



A Malaysia 97 monovalent foot-and-mouth disease vaccine (>6PD₅₀/dose) protects pigs against challenge with a variant FMDV A SEA-97 lineage virus, 4 and 7 days post vaccination



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ABSTRACT

Pigs play a significant role during outbreaks of foot-and-mouth disease (FMD) due to their ability to amplify the virus. It is therefore essential to determine what role vaccination could play to prevent clinical disease and lower virus excretion into the environment. In this study we investigated the efficacy of the double oil emulsion A Malaysia 97 vaccine (>6PD₅₀/dose) against heterologous challenge with an isolate belonging to the A SEA-97 lineage at 4 and 7 days post vaccination (dpv). In addition, we determined whether physical separation of pigs in the same room could prevent virus transmission. Statistically there was no difference in the level of protection offered by 4 and 7 dpv. However, no clinical disease or viral RNA was detected in the blood of pigs challenged 4 dpv, although three of the pigs had antibodies to the non-structural proteins (NSPs), indicating viral replication. Viral RNA was also detected in nasal and saliva swabs, but on very few occasions. Two of the pigs vaccinated seven days prior to challenge had vesicles distal from the injection site, but on the inoculated foot, and two pigs had viral RNA detected in the blood. One pig sero-converted to the NSPs. In contrast, all unvaccinated and inoculated pigs had evidence of infection. No infection occurred in any of the susceptible pigs in the same room, but separated from the infected pigs, indicating that strict biosecurity measures were sufficient under these experimental conditions to prevent virus transmission. However, viral RNA was detected in the nasal swabs of one group of pigs, but apparently not at sufficient levels to cause clinical disease. Vaccination led to a significant decrease in viral RNA in vaccinated pigs compared to unvaccinated and infected pigs, even with this heterologous challenge, and could therefore be considered as a control option during outbreaks.

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1. Introduction

Foot-and-mouth disease (FMD) directly impacts livestock production due to loss in productivity and usually affects the economy further due to quarantine and import restrictions on live animals and their products. Vaccination has been used successfully in a number of previously endemic countries to control the disease and

most countries free from FMD will consider emergency vaccination if an outbreak should occur.

Susceptible domestic species include cattle, sheep, goats, pigs and water buffalo. All may demonstrate lesions on areas of friction such as the mouth, feet and teats in lactating animals, but sub-clinical infections can also occur, especially in sheep and goats [1]. Pigs are the amplifier hosts of the disease and excrete large amounts of virus in all secretions and excretions [2,3]. For this reason it is imperative to prevent them from becoming infected or to decrease viral shedding using vaccination.

There are seven serotypes of FMD virus (FMDV—A, O, C, Asia-1, SAT 1, SAT 2 and SAT 3) and large numbers of variants exist

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within each. Since cross protection between serotypes does not exist [4,5], vaccines need to contain multiple strains to ensure immunity to more than one serotype. Even within serotypes, protection is not complete [6,7]. FMDV serotype A viruses have always been considered to be antigenically the most diverse [8,9], and have genetically been classified under three broad genotypes [10]. The Asian genotype consists of several lineages and sub-lineages with viruses belonging to the lineage A SEA-97 being endemic to South East Asia (SEA) and new clusters emerging in the region [10,11]. These viruses have recently spread beyond the SEA region to cause outbreaks in countries that were previously free of serotype A [12].

FMD is endemic in many parts of the world and occurs in most countries in SEA. Through their proximity and the amount of trade and travel, these countries pose the biggest perceived risk to Australia's livestock industries and agricultural economy. Australia's last suspected outbreak was in 1872 [13] and having FMD-free status, together with the absence of several other diseases, has provided the country with a significant trade advantage. The local pork industry is small compared to high producing countries such as China, South Korea and Japan, but the potential overall losses due to a large outbreak of FMD could reach 50 billion Australian dollars over a 10 year period [14]. For this reason it is important to determine whether the strains in the Australian vaccine bank will provide early protection in pigs against the serotype A viruses that are currently circulating in SEA.

