pfu) per mL of blood or nasal swab. The limit of detection was $\geq$0.3.

### 2.9. Analysis of IFN-γ production by activated T cells

IFN-γ expression by isolated CD4 and CD8 T cells was analyzed as per methods previously published [15]. Briefly, blood samples were collected from infected pigs on D0, D1, D3, and D5 post-infection into heparin-containing vacuum tubes. Samples were diluted 1:1 with PBS and layered onto Lymphoprep (Axis-Shield; PoC AS, Oslo, Norway). Cells were centrifuged at 20 °C for 20 min. Peripheral blood mononuclear cells (PBMCs) were collected and stimulated overnight with live FMDV $O_1$ Manisa or with a non-specific stimulator (PMA + Ca Ionophore) at 37 °C in 5% $CO_2$. Cells were then incubated with GolgiStop (BD Bioscience, Franklin Lakes, NJ) per the manufacturer's instructions, pelleted, and then resuspended in PBS + 10% FBS for flow cytometry analysis. Cells were surface stained using mouse anti-pig CD4α:FITC (BioRad, Hercules, CA) and mouse anti-pig CD8 α:AF647 (BD Bioscience). Cells were then fixed and permeabilized with BD Cytoperm/Cytofix (BD Bioscience) for 30 min at 4 °C, washed with BD Perm/Wash three times, and then stained for intracellular IFN-γ using mouse anti-bovine IFN-γ:RPE (BioRad; cross-reactive with porcine IFN-γ). Background fluorescence was determined with corresponding IgG1-RPE isotype controls (BioRad). Cells were analyzed using a FACS LSR-II Flow Cytometer (BD Bioscience). Data was analyzed using the FACSDiva Software (BD Bioscience).

## 3. Statistical analysis

Statistical significance between groups over time was evaluated by a two-way ANOVA, with a Tukey post-hoc test to determine differences between groups at a given time point. Differences were considered significant for $p < 0.05$.

## 4. Results

### 4.1. Construction of Ad5-$FMD_{Xeno}$

The Ad5-$FMD_{Xeno}$ virus displaying a xenoepitope from the PRRSV ORF-5 glycoprotein [43] were prepared by standard methods and purified by Cesium Chloride isopycnic gradient [16]. Plasmid and adenoviral DNA was sequenced and the expression of the FMD transgenes confirmed by infection of IBRS-2 cells, followed by Western blot of cell lysates. Infection with Ad5-$FMD_{xeno}$ allowed for expression of FMD proteins in similar amounts, and with normal processing when compared to the Ad5-FMD unmodified vector (Fig. 1A). Specific bands for structural VP0, VP1-3 and non-structural 3C protease proteins were detected, comparable to protein bands visible in FMDV infected cells. To investigate the potential impact of the PRRSV xenoepitope insertion on the structure of the FMDV capsid delivered by the Ad5-$FMD_{xeno}$, sequences were submitted to the QUARK server of the University of Michigan Department of Computational Medicine and Bioinformatics (Yang Zhang Lab, University of Michigan, Ann Arbor, MI). Generation of predicted FMDV capsid structures delivered by Ad5-FMD and Ad5-$FMD_{xeno}$ revealed no substantial structural changes resulting from the PRRSV epitope insertion into the VP1 G-H loop (Fig. 1B).

