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Reduction of foot-and-mouth disease (FMD) virus load in nasal excretions, saliva and exhaled air of vaccinated pigs following direct contact challenge

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Abstract

In future, a policy of "vaccinate-to-live" may be included in the repertoire of foot-and-mouth disease (FMD) control measures and in support of this approach, we have investigated the hypothesis that vaccine-induced reduction in virus replication and excretion from pigs can be correlated to the severity of clinical signs of FMD by measuring excretion of virus in natural secretions and aerosols. The other aims of this study were to verify the existence of sub-clinical infection in vaccinated pigs, to evaluate the correlation between this and seroconversion to foot-and-mouth disease virus (FMDV) non-structural protein antibodies and to re-examine the occurrence of FMDV persistence in the oro-pharynx of pigs. Therefore, pigs were vaccinated (O1 Manisa) and challenged (O1 UKG) in a manner calculated to produce a broad range of clinical outcomes and were monitored for a minimum of another 33 days post-challenge. Eighty-one percent of the early (10 days vaccinated) challenged pigs and 25% of the late (29 days vaccinated) challenged pigs were clinically infected and all other vaccinated pigs were sub-clinically infected. Although vaccination could not provide complete clinical or virological protection, it reduced the severity of the disease, virus excretion and production of non-structural FMDV antibodies in vaccinated and subsequently infected pigs. As hypothesised, vaccine-induced reduction of virus replication and excretion was found to be correlated to the severity of clinical disease. RNA copies, but no live virus was detected from the pharyngeal and soft palate tissues of a minority of vaccinated and infected pigs beyond the acute stage of the infection.

Keywords: FMD virus excretion; FMD emergency vaccination; Direct contact challenge

1. Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease caused by FMD virus (FMDV), an apthovirus within the picornavirus family that infects all cloven hoofed animals [1]. Susceptible livestock may be infected with FMDV by direct or indirect contact with infected animals. When infected and susceptible animals are in close proximity, aerial transfer of droplets and droplet nuclei is probably the most common mode of transmission [2]. Though pigs are less susceptible to FMDV infection by the airborne route when compared to ruminants, they excrete more airborne FMD virus [2,3]. Consequently, a common pattern of airborne FMD spread is from pigs to cattle, sheep and goats downwind [4,5].

Since the 2001 FMD outbreak in the UK, there has been renewed interest in Europe to use vaccination as a means of reducing reliance on culling of animals. Consequently, the new European council Directive 2003/85/EC on FMD has made provision for vaccination and the use of post-vaccination serosurveillance to detect sub-clinical infection [6]. The extent of reduction of virus load in natural secretions such as saliva and nasal fluids and in exhaled air from vaccinated and subsequently infected pigs in comparison to unvaccinated infected pigs are crucial parameters for predicting the likelihood of spread from animal to animal on

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an affected farm as well as airborne FMDV spread from pig farms to cattle, sheep and goat farms. The degree of vaccine-induced clinical protection afforded to pigs may correlate to protection against virus replication and excretion and it would be useful to know if this so, as clinical signs are relatively easily observed and could then be used to predict the likelihood of virus shedding and spread of disease.

Long-term sub-clinical infection with FMDV has also been demonstrated in ruminants in which live virus could be detected within the oro-pharynx beyond 28 days of infection and these viral "carriers" [7,8] may be found amongst vaccinated and subsequently challenged animals [7–9]. Since carriers may be considered a risk for transmitting infection [10], they must be identified by post-vaccination surveillance to substantiate freedom from infection [6,11] to regain the FMD-free status for the purpose of international trade. However, in cattle it has been seen that though the virus could be isolated up to 57 [14] or 98 [15] days post-challenge from vaccinated and challenged animals, introducing naïve cattle for direct contact with these carrier animals could not transmit the disease. Pigs are considered to clear virus rapidly and not to become carriers [10], although two publications [12,13] contest this. A proper investigation of virus persistence in oro-pharyngeal fluid (collected by probang cup) and in pharyngeal tissues of pig after 28 days post-challenge has not been reported in the literature, possibly due to the difficulty of maintaining infected pigs due to the severity of clinical signs in this species.

To address these questions, a vaccination challenge study in pigs was designed and carried out so as to produce a range of clinical outcomes and allow testing of the hypotheses that vaccinated pigs can become sub-clinically infected and that vaccine-induced reduction in virus replication and excretion can be correlated to the severity of clinical signs of FMD. The opportunity was also taken to evaluate the production of non-structural antibodies to FMDV in sub-clinically affected pigs. Use of vaccine to ameliorate the clinical effects of challenge also enabled us to keep some of the pigs alive after experimental challenge and to examine the persistence of FMDV in the oro-pharynx before and after death.

2. Materials and methods

2.1. Animals

Thirty-six large white × landrace pigs initially weighing 20–25 kg were used for immunisation and challenge in this study. All animals were housed in disease-secure accommodation at IAH, Pirbright.

2.2. Immunisation and challenge protocol

Twenty-four pigs were initially housed and vaccinated in a clean isolation unit in which FMDV is not handled and subsequently exposed to FMDV in a challenge unit, at 10 or 29 days post-vaccination. Vaccination was done with full bovine doses of 1/1 antigen payload of O1 Manisa oil adjuvant vaccine with a previously determined 50% potency for cattle (PD_{50}) of 18. Control and donor pigs were also housed in the clean unit until moved for challenge.

2.2.1. Challenge group one (Gr-1)

Sixteen pigs vaccinated 10 days previously were housed in two pens of eight animals along with two unvaccinated control pigs per pen. All twenty pigs were challenged by 9 h of direct contact with two donor infected pigs per pen that had been inoculated intradermally in the heel bulb at 48 h pre-challenge with 0.2 ml of pig passaged FMDV O UKG ($10^{5.7}$ $TCID_{50}$). After the challenge, the donors were separated and killed and the unvaccinated pigs were removed and housed separately.