2. Materials and methods

2.1. Cell lines, viruses and vaccine

Baby hamster kidney-21 (BHK-21) cells were used for all virus culture. The challenge virus (A/VIT/08/2005) belongs to the FMDV A SEA-97 topotype, circulating in Vietnam and other SEA countries, and has a relative homology (r_1) of 0.51 to the A Malaysia 97 (A/MAY/97) vaccine strain (WRL Report 2006; http://www.wrlfmd.org/ref_labs/ref_lab_reports/OIE-FAO%20FMD%20Ref%20Lab%20Network%20Report%202006.pdf). The virus was passaged three times in BHK-21 cells before preparation of the pig-derived challenge virus.

A monovalent double oil emulsion A/MAY/97 vaccine ($>6\text{PD}_{50}/\text{dose}$) was prepared by Merial, United Kingdom.

2.2. Animal ethics and pigs used in the study

The animal studies were performed according to the Australian code of practice for the care and use of animals for scientific purposes (AEC1514 and 1571). Sero-negative three-month-old cross-bred Landrace pigs were obtained from a commercial piggery in Vietnam.

2.3. Preparation of challenge virus

Five healthy pigs were used to prepare pig-derived challenge virus. Two pigs were administered 1 ml of A/VIT/08/2005 intravenously into the ear vein, 1 ml intramuscularly on the dorsal aspect just behind the left ear and 2 ml intradermally into the foot-pad of the left-hind limb at multiple sites (0.1 ml/site in each digit). The animals were monitored for the appearance of lesions for three days. A 10% (w/v) suspension of tissue homogenate was prepared in phosphate buffered saline using the epithelial tissue from the coronary band and foot lesions and three more pigs were inoculated intradermally with 0.1–0.2 ml of a 10% (w/v) suspension in the foot pad of the left-fore limb. Epithelial tissue from the coronary band and foot lesions was collected and a 10% (w/v) suspension of tissue homogenate was prepared and stored at -80°C .

2.4. Titration of A/VIT/08/2005 pig-derived virus

Four healthy pigs were used for titrating the pig-derived virus at \log_{10} dilutions (10^{-1} to 10^{-8}) in basal medium eagles (BME) cell culture medium supplemented with 1% foetal calf serum (FCS). Two pigs received 0.1–0.2 ml of inoculum dilutions -2 , -3 , -4 and -5 , whereas two other pigs were administered dilutions -4 , -5 , -6 and -7 , intradermally in the footpad. Each dilution was administered to two feet. Lesions at the inoculation sites were scored at 24, 36, 48, 60 and 72 h post inoculation. The 50% pig infective dose per ml ($\text{PID}_{50}/\text{ml}$) was calculated using the Spearman-Kärber method [15].

2.5. Pig immunisation and challenge

The experiment consisted of three groups of eight pigs each in separate rooms. One group was vaccinated intramuscularly in the mid neck region with 2 ml of vaccine (0.82 mm \times 38.1 mm) seven days prior to challenge (A-V7), another four days before challenge (A-V4) and the last group was left unvaccinated and was challenged on day 0 (A-UV). Vaccinations were staggered so that the virus challenge occurred on the same day. For each of these groups, five additional non-vaccinated pigs were kept in the same room (comprising groups A-UVC7, A-UVC4 and A-UVC), but were separated by a waist-high steel wall that prevented direct contact with the challenged animals.

Groups A-V7, A-V4 and A-UV were challenged with $10^{5.0}$ PID_{50} of the pig-derived virus by inoculation in two sites in the left-hind foot pad (0.2 ml/site). The animals were observed and sampled daily for 14 days, and rectal temperatures recorded. Clinical scores were determined by giving each site of lesion development, except the inoculation site, one point (four feet, tongue, mouth and snout); the maximum score was therefore seven. Nasal secretions, saliva and faeces were collected in duplicate using cotton swabs (diameter: 2.7 mm; length: 150 mm); one swab was used for virus isolation (0.5 ml of BME with 10% FCS and antibiotics) and the other for viral genome detection (0.5 ml of lysis buffer with carrier RNA and proteinase K; Startec Biomedical AG, Germany). Swabs were submerged in the buffer and stored at -80°C . Clotted blood for serum was collected on -7 , -4 , 0, 5, 7, 10 and 14 days post-challenge (dpc). Whole blood was collected in EDTA tubes on 0, 1–7, 9, 10 and 14 dpc.