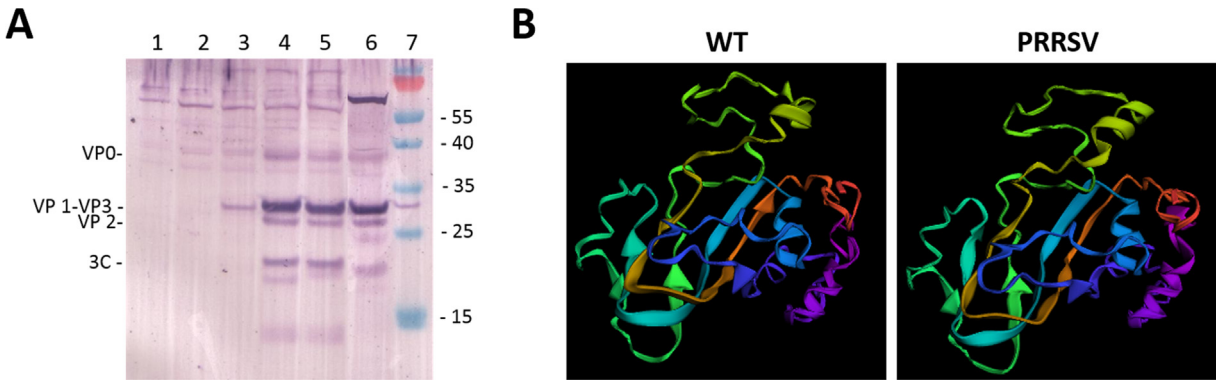


**Fig. 1.** A. Western blot analysis of FMDV protein expression. IBRS2 cells were infected at a MOI = 20 with Ad5-blue or Ad5-FMD displaying a pig-toleragen xenoepitope, HIV xenoepitope, or PRRSV xenoepitope in VP1. A control infection was performed with FMDV $O_1$ Manisa at MOI = 10 for 4 h to verify polyprotein processing. At 24 h cell lysates were prepared and analyzed by Western blot using polyclonal antibodies [16]. Lane 1 = Mock IBRS2 cells, lane 2 = Ad5-Blue (empty vector), lane 3 = Ad5-$FMD_{xeno}$ (pig toleragen), lane 4 = Ad5-$FMD_{xeno}$ (HIV), lane 5 = Ad5-$FMD_{xeno}$ (PRRSV), lane 6 = FMDV O1Manisa infected cell lysates, lane 7 = ladder. B. Predicted structure of VP1-Ad5-FMD (left) and VP1-Ad5-$FMD_{xeno}$ (right) generated via QUARK shows minimal structural changes in VP1 after substitution of the PRSSV Xenoepitope (Yang Zhang Lab, University of Michigan, Ann Arbor, MI).

### 4.2. FMDV serum neutralizing titers

After verification of protein expression *in vitro*, groups of 4 pigs were inoculated with either the Ad5-$FMD_{Xeno}$, WT Ad5-FMD, or PBS as a control. PBS was used in place of Ad5-Blue, as previous studies by our group have compared the immunogenicity of both controls and found no induction of or significant difference in the host adaptive immune response [15,16,38]. Two additional groups of pigs were injected with HIV epitope (GPGRAF)-Ad5-$FMD_{xeno}$ or pig toleragen Ad5-$FMD_{xeno}$ vaccines, but no VNTs were elicited by these pigs, so they were not included in the challenge study (data not shown). Serum was collected from pigs on day 0, and every 7 days thereafter until day 42. At each time-point, serum collected from each animal was used to determine VNT using the plaque reduction assay (PRA) (Fig. 2A). While WT Ad5-FMD vaccine induced virus neutralizing titers at approximately a 1:32 dilution after a single prime/boost inoculation regimen (42 days post vaccination), a reduced neutralizing titer (about 1:8) was detected in animals vaccinated and boosted with Ad5-$FMD_{xeno}$ vaccine. Ad5-FMD titers were significantly different from Ad5-$FMD_{xeno}$ and PBS at all time points post-vaccination, but Ad5-$FMD_{xeno}$ and PBS were not significantly different at any time point.

### 4.3. FMDV total antibody assay (LPBE)

Liquid Phase Blocking ELISA (LPBE) was used to analyze seroconversion of vaccinated animals. Sera from vaccinated animals was run against inactivated FMD virions, and competitive binding was assessed. Results indicated that there was no significant difference in antibody titers from animals vaccinated with Ad5-FMD or Ad5-$FMD_{xeno}$ at 0 or 14 dpv. Surprisingly, by 28 dpv LPBE titers were significantly higher in Ad5-$FMD_{xeno}$ vaccinated animals in comparison to Ad5-FMD vaccinated controls ($p < 0.0001$) and PBS vaccinated controls ($p < 0.0001$). As expected Ad5-FMD vaccinated pigs had antibody titers significantly higher than PBS vaccinated pigs ($p < 0.01$) (Fig. 2B). Although the overall antibody titer decreased with time, a similar profile was observed until the day of challenge (42dpv, Ad5-$FMD_{xeno}$ $p < 0.0001$ vs. AD5-FMD and PBS; Ad5-FMD vs. PBS trended towards significance ($p = 0.06$)). Taken together, the data suggests that while Ad5-$FMD_{xeno}$ does not appear to induce a robust neutralizing antibody response, it does induce a higher amount of antibodies than the unmodified Ad5-FMD. These results suggest that introduction of the xenoepitope into the G-H loop of VP-1 does not prevent immune recognition of the modified virion, but rather redirects the focus of the immune response toward other non-neutralizing epitopes.