2.2.2. Challenge group two (Gr-2)

The procedure was as for Gr-1 except that a single pen was used containing eight pigs vaccinated 29 days before challenge with two unvaccinated control pigs. Out of a total of 24 vaccinates and six control pigs, eight vaccinates and all of the controls had to be killed humanely on ethical grounds, within the first week of challenge, due to the onset of severe signs of FMD. Shedding of hooves was considered as end point termination. The remaining 16 vaccinated pigs were monitored for at least 57 days post-vaccination. Rectal temperatures and clinical scores were recorded for up to 9 days post-challenge. Elevated temperature more than 39.5 °C and congestion of skin in the inter-digital space and coronary band region were scored as 1 whereas fresh lesions on the tongue, snout or feet were scored as 2. Severe lesions were scored as 3 whereas healed lesions were scored as 1.

2.3. Challenge virus

Challenge virus O UKG FMDV 34/2001 was prepared as previously described [16].

2.4. Sample collection and processing

Heparinised and clotted blood, saliva, nasal and oro-pharyngeal fluids and exhaled air samples were collected from the pigs for detection of virus and/or antibodies. Small-sterilised cotton buds were used to collect nasal and saliva secretions daily up to 16 days post-challenge and thereafter weekly intervals in 1 ml of PBS or 0.5 ml of Trizol (Gibco BRL) for virus isolation and real-time RT-PCR, respectively. To detect viraemia, 0.2 ml of heparinised blood was mixed with 0.3 ml of lysis buffer (Roche) for analysis by real-time RT-PCR and stored at −70 °C together with 1 ml of untreated heparinised blood for virus isolation. Oro-pharyngeal fluids were collected from the upper oesophagus and pharynx with a probang sampling cup at 28, 33, 41 and 48 days post-challenge from the Gr-1 pigs and at 29 and 33 days post-challenge from the Gr-2 pigs. The probang cup used

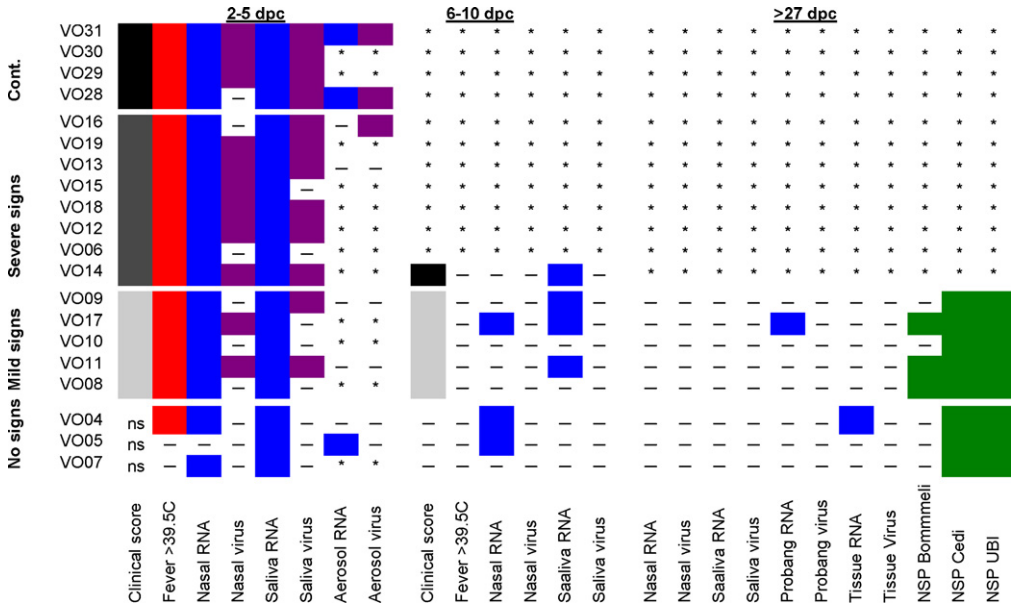

Fig. 1. Comparative clinical and virological results of Gr-1 pigs following challenge. ns: no clinical signs; (*) samples not collected; (−) negative. Black colour indicates severe clinical signs; gray: mild clinical signs. Red, blue, purple and green colours indicate temperature >39.5 °C, detection of viral RNA, detection of live virus and non-structural seroconversion, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

for cattle was modified so as to have a smaller cup for the proper movement inside the throat of pigs. Pigs were sedated with meloxicam (Metacam® 20 mg/ml, Boehringer Ingelheim) at an intramuscular dose of 0.4 mg/kg, 15–30 min before probang sample collection. The samples were collected by introducing the probang cup in to the pharynx of the standing animal. Immediately after collection, 0.2 ml of oropharyngeal fluid (OP fluid/probang) was mixed with 0.3 ml of lysis buffer (Roche) and stored at −70 °C for future analysis by real-time RT-PCR. The rest of the OP fluid was stored untreated at −70 °C for virus isolation. At the termination of the experiment (48 days post-challenge for the Gr-1 pigs and 33 days post-challenge for the Gr-2 pigs), 20 mg tissue samples from 10 representative areas of pharynx or of dorsal and ventral soft palate were collected in 1 ml of RNA later (Ambion, Warrington, UK) for RT-PCR to identify the persistence of virus in the these tissues. Parallel sets of tissues were also frozen in maintenance medium at −70 °C for subsequent virus isolation.

2.5. Air sampling

Four FMD aerosol measurements were taken in the pens on the day of challenge to measure the amount of challenge virus excreted from donor pigs. Sampling was done by using a glass cyclone sampler [17] and a porton glass impinger [18].