The animals in groups A-UVC7, A-UVC4 and A-UVC were observed and sampled as described above. Clotted blood for serum was collected on days 0, 5, 7, 10 and 14 dpc. Whole blood was collected in EDTA tubes on 0, 3, 5, 7, 9, 10 and 14 dpc.

2.6. Quantitative real-time reverse transcriptase PCR (RT-qPCR) for detection of FMD viral RNA

Total RNA from samples was extracted using the InviMag Virus RNA Mini kit/KF96 (Strattec Molecular, Germany) on an automated nucleic acid extraction system (KingFisher Flex Magnetic Particle Processor, ThermoFisher Scientific, USA) following the manufacturer's protocol. RT-qPCR was carried out using Ambion AgPath-ID MasterMix (Life Technologies, USA) using the assay previously described by [16].

In vitro transcribed RNA was prepared using the Megascript T7 kit (Ambion, USA) from a pBluescript KS+ plasmid containing the FMDV IRES region [17]. The RNA was purified and checked for integrity by RT-PCR using the specific primers that would be used for the RT-qPCR [18], and by sequencing. RNA standards were prepared to determine a standard curve for each RT-qPCR run.

2.7. Serology to detect antibodies to FMDV structural proteins

A liquid phase blocking ELISA (LPBE) was performed as described by Hamblin et al. [19] using A/MAY/97-specific reagents. Antibody titres were expressed as the 50% end-point titre, i.e. the dilution at which the reaction of the test sera resulted in an optical density equal to 50% inhibition of the mean optical density of the reaction (antigen) control wells [15]. Sera showing a titre of log 1.20 were considered positive.

2.8. Serology to detect antibodies to FMDV non-structural proteins

A competitive ELISA (c-ELISA) was performed on serum samples at 1:5 dilutions [20]. The final OD values were expressed as percentage inhibition relative to the mean OD of the OD Max control wells representing the no serum controls i.e. $100 - (100 \times (\text{OD test serum mean} / \text{OD Max control mean}))$ where a positive result was recorded for samples that were $\geq 50\%$ inhibition of the OD max control.

2.9. Statistical analysis

Quantitative data were assessed for normality by calculating descriptive statistics, plotting histograms, and performing the Anderson–Darling test for normality using commercially available software (MINITAB Statistical Software, Release 13.32, Minitab Inc, State College, Pennsylvania, USA). Data were transformed using the natural logarithm when necessary to improve the distributional form prior to statistical analysis. Data were descriptively presented as the median and range. A linear mixed models approach was used to estimate the effect of treatment group on viral genome quantity determined using RT-qPCR. Independent models were fitted for the four types of PCR specimen (whole blood and oral, nasal, and faecal swabs) in addition to a combined analysis of all sample types. All models included a random effect term for pig identification to account for the repeated measurements and also

included fixed effect terms for treatment group, and experimental day. Bonferroni correction was used to adjust *P* values for multiple post-hoc comparisons. Statistical analyses were performed in commercially available software (IBM SPSS Statistics Version 22, International Business Machines Corp., Armonk, New York, USA) and results interpreted at the 5% level of significance.

3. Results

3.1. Adaptation and titration of A/VIT/08/2005 in pigs

The challenge virus was passed through pigs twice and had a titre of approximately 10^6 PID₅₀/ml when titrated in pigs.

3.2. Vaccine efficacy study

All eight pigs in group A-UV developed generalized disease between 2 and 5 dpc with lesions on all four feet, snout, lower lip and tongue. In total five out of eight pigs had temperatures $\geq 40^\circ\text{C}$ between 3 and 5 dpc on one or more days. Pig #5, which had lesions at all sites, had an elevated temperature also at 8 dpc (Table 1).

In group A-V4, none of the eight pigs showed generalized disease with secondary lesions or increased temperatures, but four of the pigs developed vesicles at the site of inoculation between 2 and 5 dpc (Table 1).