### 4.4. Vaccine challenge with FMDV Manisa strain

Twenty-one days after completion of the vaccination regimen, all animals were intradermally challenged in the heel bulb with $10^4$ $TCID_{50}$ of the FMDV $O_1$ Manisa strain. The clinical score (Fig. 3A) and rectal temperature (Fig. 3B) of each animal was recorded daily for 7 days post-infection. WT Ad5-FMD vaccinated

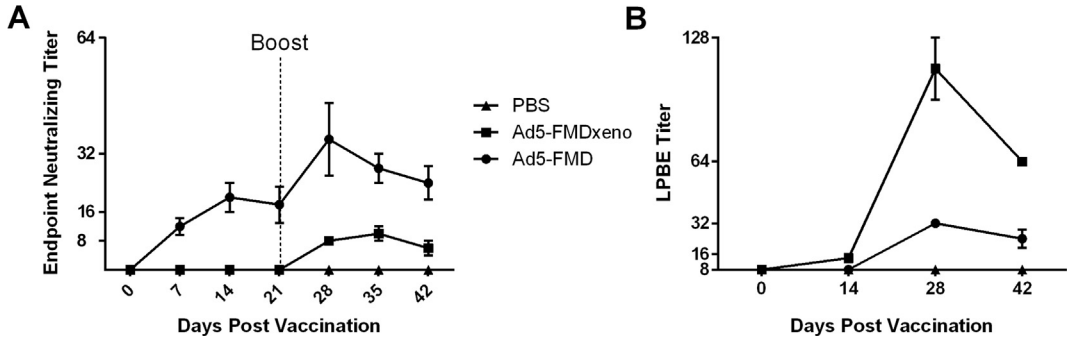


**Fig. 2.** A. Virus Neutralizing Titer Assays on pig serum after vaccination demonstrated that Ad5-$FMD_{xeno}$ induces neutralizing antibodies against homologous challenge, but to a lesser extent than WT Ad5-FMD. Titers in Ad5-FMD vaccinated pigs were significantly higher than Ad5-$FMD_{xeno}$ and PBS at all post-vaccination time points, but Ad5-$FMD_{xeno}$ and PBS were not significantly different at any time point. B. Liquid-Phase Binding ELISA (LPBE) analysis of antibody induction in response to Ad5-FMD (●), Ad5-$FMD_{xeno}$ (■), and PBS (▲). At 28 dpv animals vaccinated with Ad5-$FMD_{xeno}$ displayed approximately 4-fold higher antibody titers in comparison to animals vaccinated with Ad5-FMD. Ad5-$FMD_{xeno}$ had statistically significantly higher antibody titers at 28 dpv. when compared to both Ad5-FMD ($p < 0.0001$) and PBS controls ($p < 0.0001$). At 42dpv, Ad5-$FMD_{xeno}$ $p < 0.0001$ vs. AD5-FMD and PBS; Ad5-FMD vs. PBS trended towards significance ($p = 0.06$).