Air samples were also collected from individual challenged pigs that had been vaccinated or were unvaccinated (for individual animals see Figs. 1 and 2), by introducing each pig into a 610 L air sampling cabinet [19] located in

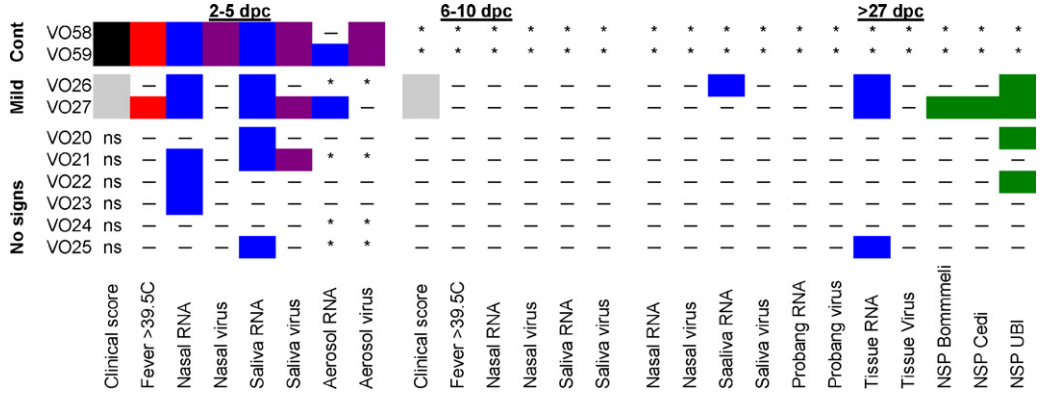

Fig. 2. Comparative clinical and virological results of Gr-2 pigs following challenge. ns: no clinical signs; (*) samples not collected; (−) negative. Black colour indicates severe clinical signs; gray: mild clinical signs. Red, blue, purple and green colours indicate temperature >39.5 °C, detection of viral RNA, detection of live virus and non-structural seroconversion, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

a separate pen. Measurements of virus/ RNA copy were as described [4,20,21].

2.6. Virus isolation

Samples for virus isolation were inoculated onto BTY cells [22] in roller drums. Antigen ELISA was used to confirm the presence of FMD virus in cultures. For isolation of virus from air samples, a similar protocol was followed after titrating samples in a 10-fold dilution series such that 3 BTY monolayer tubes were inoculated with neat (undiluted), 10^{-1} and 10^{-2} dilutions of air samples.

2.7. RNA extraction from liquid samples

Total nucleic acid was extracted from liquid samples with MagNA pure LC total nucleic acid isolation kits (Roche) using an automated nucleic acid robotic work station (Roche) [24]. QIAamp® MinElute® Virus Spin kits (Qiagen) were used for RNA extraction from air samples according to the manufacturer's instructions. Briefly, RNA was extracted from 200 μl of the original samples and a final volume of 18 μl RNA was recovered at the end of the process. This material was used for real-time RT-PCR as described below.

2.8. RNA extraction from tissues

Twenty milligrams of tissues were homogenized by placing them in 600 μl of tissue lysis buffer (Roche) in Lyzing Matrix D tubes (Q-Biogene, Cambridge, UK) and homogenizing them at 6500 revolutions per minute for 45 s, three times in a FastPrep FP120 homogenizing machine (Q-Biogene). RNA was extracted and eluted in 50 μl elution buffer using the MagNA Pure LC RNA isolation kit III (Roche) with an automated robot as described above. Extracted RNA was stored at −80 °C until used.

2.9. Real-time RT-PCR

Viral RNA in samples was reverse transcribed [23] using random hexamers and quantified by real-time RT-PCR using primers and a probe from the internal ribosomal entry site (IRES) of FMDV O UKG 34/01[24]. A Stratagene MX4000 PCR machine was used. The c-DNAs obtained from the tissues were reanalysed for real-time PCR by using the probe and primers from the 3D region of FMDV [25,26] following the above described methods without use of standards.

2.10. Non-structural protein serology

Sera were examined for antibody against non-structural FMDV proteins by three commercial tests, i.e. Cedi test FMDV-NS, Cedi-Diagnostics [27]; the UBI FMDV NSP ELISA, United Biochemical Incorporated [28] and the CHEKIT-FMD-3ABC, Bommeli Diagnostics [29].

2.11. Virus neutralising antibody test (VNT)

Titres of neutralising antibodies against FMDV O1 Manisa and O1 UKG viruses were measured by micro-neutralisation assay as described in the OIE Manual of Diagnostic Tests and vaccines [30].

2.12. Statistics

The RNA copy numbers in different groups (control, severe signs, mild signs and no signs) of pigs were compared at each time point using a Kruskal–Wallis test and, where significant differences ($P < 0.05$) were found, using multiple contrasts. Animals vaccinated 10 days before challenge were also compared with those vaccinated 29 days before challenge, irrespective of the severity of clinical signs.

All vaccinated and control pigs ($n = 30$) were regrouped according to severity of infection (controls, vaccinated with severe signs, vaccinated with mild signs and vaccinated with no signs) and a Fisher exact test was used to detect differences between the proportion of pigs in each group for which RNA or virus was detected in the nasal fluids, saliva and aerosol, or which were positive by NSP Bommeli test.

The results of the virus neutralisation (VN) test for animals vaccinated 10 days prior to challenge were compared with those vaccinated 29 days prior to challenge at each time point using a Wilcoxon rank sum test.

3. Results

3.1. Development of clinical FMD

The six needle challenged donor pigs in both the groups showed clinical disease within 1–2 days post-infection. Unvaccinated control pigs succumbed to disease within 1–2 days of contact (Tables 1 and 2). The majority ($n = 13$) of directly challenged vaccinated pigs of Gr-1 were clinically infected at the same time as their controls within 1–2 days of contact challenge (Tables 1 and 2). However, the clinical scoring in these vaccinated clinically infected pigs was always lower than the control pigs (Table 1) except one unvaccinated animal (VO28). According to degree of severity of disease, the vaccinated pigs were regrouped as severe ($n = 8$), mild ($n = 5$) and no signs ($n = 3$) (Tables 1 and 2, Figs. 1 and 2). Increase of temperature was observed on the first day of challenge and 1 day thereafter in Gr-1 vaccinated pigs (Table 2). By the fourth day of challenge temperatures had returned to normal in all animals except VO12 and 19.