Two of the pigs in group A-V7 (#29 and #31) developed vesicles at the site of inoculation while pigs #28 and #29 showed secondary lesions on the coronary band of the inoculated feet 7 and 5 dpc, respectively (Table 1). No other lesions were detected. None of the contact pigs (groups A-UVC, A-UVC4 and A-UVC7) developed any lesions or elevated temperatures (data not shown).

3.3. Detection of viral RNA in swabs

All eight pigs in group A-UV had viral RNA in nasal and saliva swabs by 2 dpc that was detected in most pigs up to 5 dpc (Table 2; Supplementary Tables 1 and 2). By 6 dpc, only four pigs tested

Table 1

Summary of the clinical outcome in pigs after challenge with A/VIT/08/2005. The in-contact groups did not show any signs of clinical disease or temperature.

Group	Animal ID	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc	6 dpc	7 dpc	8 dpc	9–13 dpc	14 dpc ^a
A-UV	1	–	◆	6	6	6	–	–	–	–	–
	2	–	4	4	4	4	–	–	–	–	–
	3	–	–	3	4	4	–	–	–	–	–
	4	–	2	2	2	2	–	–	–	–	–
	5	◆	4	4	6	6	–	–	–	–	–
	6	–	4	4	6	6	–	–	–	–	–
	7	–	–	–	6	6	6	6	6	–	–
	8	–	–	–	–	2	4	4	–	–	–
A-V4	14	–	–	–	–	◆	◆	–	–	–	–
	15	–	◆	◆	◆	◆	–	–	–	–	–
	16	–	–	–	–	–	–	–	–	–	–
	17	–	–	–	–	–	–	–	–	–	–
	18	–	◆	◆	◆	–	–	–	–	–	–
	19	–	–	–	–	–	–	–	–	–	–
	21	–	◆	◆	◆	–	–	–	–	–	–
A-V7	27	–	–	–	–	–	–	–	–	–	–
	28	–	–	–	–	–	–	1	1	–	–
	29	–	◆	◆	◆	◆1	–	–	–	–	–
	30	–	–	–	–	–	–	–	–	–	–
	31	–	◆	◆	◆	–	–	–	–	–	–
	32	–	–	–	–	–	–	–	–	–	–
	33	–	–	–	–	–	–	–	–	–	–
	34	–	–	–	–	–	–	–	–	–	–

◆ Inoculation site positive; Clinical score was determined as follows: 1 for each affected foot (lesion at the site of inoculation was not counted), tongue, mouth and snout. The maximum score is therefore 7; shaded boxes indicate temp $\geq 40^\circ\text{C}$.

^a All pigs were euthanized.

Table 2
Viral RNA detected in nasal, saliva and faecal swabs as well as EDTA blood using RT-qPCR.