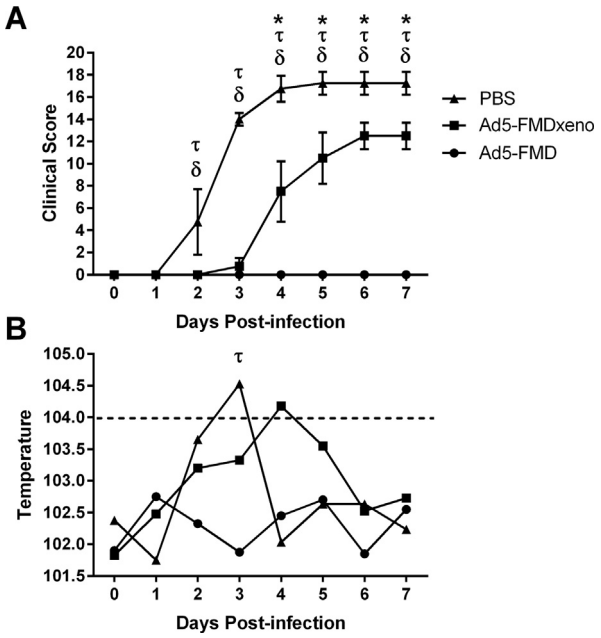


**Fig. 3.** Clinical scores (A) and rectal temperature (B) of pigs vaccinated and challenged with FMDV $O_1$ Manisa. * = significant difference between Ad5-FMD and Ad5-$FMD_{xeno}$, τ = significant difference between Ad5-$FMD_{xeno}$ and PBS, δ = significant difference between Ad5-FMD and PBS.

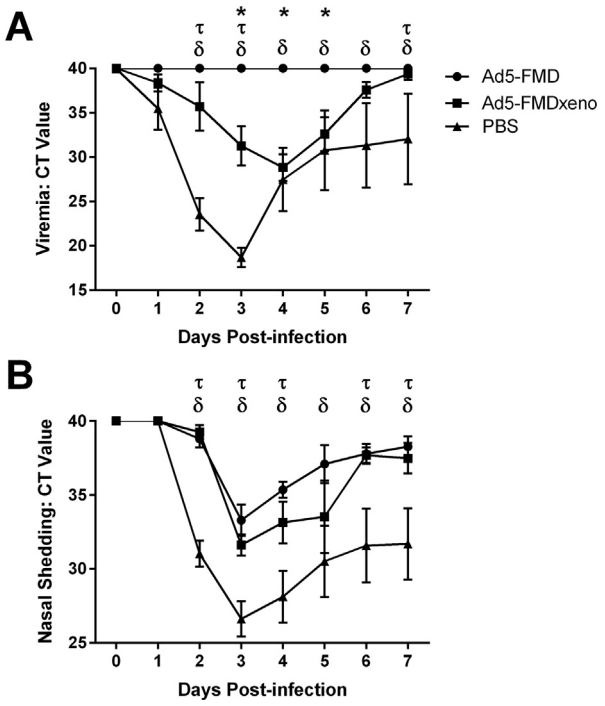


**Fig. 4.** Viremia (A) and nasal shedding (B) of pigs vaccinated and challenged with FMDV $O_1$ Manisa indicate that use WT Ad5-FMD resulted in significantly lower viremia in vaccinated swine in comparison to Ad5-$FMD_{xeno}$. Comparison of nasal shedding of FMDV revealed that WT Ad5-FMD and Ad5-$FMD_{xeno}$ had similar impacts on viral shedding in vaccinated animals. Significance indicators are the same as in Fig. 3.

animals displayed no clinical signs and had normal temperatures (rectal temperature below 103°F) for the entire 7-day period. Conversely, Ad5-$FMD_{Xeno}$ vaccinated animals displayed clinical signs and fever during the 7-day period, though these were delayed, lower in severity, and shorter in duration when compared to PBS vaccinated controls (Fig. 3B).

### 4.5. Duration of viremia and viral shedding

Pigs vaccinated with Ad5-FMD showed no signs of viremia during the 7 days post-infection, but did shed virus in nasal secretions. The Ad5-$FMD_{Xeno}$ vaccine markedly reduced viremia when compared to PBS controls (Fig. 4A), but nonetheless did not prevent virus from reaching the bloodstream. Ad5-$FMD_{Xeno}$ animals did not show statistically different shedding characteristics in comparison to their WT Ad5-FMD counterparts, but both vaccinated groups shed considerably lower amounts of virus than did unvaccinated controls (Fig. 4B).

### 4.6. Cross neutralization titers

In a previous study we showed that vaccination with Ad5-FMD ($O_1$ Manisa) induced significant amounts of cross-reacting antibody titers against FMDV O1-UK-2001 [16]. A similar analysis was performed in this experiment. Sera from animals vaccinated with Ad5-$FMD_{xeno}$ was used to neutralize a panel of related FMDV strains. Interestingly, despite the difference in efficacy and specific neutralizing antibody titers, no significant differences in cross neutralizing antibody titers were detected between the two (Ad5-FMD (Fig. 5A) and Ad5-$FMD_{xeno}$ (Fig. 5B)) vaccinated groups. These results suggest that xenoepitope substitution did not broaden vaccine immunity.