Out of eight vaccinated pigs in group two (Gr-2), only two (VO26 and 27) were clinically affected with mild signs, observed on or after the third day of contact challenge; a delay of 1–2 days compared to clinically affected Gr-1 vaccinated pigs.

High temperatures preceded the development of lameness that was associated with congestion in the inter-digital space

Table 1
Total clinical scoring of vaccinated and unvaccinated pigs (Gr-1 and -2) following direct contact challenge

Category	Animal no.	Group 1					Animal no.	Group 2				
		0 dpc	1 dpc	2 dpc	3 dpc	4 dpc		0 dpc	1 dpc	2 dpc	3 dpc	4 dpc
Unvaccinated (control)	VO31	0	3	17	15	16	VO58	0	3	13	12[b]	
	VO30	0	1	13	14	16	VO59	0	3	13	12[b]	
	VO29	0	0	13	13	16						
	VO28	0	1	10	7	7						
Vaccinated												
Severe	VO16	0	3	10	10	14						
	VO19	0	1	6	12	13						
	VO13	0	2	8	9	13						
	VO15	0	0	7	7	9						
	VO18	0	0	2	5	9						
	VO12	0	0	5	5	8						
	VO06	0	1	4	4	7						
	VO14	0	3	3	5	6						
Mild signs	VO09	0	2	2	4	4	VO26	0	0, 1[a]	1[a]		3
	VO17	0	0	2	3	4	VO27	0	0, 1[a]		3	3
	VO10	0	1	1	2	2						
	VO11	0	2	2	1	2						
	VO08	0	0	1	1	2						
No signs (Protected)	VO04	0	0	0	0	0	VO20	0	0	0	0	0
	VO05	0	0	0	0	0	VO21	0	0	0	0	0
	VO07	0	0	0	0	0	VO22	0	0	0	0	0
							VO23	0	0	0	0	0
							VO24	0	0	0	0	0
							VO25	0	0	0	0	0

Clinical scoring was carried out by considering the following. Rectal temperature more than 39.5 °C, congestion of skin in inter-digital space and coronary band, healed lesions in each foot were scored as 1; fresh lesions in tongue, snout, each foot were scored as 2; severe lesions in tongue, snout and in each foot were scored as 3. Total scoring was calculated by adding individual scores. dpc: days post-challenge.

[a] The clinical scoring is due to lameness without showing any lesions.

[b] The animal was killed.

and on the coronary band. Vesicular lesions appeared a few hours later. Foot lesions appeared before those in the mouth, tongue and snout and were more severe.

3.2. Detection of virus/genome in nasal, saliva and blood samples

Detection of virus/genome, as determined by virus isolation and/or RT-PCR on nasal fluids, saliva or blood samples, was evident in all of the unvaccinated and vaccinated pigs of both the groups (Figs. 1 and 2, Table 3). From one animal (VO24) in Gr-2, no viral RNA was recovered from either nasal (Table 3) or saliva swabs, but live virus was isolated from blood (data not shown). From nasal swabs collected from the Gr-1 pigs, virus could be isolated up to day 3 after challenge, irrespective of vaccination status whereas viral RNA was detected for longer; up to 13 days in one vaccinated pig (VO4, Table 3). Although virus could be isolated up to 3 days after challenge from nasal swabs from both the control pigs in Gr-2, no virus was recovered from nasal swabs of vaccinated animals (Fig. 2). Viral RNA was detected less frequently in nasal swabs of Gr-2 vaccinated pigs than the nasal swabs of Gr-1 vaccinated pigs and the frequency of detection of viral RNA was found to be decreased in both the groups after the first week of challenge (Table 3). However, viral RNA from nasal swab samples was detected at 11 and 21 days post-challenge in vaccinated pigs VO22 and VO25, respectively (Table 3).

The similar quantities of viral RNA initially detected from nasal swabs of all vaccinated and control pigs in Gr-1 (no significant difference on day of challenge, $P > 0.05$) demonstrated that similar amounts of virus had been inhaled during the first 4 h of contact challenge (Fig. 3). By the day after challenge, only significant differences were found ($P = 0.04$), with the vaccinated, mild clinical signs group having a significantly higher copy number than the vaccinated, clinically protected group. At 3 days post-challenge the unvaccinated group had a significantly higher copy number than the vaccinated, clinically protected group ($P = 0.02$). Finally, significant differences were found at 4 days post-challenge ($P = 0.02$), but were not confirmed by the multiple contrast analysis. Viral RNA copy number was reduced in all vaccinated animals irrespective of severity of clinical signs by 4 days post-challenge, except for the unvaccinated control group where the RNA copy number was increased (Fig. 3).

Again, there were no significant differences in RNA copy numbers at 0 day post-challenge ($P > 0.05$) in Gr-2 pigs. Although a higher copy number of RNA was detected in

Table 2
Rectal temperature of vaccinated and unvaccinated pigs (Gr-1 and -2) following direct contact challenge