Groups	Pig ID	Days post challenge														
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A-UV	1	-	-	SP	SP,NP	SP,NP	SP,NP	NP	-	-	-	-	-	-	-	-
	2	-	-	SP,NP	SP,NP	SP	-	-	-	-	-	-	-	-	-	-
	3	-	-	NP,FP	SP,NP	SP	SP	-	-	-	NP	-	-	-	-	-
	4	-	-	SP,NP,FP	SP,NP	SP,NP	NP	-	-	-	-	-	-	-	-	-
	5	-	-	SP,NP	SP,NP	NP	NP	-	-	-	-	-	-	-	-	-
	6	-	-	SP,NP	SP,NP	SP	SP	SP	-	-	NP	-	-	-	-	-
	7	-	-	SP	-	SP	SP,NP	NP	-	-	-	-	-	-	-	-
	8	-	-	NP	-	SP,NP	SP	SP	-	SP,FP	NP	-	-	-	-	-
A-UVC	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A-V4	14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	16	-	-	-	SP	-	-	-	-	-	-	-	-	-	-	-
	17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	19	-	-	-	-	NP	-	-	-	-	-	-	-	-	-	-
	20	-	-	-	-	-	-	-	SP	-	-	-	-	-	-	-
	21	-	-	-	NP	-	-	-	-	-	-	-	-	-	-	-
A-UVC4	22	-	-	-	NP	-	-	-	-	NP	-	-	-	-	-	-
	23	-	-	-	-	NP	NP	-	-	-	-	-	-	-	-	-
	24	-	-	-	NP	NP	-	-	NP	NP	-	-	-	-	-	-
	25	-	-	-	NP	-	-	-	-	-	NP	-	-	-	-	-
	26	-	-	-	-	NP	NP	-	-	-	-	-	-	-	-	-
A-V7	27	-	-	-	SP,NP	SP,FP	SP,NP	NP	-	NP	-	-	-	-	-	-
	28	-	-	-	SP,NP,FP	NP	-	-	-	NP	-	-	-	-	-	-
	29	-	-	-	SP,NP	-	SP	-	-	-	-	-	-	-	-	-
	30	-	-	-	SP,NP,FP	SP,FP	SP	-	-	-	-	-	-	-	-	-
	31	-	-	-	SP,NP	SP	SP	-	SP	-	-	-	-	-	-	-
	32	-	-	-	SP,NP	SP	SP,NP	-	SP	-	-	-	-	-	-	-
	33	-	-	-	SP,FP	-	SP,FP	-	-	-	-	-	-	-	-	-
	34	-	-	-	NP	SP	SP	-	NP	-	-	-	-	-	-	-
A-UVC7	35	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	36	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	37	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	38	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	39	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

SP—saliva swab positive by PCR, NP—nasal swab positive by PCR, FP—faecal swab positive by PCR.

Shaded boxes indicate viral RNA detected in blood (animals in groups A-UV, A-V4 and A-V7 were tested on 0-7, 9, 10 and 14 dpc; animals in groups A-UVC, A-UVC4 and A-UVC7 were tested on 1, 3, 5, 7, 9, 10 and 14 dpc). The actual values are indicated in Supplementary Tables 1–4).

positive with three of the nasal swabs positive at 9 dpc. In contrast, viral RNA was only detected in faecal swabs in three samples (in two pigs at 2 dpc and in one pig at 8 dpc; Table 2; Supplementary Table 3). No RNA was found in samples collected from pigs in group A-UVC.

In group A-V4, four of the eight pigs had positive samples: one nasal and one saliva sample tested positive at 3 dpc, followed by another nasal swab at 4 dpc and, finally, a saliva sample at 7 dpc. No RNA was detected in the faecal swabs. In contrast, RNA was detected in only the nasal swabs of all five indirect contact pigs (group A-UVC4) between 3 and 10 dpc (Table 2; Supplementary Table 1).

Viral RNA was present in all pigs of group A-V7 at 3 dpc, and two pigs had RNA in their nasal swabs at 8 dpc. Four pigs had low levels of viral RNA in their faecal swabs 3–5 dpc (Table 2, Supplementary Tables 1–3). All swabs collected from the contact pigs in group A-UVC7 were negative.

3.4. Detection of viral RNA in the blood

Viral RNA was detected only in pigs of groups A-UV and A-V7 (Table 2; Supplementary Table 4). In seven of the eight

unvaccinated and infected pigs (A-UV), RNA was detected between 2 and 10 dpc, while pig #1 tested negative, although it had clinical disease (Table 1). In group A-V7 viral RNA was detected in six of the pigs between 1 and 10 dpc with only two animals having a number of consecutive days positive. The infected pigs in group A-V4 and those in contact groups A-UVC, A-UVC4 and A-UVC7 did not have detectable RNA in the blood.

3.5. Serological response in the study animals

All the pigs were sero-negative on the day of challenge as determined by the LPBE except three of the eight animals that were vaccinated seven days prior to challenge (Group A-V7; Fig. 1). By 5 dpc, seven of the unvaccinated pigs had antibodies, five of the pigs in group A-V4 and all eight in group A-V7. All the infected animals were positive by 10 dpc. The indirect contact animals did not have any detectable antibodies (results not shown).