### 4.7. IFN-γ production by activated T cells

T cell activation was measured for 5 d.p.i by quantification of IFN-γ producing CD4 and CD8 positive T cells, as a measure of anti-viral activity in the wake of vaccination and challenge. Ad5-FMD vaccinated animals had a higher percentage of IFN-γ-expressing CD4 positive T cells at days 1 and 3 post-infection, reaching a peak of approximately 1%, in comparison to Ad5-$FMD_{xeno}$ and PBS vaccinated animals. IFN-γ-expressing CD4 positive T cells from Ad5-$FMD_{xeno}$ vaccinated animals generally trended with PBS vaccinated control animals, with Ad5-$FMD_{xeno}$ peaking at approximately 0.2% on day 1 post-infection (Fig. 6A). Additionally, Ad5-FMD vaccinated animals had higher percentages of IFN-γ-expressing CD8 positive T cells at all time points measured post-infection, peaking at approximately 1.2% at 1 d.p.i. In comparison, IFN-γ-expressing CD8 positive T cells from Ad5-$FMD_{xeno}$ vaccinated animals once again trended with PBS vaccinated controls, peaking at approximately 0.4% at 5 d.p.i. (Fig. 6B).

## 5. Discussion

Our data demonstrates that substitution of an 8-amino acid span of the HV epitope of the VP1 G-H loop of FMDV type O with a xenoepitope derived from PRRSV reduces, but does not eliminate, the protective efficacy of the Ad5-FMD vaccine in response to homologous challenge. Our results suggest that the G-H loop HV epitope contributes to protective immunity against FMDV in swine. Pigs vaccinated with Ad5-$FMD_{xeno}$ displayed higher serum antibody titers to FMDV capsid proteins than those vaccinated with Ad5-FMD. However, higher titer did not correlate with better protection against FMDV challenge suggesting that while substitution of the G-H loop increases the immunogenicity of the vaccine, it comes at the cost of efficacy. This presents an intriguing new piece of information about the role of different regions within the G-H loop with regard to virus neutralization. As previous research has suggested, this region is an immunodominant antigen for induction of virus neutralizing antibodies [2], yet in some circumstances

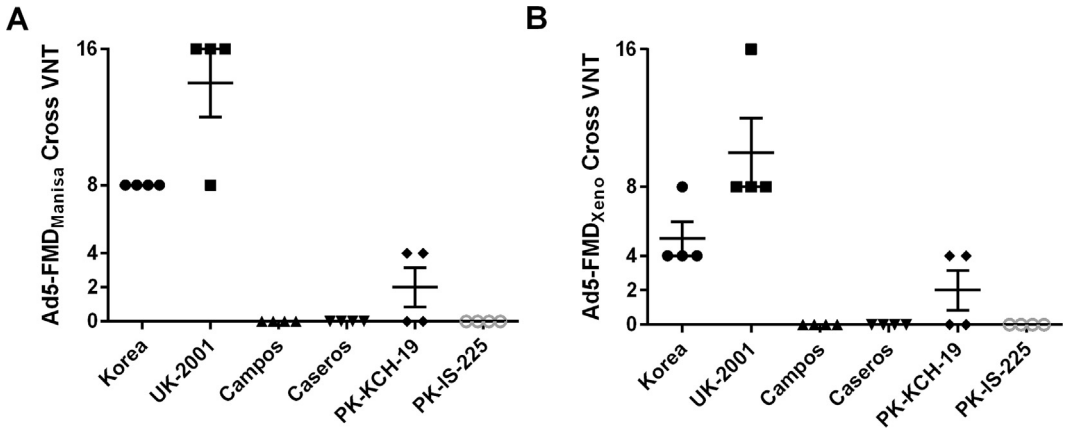


**Fig. 5.** Analysis of cross-neutralizing antibody titers for Ad5-FMD and Ad5-FMD$_{xeno}$ vaccinated animals revealed that antibodies induced by both constructs were similarly capable of neutralizing type O FMDV strains.