Category	Animal no.	Group 1					Animal no.	Group 2				
		0 dpc	1 dpc	2 dpc	3 dpc	4 dpc		0 dpc	1 dpc	2 dpc	3 dpc	4 dpc
Unvaccinated (control)	VO31	38.6	39.6	40.6	38.4	38.5[b]	VO58	38	38.8	40	40.5[a]	
	VO30	38.7	40	40.5	39.1	38.8[b]	VO59	38.4	38.7	40.3	40[a]	
	VO29	38.6	38.6	39.9	37.5	38.6[b]						
	VO28	38.7	38.7	40.5	38.5	38.5[b]						
Vaccinated												
Severe	VO16	38.2	40.7	39.5	38.5	38.9[b]						
	VO19	38.5	38.8	40.4	40.3	39.7[c]						
	VO13	38.5	39.8	39.6	40.1	38.4[b]						
	VO15	38.6	40	40	39.9	38.6[c]						
	VO18	38.4	39.9	39.9	40.2	38.9[c]						
	VO12	38.5	40	40.3	40.3	39.2[c]						
	VO06	38.6	40.7	39.5	38.4	38.2[c]						
	VO14	38.5	39.7	40.1	40.5	38.5[d]						
Mild signs	VO09	38.6	39.8	40	38.4	38.8	VO26	38.5	39.2	39	38.7	38.5
	VO17	38.5	40.2	40.2	38.7	38.8	VO27	38.3	38.3	40	40	38.6
	VO10	38.5	39.9	39.5	37.5	38.3						
	VO11	38.6	39.3	40.2	38.7	38.5						
	VO08	38.4	39.6	40	37.7	38.2						
No signs (Protected)	VO04	37.9	40	38.5	37.5	37.9	VO20	38	39.4	38.5	38.7	38.3
	VO05	38	39.4	39.5	38.5	38	VO21	38.2	38.2	38.8	38.5	39
	VO07	38.6	39	39	39	39.3	VO22	38.2	38.4	38.7	38.3	38.8
							VO23	38	38	38.8	39	38.7
							VO24	38.5	38.9	38.6	38	38.9
							VO25	38.5	39.5	39.2	39.1	39.5

[a] Indicates the animal was killed on 3 dpc.
[b] Indicates the animal was killed on 4 dpc.
[c] Indicates the animal was killed on 5 dpc.
[d] Indicates the animal was killed on 6 dpc.

the nasal swabs of control animals than of the vaccinated Gr-2 pigs (Table 3), no significant differences ($P > 0.05$) in RNA copy numbers were found at 1 or 3 days post-challenge. At 2 days post-challenge, significant differences were found ($P = 0.04$), with the unvaccinated group having a significantly higher copy number than both the vaccinated, clinically protected and the vaccinated, mild clinical signs groups.

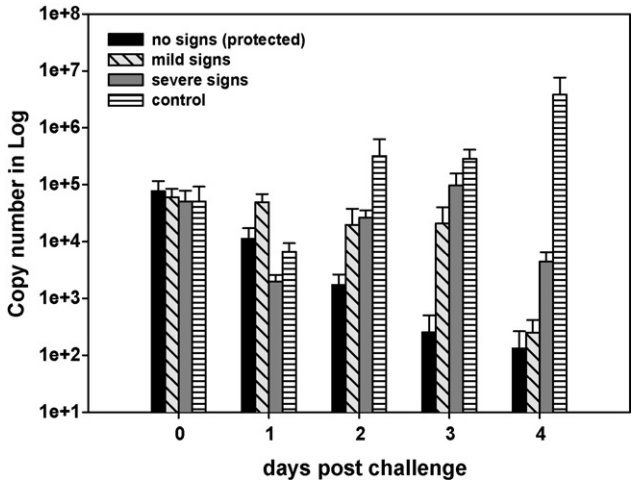

Fig. 3. Mean FMDV RNA copy number detected over time by real-time RT-PCR from cotton bud samples collected from nose of Gr-1 pigs.

When comparing the viral RNA copy number for animals vaccinated 10 days prior to challenge with those vaccinated 29 days prior to challenge, significant differences ($P < 0.01$) were found at 2 and 4 days post-challenge, with the copy number for Gr-1 vaccinated pigs higher than for Gr-2 vaccinated pigs. No significant differences ($P > 0.1$) were found at 1 and 3 days post-challenge.

Live FMD virus could not be detected in saliva samples from vaccinated animals irrespective of clinical signs beyond the second day after challenge except on the fourth day from two pigs in Gr-2 from a vaccinated and clinically affected pig (VO27) and a vaccinated clinically protected pig (VO21) (Fig. 2). Beyond 4 days after challenge, all vaccinated clinically protected animals in Gr-1 and 2 were negative for viral RNA in saliva samples (Figs. 1 and 2). RNA copy/virus was detected only in vaccinated clinically affected animals up to 1 week after challenge in Gr-1 pigs where as up to 4 days after challenge in Gr-2 vaccinated pigs. On two occasions, viral RNA could be detected from convalescent saliva swabs at 21 days post-challenge and 28 days post-challenge from animals VO22 (data not shown) and VO26 (Fig. 2), respec-

Table 3
RNA copy number calculated in real-time PCR per one cotton bud sample from the nose of different groups of pigs

		1dp	2dp	3dp	4dp	5dp	6dp	7dp	8dp	9dp	10dp	11dp	12dp	13dp	14dp	15dp	16dp	21dp	28dp	30dp
Controls	**Animal ID**																			
	VO28	3.3	3.7	4.2	2.7															
	VO29	3.3	3.8	5.7	7.2															
	VO30	4.1	6.1	5.7	3.9															
	VO31	4.0	4.4	5.1	3.3															
	VO58*	5.0	5.3	4.8																
	VO59*	5.0	5.6	4.9																
Severe signs																				
	VO06	3.6	0.0	0.0	3.2	0.0														
	VO12	3.6	4.3	4.4	3.9	3.2														
	VO13	3.5	3.8	3.8	3.2															
	VO14	3.3	4.2	5.1	3.8	0.0	0.0													
	VO15	0.0	4.1	4.9	4.2	4.2														
	VO16	3.2	4.9	3.6	0.0															
	VO18	2.9	4.6	4.4	3.0	0.0														
	VO19	0.0	4.6	5.7	2.0	0.0														
Mild signs																				
	VO08	5.1	3.5	2.9	2.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO09	4.7	0.0	3.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO10	4.6	0.0	0.0	2.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO11	4.5	3.4	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO17	3.4	5.0	5.0	0.0	3.6	3.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO26*	2.4	0.0	0.0	0.0	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO27*	4.1	0.0	0.0	0.0	3.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
No signs																				
	VO04	0.0	3.3	2.9	2.6	0.0	0.0	0.0	0.0	0.0	3.8	0.0	0.0	1.6	0.0	0.0	0.0	0.0	0.0	0.0
	VO05	4.1	0.0	0.0	0.0	0.0	0.0	3.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO07	4.3	3.5	0.0	0.0	3.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO20*†	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO21*	0.0	0.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO22*	4.0	0.0	4.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO23*	0.0	3.3	3.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO24*††	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VO25*	4.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.3	0.0	0.0