None of the pigs had antibodies to the non-structural proteins using the c-ELISA until 7 dpc when five of the pigs in group A-UV and one in A-V4 sero-converted (Fig. 1). By 14 dpc six of those in group A-UV were sero-positive, and two more in A-V4 demonstrated antibodies as well as one in group A-V7.

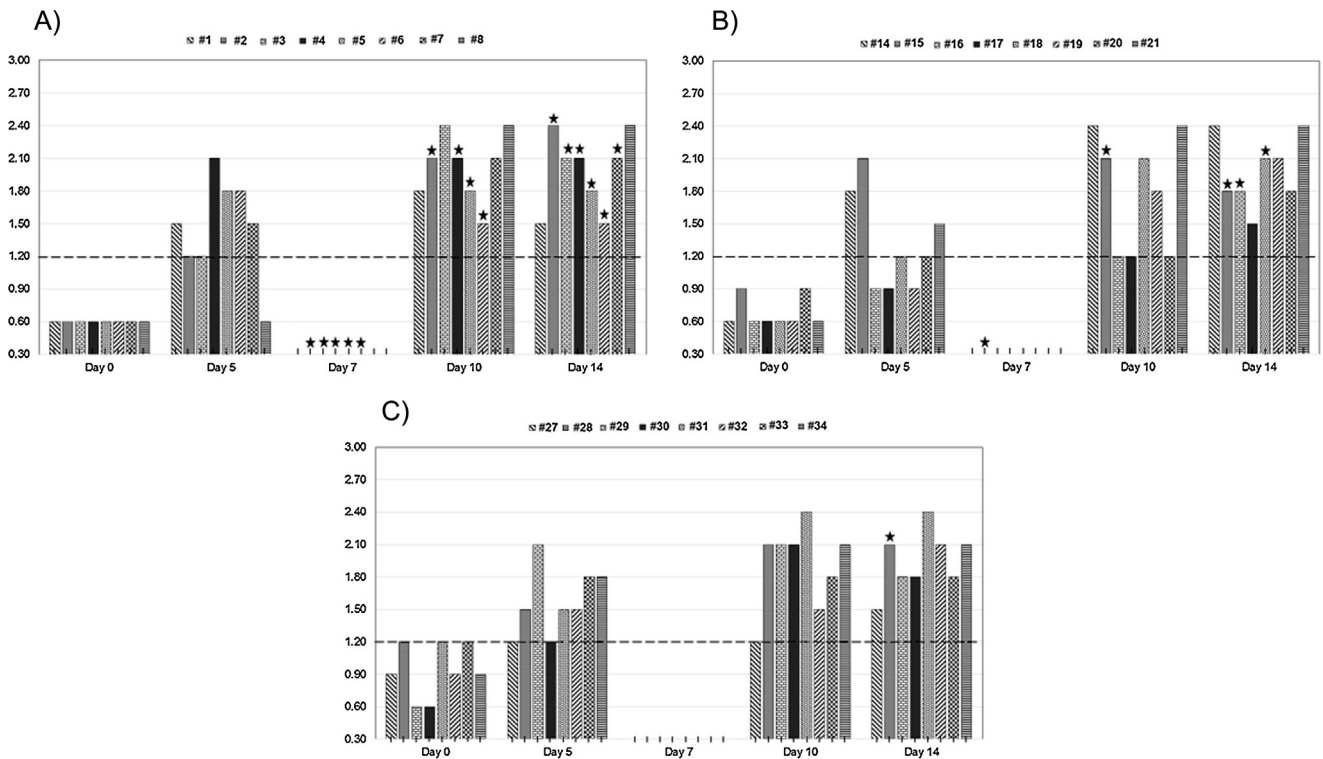


Fig. 1. Serological results showing the antibody titres determined by LPBE for pigs that were (a) unvaccinated and challenged (A-V4); (b) vaccinated and challenged 4 dpv (A-V4); and (c) vaccinated and challenged 7 dpv (A-V7). The broken line indicates the cut-off value for declaring positive samples according to the guidelines in the OIE Manual (2012). No antibodies were detected in pigs from groups A-UVC, A-VC4 and A-VC7. Stars indicate animals that had antibodies to the non-structural proteins. LPBE was not performed on Day 7 samples.