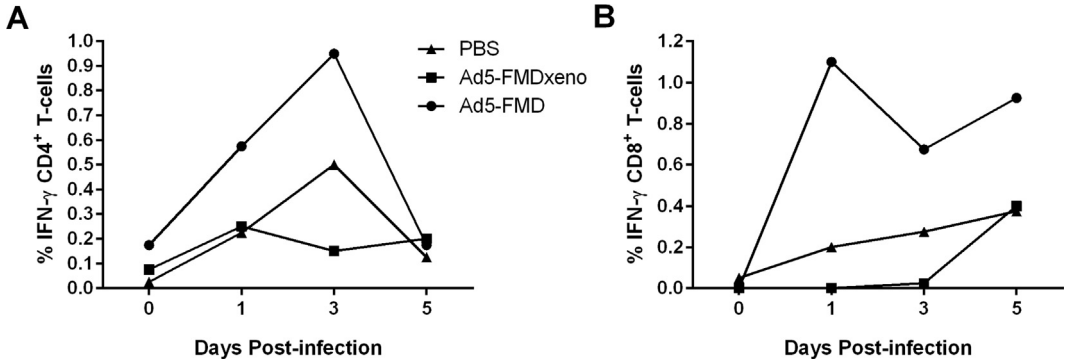


**Fig. 6.** Analysis of IFN-γ production by activated CD4 (A) and CD8 (B) T cells after vaccination with Ad5-FMD, Ad5-FMD$_{xeno}$, and PBS showed diminished induction of cell-mediated immunity in Ad5-FMD$_{xeno}$-vaccinated animals compared with Ad5-FMD. Data are pooled samples from vaccinated animals.

it may be dispensable [17,30,40,47,48]. The highly conserved RGD motif present within this region [18], responsible for integrin binding during host cell entry, is thought to be heavily implicated in antibody-mediated neutralization of FMDV. However, studies have indicated that while RGD is involved in induction of effective virus neutralizing antibodies, it is not the sole motif involved in the blocking of viral entry into host cells. Indeed, virions with mutated RGD motifs are still capable of entering and infecting cells *in vitro*, and display similar growth kinetics to virions with intact RGD motifs [3,6,18,37,39,46]. The capability of RGD$^-$ mutant virions to infect cells provides evidence to suggest that there are other mechanisms involved in cell entry and virus neutralization.

The data presented in this study suggests that the G-H loop HV epitope is important for protective immune responses against FMDV, and therefore this site may be involved in antibody-mediated neutralization of the virus. In a recently published study by Zhu et al., insertion of a FLAG domain 4 amino acids upstream of the RGD motif resulted in immunogenic vaccines with similar host cell binding capabilities to the WT parental O/CHA/90 strain [50]. In our study, when an overlapping region was replaced by the PRRSV xenoepitope, vaccinated pigs mounted measurable immune responses, but less vigorously than pigs vaccinated with Ad5-FMD. Upon challenge, Ad5-FMD$_{xeno}$ pigs displayed higher viremia, clinical score, and fever than those vaccinated with Ad5-FMD, but had less severe clinical course of disease than PBS control groups. In contrast, when Zhu et al. inserted (as opposed to substituting) a FLAG tag 4 amino acids upstream of RGD, they found that vaccinated pigs were protected. This insertion overlapped with the final 2 amino acids of our PRRSV xenoepitope insertion site, but yielded dramatically different results [50]. This suggests that within the HV region itself, there may be a sub-epitope that is critically important for inducing protective immunity. It is also possible that substitution by the PRSSV xenoepitope affected the induction of neutralizing antibodies that specifically recognize conformational epitopes involved in interactions between VP1 and other viral proteins. Additionally, the xenoepitope substitution may have affected the avidity by which the antibodies bind to their viral target, or the kinetics of B cell class switching [5,29]. Further studies to measure these parameters are warranted. Based on our findings and the data presented by Zhu et al., it is possible that the region of the HV epitope closer to the N-terminal region of our PRRSV xenoepitope substitution is an immunologically important motif that contributes to protective immunity during vaccination.