RNA copy numbers are expressed in $\log_{10}$ copies per cotton swab. *The animals from Gr 2 pigs, dpc indicates days post-challenge, gray shadings indicates the animals were killed. †Indicates the animal is found positive for detection of RNA in saliva swab sample. ††The animal was found virus isolation positive from blood.

tively. The proportion of animals detected positive in PCR was less in saliva samples than from the nasal samples (data not shown).

3.3. Detection of virus/genome in exhaled air samples

Air sampling within the challenge pens followed by virus isolation and real-time RT-PCR revealed a consistent challenge in the two experiments. Total output over the 9 h of challenge was 5.5 log $TCID_{50}$ virus and 7.1 log copies of viral genome from the two donor pigs for Gr-1 and 5.4 log $TCID_{50}$ virus and 6.7 log copies of viral genome for Gr-2.

For Gr-1, individual pig sampling using a cabinet and a Porton sampler detected viable virus in both control pigs at 2 days post-challenge (dpc) at levels of 4.4 log $TCID_{50}$/24 h in pig VO28 and 6.1 log $TCID_{50}$/24 h in pig VO31. RT-PCR detected 7.0, 7.8, 6.2 log RNA copies/24 h of FMD virus in control pig VO28 on days 2–4 post-challenge and 7.8 log copies/24 h at 2 dpc only from control pig VO31. In contrast, viable virus was detected only from one of the vaccinated pigs (VO16-severely affected), whilst 3.6 log RNA copies/24 h were detected at day 2 post-challenge from another vaccinated and subsequently infected pig (VO5).

For Gr-2, viable virus was detected in one of the control pigs (VO58) at day 2 post-challenge (4.2 log $TCID_{50}$/24 h) and on days 1 and 2 post-challenge in the other control pig (VO59: 5.2, 4.2 log $TCID_{50}$/24 h). RNA copies of virus were detected in only one control pig (VO59) on 1 and 2 dpc (7.4, 7.3 log copies/24 h). No viable virus was detected from any of the vaccinated pigs, whilst RNA copies were detected at 4 dpc from one vaccinated clinically affected pig VO27 (5.4 log $TCID_{50}$/24 h).

3.4. Detection of virus/genome in oro-pharyngeal fluid (probang) and tissues for identification of FMD virus carriers

Oro-pharyngeal fluids obtained by probang cup on or after 28 days of challenge up to termination of the experiment were analysed by virus isolation and RT-PCR. No virus/RNA was obtained from cell culture/real-time RT-PCR from any of the samples except on one occasion from one of the Gr-1 animals (VO17) where viral RNA (2.37E+04 copies) was found by real-time PCR at 28 days post-challenge. The RNA extraction and RT-PCR was repeated twice with similar results.

Extracted RNA from tissues of pharynx and dorsal and ventral soft palate of both Gr-1 and Gr-2 vaccinated challenged pigs ($n = 16$) were used for real-time RT-PCR using the primers and probe from the IRES region of the FMDV. Viral RNA was detected from only four pigs. Viral RNA (9.63E + 02) was detected from the dorsal soft palate of one Gr-1 pig (VO4), killed at 48 days post-challenge. Viral RNA (1.14E + 03 and 1.25E + 03) was also detected from the nasopharynx of two vaccinated and clinically affected Gr-2 pigs (VO26 and 27), killed at 33 dpc. Viral RNA (1.54E + 01 and 1.86E + 01) was also detected in the dorsal soft palate of both of these pigs. Viral RNA was detected at 33 dpc (4.4E + 01) from dorsal soft palate of a fourth animal (VO25). All RT-PCR tests were repeated three times with same results. However, we were not able to recover live virus from any of these tissues when extracts from frozen tissues were inoculated repeatedly, at least with two further passages in BTY cells. When the tissue-derived RNA or c-DNA samples were re-examined with another set of primers and a probe specific for the 3D region of FMDV, no amplified product was found for these samples whereas a strong positive signal (CT value of 22) was obtained for positive control material. Same results were also obtained on repetition of this experiment.

3.5. Detection of clinical and sub-clinical infection by non-structural antibody assay

All three tests detected antibodies to FMDV NSPs in vaccinated and clinically affected pigs (Figs. 1, 2 and 4) and all seven animals were scored positive at some stage by both the Cedi and UBI tests. The UBI test started to detect infection from 4 days post-challenge and scored all affected pigs positive from 13 days post-challenge up to the end of the experiment except on one occasion at 33 days post-challenge (Fig. 4). Eighty-six percent of the clinically

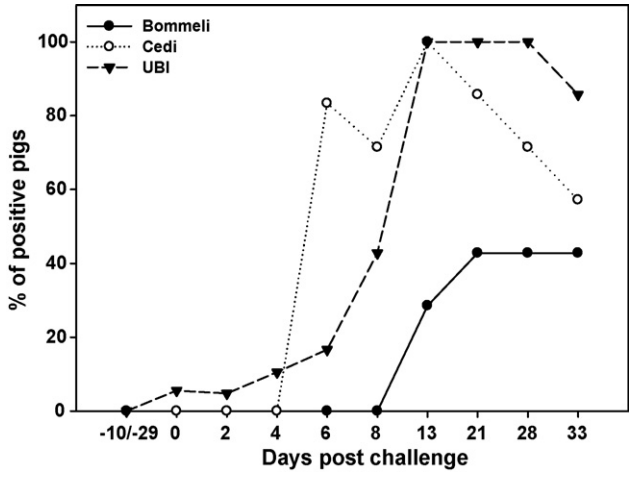

Fig. 4. Detection of non-structural antibodies in clinically infected recovered pigs of Gr-1 and Gr-2. (●) indicates % of positive pigs detected in Bommeli NSP test, (◯) indicates % of positive pigs detected in Cedi NSP test and (▼) indicates % of positive pigs detected in UBI NSP test.