3.6. Comparison of the amount of viral RNA in various excretions

Pigs in group A-UV had significantly more virus than all other groups in nasal ($p < 0.05$) and saliva ($p < 0.05$) swabs. Viral RNA in blood was infrequently identified, but group A-UV had significantly more compared to A-V4 ($p < 0.05$). Group A-V7 also had significantly more RNA in the blood compared to all groups, except A-UV ($p < 0.05$). There were no significant differences among groups in the amount of viral RNA detected in faecal samples. Group A-UV had significantly more RNA compared to the other groups when evaluated over all specimen types (Table 3). Faecal samples had significantly less viral RNA compared to saliva and nasal swabs when evaluated over all groups.

4. Discussion

In the present study, a $>6 \text{ PD}_{50}/\text{dose}$ A/MAY/97 vaccine was used to determine its protective ability in pigs 4 and 7 dpv against the variant A SEA-97 strain, A/VIT/08/2005. Vaccine efficacy against this variant virus had not been tested previously. Generalisation to other sites, such as the uninoculated feet, or mouth, was not observed in any of the vaccinated pigs, indicating that the vaccine protected against clinical disease. Two of the pigs vaccinated 7 days prior to infection had lesions on the coronary band of the inoculated feet at 5 and 7 dpc, respectively, but no other lesions were noted. The convention when reading protection during vaccine challenge studies is to only score lesions on un-inoculated feet, or the mouth. Therefore, it is uncertain whether the lesions on the coronary band, away from the inoculation site, represented generalised disease. Only one of these pigs (#29) had viral RNA in the blood. The clinical scores for both these two pigs were 1, compared to the unvaccinated and challenged pigs where the scores ranged from 2 to 6. Six of the pigs in group A-V7 had viral RNA in the blood. Three of the

pigs only had detectable RNA in the blood at 5–7 dpc, suggesting infection by cohorts, rather than by direct inoculation. None of the pigs vaccinated four days prior to challenge had any viral RNA in the blood.

Only three of the pigs vaccinated seven days prior to challenge had detectable antibodies using the LPBE at the time of challenge. All the other pigs were sero-negative. Protection has been observed in other cases with low or undetectable antibody levels [21–24]. Barnett et al. [22] argued that in the absence of specific antibodies, innate immune responses could be the first line of defence against viral intrusion since the majority of viral infections preferentially induce the production of Type 1 interferons. However, there is also evidence that the swine innate response is inhibited by FMDV infection [25–27]. This study did not investigate the role of innate and early adaptive immune responses post vaccination and infection, but these could explain why pigs that were vaccinated four days prior to challenge were protected in the absence of antibodies [23,28,29]. Guzman et al. [30] suggested that such animals may be protected because of cell-mediated immune responses. Levels of several cytokines (IL-6, IL-8 and occasionally IL-12) increase soon after a single application of a high potency vaccine in pigs [31,28] and some evidence exists that IL-6 might increase the odds of protection against challenge [32]. In addition, it was previously shown that IgM peaked in vaccinated pigs by 7 dpv [33], and it is therefore possible that the protection we observed could in part have been contributed to this low specificity and high avidity arm of the adaptive immune response.

High levels of virus excretion in unvaccinated pigs infected by intra-dermal injection with a high challenge dose, causing severe clinical signs, are common in challenge infections in pigs [34]. Whereas pigs vaccinated with a regular vaccine ($3 \mu\text{g}/\text{dose}$ payload of O Taiwan 146S antigen) were protected by 14 dpv [35]. In our study there was no clinical or serological evidence to

Table 3
Multivariable model results evaluating the effect of treatment group on the quantity of viral RNA recovered from whole blood and saliva, nasal, and faecal swabs.