Interestingly, measurement of total antibodies by LPBE demonstrated that Ad5-FMD$_{xeno}$ induced higher antibody titers than Ad5-FMD or PBS. Taken together in the context of SNT data, the data suggests that introduction of the xenoepitope in the Ad5- delivered FMD capsid, refocused the immune response but at the cost of neutralizing protection against challenge. Moreover, increased concentrations of total anti-FMDV antibodies did not lead to increased serum cross-reactivity. Presumably, the antibodies induced by the Ad5-FMD$_{xeno}$ vaccine were directed at secondary non-neutralizing sites, rather than at the highly immunogenic VP1. Characterization of these antibodies and their epitopes, warrants further study to better understand the effects of xenoepitope substitution on immune refocusing in response to variant FMDV virions.

Despite its similarity to the WT Ad5-FMD vaccine, our Ad5-$FMD_{Xeno}$ vaccine displayed lower efficacy than the WT vaccine in a number of clinically relevant areas, including the prevention of fever (Fig. 3), prevention of viremia (Fig. 4), and severity of clinical score (Fig. 3). This decreased efficacy may be due in part to the inherent instability of empty capsids containing the modified G-H loop. Increasing the stability of the modified capsids may improve the activity of the vaccine in future experiments. It may also be possible that with higher priming doses, booster immunizations and/or the addition of an adjuvant, the magnitude of these differences could be improved, and thus the Ad5-$FMD_{Xeno}$ may still be a useful vaccine for diminishing the effects of viral evolution by stabilizing a known HV epitope. Despite its lower overall efficacy, the Ad5-$FMD_{Xeno}$ vaccine was not significantly different from the WT vaccine in prevention of viral shedding via the nasal route (Fig. 4). Additionally, xenoepitope substitution of the G-H loop HV epitope broadened immunity and retained FMDV binding properties in a mouse model [43], proving the utility of the technique in model species, despite failure of a similar construct to induce increased cross-neutralization in swine (Fig. 5). However, our data points to the conclusion that, as interruption of the hypervariable epitope did not significantly broaden immunity induced by the vaccine in swine, strain-matching must remain a critical facet of FMDV vaccine development. The use of vaccine matching to predominant circulating strains may mitigate the effects of viral evolution and NEV formation, although this does not preclude the use of other strategies, including changes to vaccine design and increased potency through modulation of host immunity during vaccination.

Investigation of T-cell activation in response to vaccination with Ad5-FMD, Ad5-$FMD_{xeno}$, and PBS suggested that both Ad5-$FMD_{xeno}$ and PBS induce weaker IFN-$\gamma$ expression by CD4 and CD8 T cells when compared to Ad5-FMD (Fig. 6A/B). Values observed in PBS vaccinated animals likely represent a viable background T cell activation threshold for the assay utilized, suggesting that both Ad5-$FMD_{xeno}$ and PBS did not induce measurable cell-mediated immunity in vaccinated animals in this study. Intriguingly, the diminished cell-mediated immune response to Ad5-$FMD_{xeno}$ mirrors the decreased neutralizing antibody titers seen in the Virus Neutralization Assay (Fig. 5). In a study by Carr et al., antigen-specific CD4 T cell responses were found to correlate with neutralizing antibody titers to FMDV antigen, as depletion of the CD4 T cell pool substantially decreased neutralizing antibody production by activated B cells [9]. The decreased neutralizing antibody titers in response to Ad5-$FMD_{xeno}$, as well as the decreased CD4 and CD8 cell activation in response to the modified vaccine, suggest that the VP1 G-H loop hypervariable region may contain T cell epitopes involved in the host immune response to the FMDV capsid. It is unclear whether CD4 and CD8 T cells respond to the same sequence within this region, and further study is necessary to characterize G-H loop T cell epitopes and their role in the immune response to FMDV vaccines.

The data from this study suggests that replacement of the VP1 G-H loop HV epitope with a stable epitope derived from PRRSV decreases, but does not eliminate, virus neutralization activity and therefore implicates the G-H loop HV region as a significant immunological epitope in vaccination against FMDV. While our vaccine design was shown to elicit broadened immunity in mouse models, it appears that the modified vaccine is less capable of providing protection against challenge with homologous type O FMDV in swine. Thus, it appears that in swine, the VP1 G-H loop HV epitope contributes to the formation of robust immune responses to FMDV. It is nevertheless clear that more work must be done to fully characterize this immunologically important epitope and to investigate which specific residues are relevant to host immune recognition and response to FMDV.

## Declaration of Competing Interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.05.019.

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