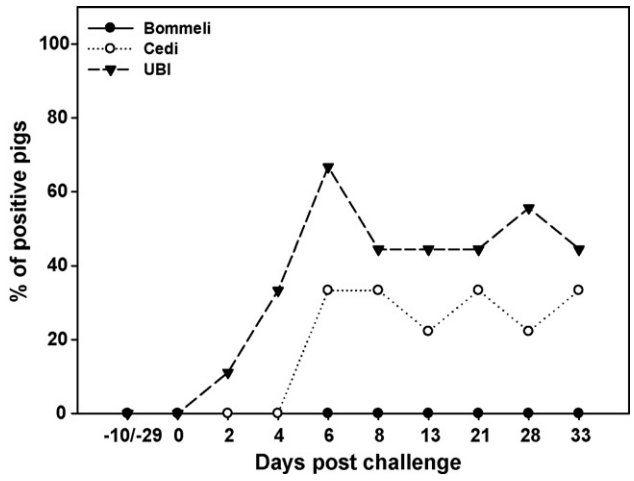

Fig. 5. Detection of sub-clinical infection in vaccinated and challenged pigs using various non-structural antibody assays. (●) % of positive pigs detected in Bommeli NSP test, (◯) % of positive pigs detected in Cedi NSP test and (▼) % of positive pigs detected in UBI NSP test.

affected animals were detected by the Cedi test at 6 days post-challenge and as for the UBI test 100% of samples initially scored positive from 13 days post-challenge. However after 21 days, the detection rate fell and by 33 days post-challenge only 57% of affected pigs scored positive (Fig. 4). The Bommeli test detected 29% of affected pigs at 13 days post-challenge, peaking at 43% at 21 days post-challenge (Fig. 4).

The Bommeli test did not detect any sub-clinical infection (Figs. 1, 2 and 5). With the UBI test, detection of sub-clinical infection was first possible at 2 days post-challenge (11% positive) and peaked at 6 days post-challenge (66%, Fig. 5). However, some non-specificity was observed in this assay during the pre-challenge period (Fig. 4). The Cedi test started to detect sub-clinical infection at 6 dpc and detected up to 33% of sub-clinical infection. On the 40th and 47th day of challenge all three protected but sub-clinically infected pigs were detected by the UBI test whereas only two were detected by the Cedi test (data not shown).

3.6. Virus neutralising antibody

Serum antibody responses against O1 Manisa were measured by virus neutralisation test before and after challenge for vaccinated animals in both groups of pigs (Fig. 6). The Gr-2 pigs had on average more neutralising antibody on the day of challenge as well as after challenge throughout the experiment. Differences were significant ($P < 0.05$) on the day of challenge, and at 21 and 28 days post-challenge.

When the clinically protected and clinically affected pigs were regrouped for their mean VNT titre separately within the groups, VNT titres were higher on the day of challenge in clinically protected animals than in clinically affected pigs, particularly in group two pigs (Fig. 7). Immediately after challenge, the clinically affected pigs had higher VNT titres than the clinically protected animals due to anamnestic

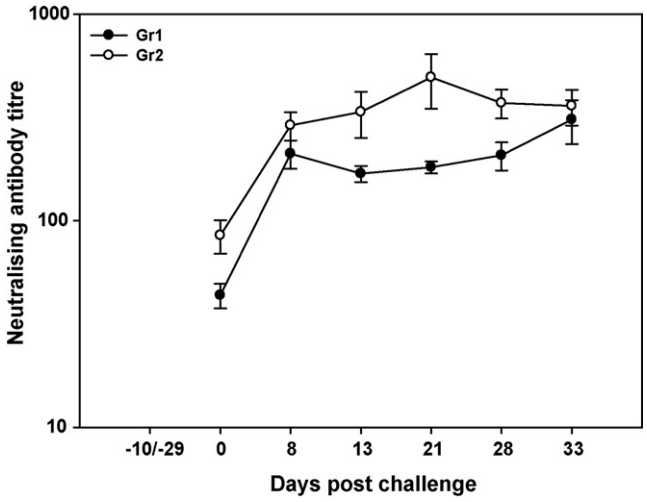

Fig. 6. Mean neutralising (O1 Manisa) antibody titres in vaccinated pigs following challenge. The pigs were vaccinated with full bovine dose of O1 Manisa oil adjuvant vaccine and challenged with O UKG 2001 on day 10 (Gr-1) and day 29 (Gr-2) post-vaccination. (●) Mean neutralising (O1 Manisa) antibody titres for Gr-1 pigs and (◯) mean neutralising (O1 Manisa) antibody titres for Gr-2 pigs.

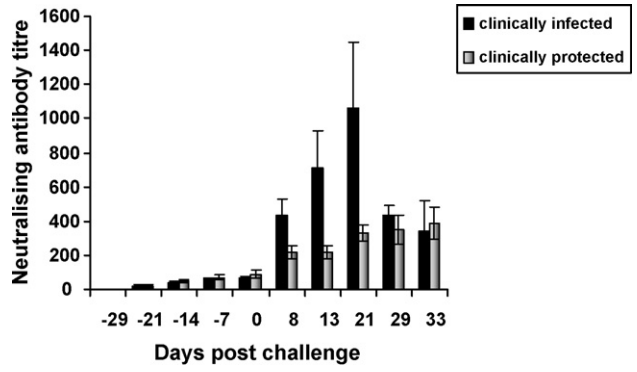

Fig. 7. Mean neutralising (O1 Manisa) antibody titres in Gr-2 vaccinated clinically infected and clinically protected pigs following challenge. The pigs were vaccinated with full bovine dose of O1 Manisa oil adjuvant vaccine and challenged with O UKG 2001 on day 29 post-vaccination. Gray bars indicate mean neutralising (O1 Manisa) antibody titres for clinically protected pigs and black bars indicate mean neutralising (O1 Manisa) antibody titres of clinically infected pigs.

responses, which declined later in comparison to protected animals (Fig. 7).