Variable	Estimate (95% CI)	t statistic	P value
Experimental group			
Contact with unvaccinated (A-UVC)	−1.41 (−1.70, −1.11)	−9.401	<0.001 [†]
Contact with vaccinated after 4 days (A-UVC4)	−1.23 (−1.53, −0.94)	−8.238	<0.001
Contact with vaccinated after 7 days (A-UVC7)	−1.41 (−1.70, −1.11)	−9.401	<0.001
Vaccinated 4 days prior to exposure (A-V4)	−1.40 (−1.65, −1.15)	−10.934	<0.001
Vaccinated 7 days prior to exposure (A-V7)	−0.59 (−0.84, −0.34)	−4.622	<0.001
Unvaccinated exposed (A-UV)	Referent		
Sample type			
Nasal swab	−0.05 (−0.31, 0.21)	−0.378	0.706
Saliva swab	0.07 (−0.19, 0.33)	0.528	0.598
Faecal swab	−0.54 (−0.80, −0.28)	−4.129	<0.001
Whole blood	Referent		

CI = confidence interval.

[†] Overall test for a difference among all treatment groups. Other P values represent the comparison of individual groups to the referent.

suggest that transmission occurred between the infected pigs and the unvaccinated indirect contact pigs in the same room. The challenged animals in groups A-UV, A-V4 and A-V7 were excreting viral RNA, but at significantly different levels ($P < 0.05$) between 2 and 9 dpc. No detectable RNA could be found in pigs of groups A-UVC or A-UVC7. On only four occasions were low levels of RNA found in oral and nasal swabs of group A-V4 between 3 and 7 dpc, while all five contact pigs had very low levels of RNA in their nasal swabs between 3 and 10 dpc in the absence of clinical disease or sero-conversion (Supplementary Table 1). This could indicate that the pigs were inhaling virus, but not sufficient quantities to cause infection.

Pigs are known to be refractory to infection via the respiratory route and an infectious aerosol dose of 2,500 TCID₅₀ is required to establish disease in close to 100% of experimentally infected pigs [36]. Recently, Gonzales [37] concluded that an infectious aerosol dose of 3300 and 3900 TCID₅₀ is needed to establish infection and disease, respectively, in pigs. Transmission is therefore not expected to occur if physical barriers are used to prevent infected pigs from making direct contact with susceptible pigs, and if measures are taken to prevent the mechanical transfer of virus [36]. In our study, vaccinated pigs excreted 100-fold less viral RNA for a short duration (3–6 dpc) when compared to the unvaccinated pigs (2–9 dpc), and there was no transmission to the indirect contact pigs, probably because of the physical separation and strict biosecurity measures regarding personnel movements and fomite transmission. Similar observations were made with pigs that were vaccinated with O₁ Manisa vaccine and challenged with an O Mya-98 lineage virus, where vaccinated animals did not transmit disease to neighbouring pigs despite RNA being detected in the oral and nasal swabs of the in-contact pigs [38]. Eble et al. [39] also found that separation of pigs lowered the transmission rate. This clearly shows that vaccination, along with efficient biosecurity measures, should prevent transmission of FMD between pens if the animals are not in direct contact.

The results of the statistical model suggested that the amount of viral RNA detected in all in-contact groups was independent of vaccination status. However, vaccination was effective at reducing the amount of viral RNA detected relative to unvaccinated controls. Therefore, this is further evidence that the aerosol route of infection might not be important for FMDV spreading among pigs. The model also suggested that the amount of viral RNA shedding in A-V4 was significantly less than A-V7 (based on non-overlapping 95% confidence intervals). The reasons for this observation are difficult to explain and might have been due to individual variability among the susceptibility of pigs, challenge dose administration, or vaccination. This relative effect was observed in all specimen types suggesting that data management or laboratory errors were unlikely the reason for this finding. The statistical model also

suggested that whole blood, oral swabs and nasal swabs contained similar levels of viral RNA, which was higher than what was detected in faecal swabs. All specimen types, other than faecal swabs, would therefore appear to be suitable for monitoring the infection status of pigs.

Extrapolation of experimental results to the field is always difficult and the efficacy of a given vaccine may differ from what is observed experimentally [40]. Considering this, when used in conjunction with biosecurity and movement restrictions, a single vaccination with A/MAY/97 may be effective under field conditions in pigs challenged with the A SEA-97 lineage to lower virus excretion and assist in eradication of the virus.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2015.07.014>

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