3.7. Correlation of vaccine-induced reduction in viral replication and excretion with severity of clinical signs

All vaccinated and control pigs ($n = 30$) were regrouped according to the severity of clinical signs (unvaccinated controls, vaccinated with severe signs, vaccinated with mild signs and vaccinated with no signs) and a Fisher exact test was used to detect differences between the proportion of pigs in each group for which RNA or virus was detected in the nasal fluids, saliva and aerosol within the first 5 days of challenge, or which were positive by Bommeli NSP test after 27 days post-challenge (Table 2). The Bommeli test results were used for this purpose since the test had a low sensitivity allowing discrimination between extensive and low level replication of virus. As control animals as well as infected animals with severe signs were killed within 1 week of challenge, seroconversion rate was only compared between pigs with mild or no signs of disease. The main difference found between unvaccinated controls and the vaccinated severely infected group was in recovery of aerosolised virus: always control groups excreted more. However, the mild signs and no signs groups had a lower rate of infectious viral recovery from nasal and saliva swabs, whilst no sign (sub-clinically infected/protected) group had the lowest indicators of viral RNA/ virus excretion and replication (NSP seroconversion). The proportion of pigs which were positive in each group (Table 4) differed significantly ($P < 0.05$) for all the tests (nasal, saliva and aerosol RNA or virus, and NSP test); animals in the control group or with more severe clinical signs were more likely to be positive for any of the tests.

Table 4
Establishment of correlation between vaccine-induced reduction in FMD virus replication and excretion with severity of clinical signs

	N RNA	N virus	S RNA	S virus	A RNA	A virus	B NSP
Signs	P/T	P/T	P/T	P/T	P/T	P/T	P/T
Control	6/6	5/6	6/6	6/6	4/4	4/4	NA
Severe	8/8	6/8	8/8	6/8	0/2	1/2	NA
Mild	7/7	2/7	7/7	3/7	1/3	0/3	4/7
No signs	5/9	0/9	6/9	1/9	1/5	0/5	0/9

P/T: no. of positives/total no. of samples tested, NA: sample not available, N: nasal, S: saliva, A: aerosol and B: Bommeli.

4. Discussion

Pigs are important 'amplifiers' of FMDV because of the abundance of infectious material excreted in their breath [31]. Intensification of agricultural systems has led to a massive increase in the size and density of pig populations which when juxtaposed with other susceptible livestock provides potential for large scale and rapid spread of FMDV [32]. This study is aimed to define the efficacy of a single application of vaccine in providing protection to pigs from infection, virus replication, virus excretion and clinical disease. Use of a semi-heterologous 9 h direct contact challenge simulated a 'worse-case' scenario encounter with FMDV, from which extrapolations can be made to less severe challenges. The reduction in virus replication and excretion afforded by vaccination was quantified and shown to be correlated to the severity of clinical signs of FMD. The study also provided insights into the ability to detect infection, particularly sub-clinical infection in vaccinated pigs, by means of NSP serology; an important approach in substantiating freedom from infection in a post-vaccination situation. Finally, the study found evidence of viral RNA but not infectious virus

in the oro-pharynx of vaccinated pigs recovered from acute infection.

In our severe challenge model, vaccination could not fully protect pigs against clinical disease even when challenge was delayed until 29 days later. However, the disease seen was less severe compared to that in pigs challenged 10 days after vaccination, and vaccinated pigs were less severely affected than controls. Although vaccinated pigs all appeared to have become infected to some degree, vaccination significantly reduced excretion of live virus, which is likely to reduce the risk of onward viral transmission to other naïve, or vaccinated animals. Even better protection might be observed after less severe FMDV challenge, e.g. following indirect exposure to virus in the field after animal movement restrictions are in place [33,34], demonstrated protection against clinical signs as early as 4 days after vaccination, when vaccinated pigs were exposed for 1–4 h to indirect/direct contact challenge using infected donor pigs.

Eble et al. [35,36] challenged three groups of pigs with O Taiwan virus 7 days after vaccination with O Taiwan or 14 days after vaccination with either O1 Manisa or O Taiwan vaccines. The challenge was done by inoculating half of the vaccinated pigs in each group of 10, giving a chance of direct contact challenge for the other half of the vaccinated pigs in each group. None of their pigs vaccinated 14 days prior to challenge developed generalised FMD, whereas even challenge at 29 days post-vaccination resulted in generalised FMD in two out of eight pigs in our study. Possibly, direct heel-bulb inoculation of pigs with FMDV represented a less severe challenge than direct exposure for 9 h to unvaccinated donor pigs exhibiting clinical disease. In addition viral strain, dose, age of animals might have influence for which it is difficult to compare the results between the experiments. Eble et al. also found that five inoculated and three in-contact pigs developed FMD in their group vaccinated 7 days prior to challenge. Assuming that 2 or 3 days would be needed before the inoculated pigs were ready to transmit, the in-contact pigs would by then have been at around 10 days post-vaccination, as for Gr-1 of our own study, in which 9 h of direct contact challenge was able to induce disease in a similar proportion of animals (13 out of 16 pigs). Eble et al. [36] reported that the titres of virus in OP swabs from their 7 day vaccinated pigs were no less than from their unvaccinated controls. However, due to the lack of aerosol measurements in their experiments and the different way of collecting and quantifying virus in the oro-pharynx, a direct comparison of viral excretion between the two studies cannot be made. The present study is the first to quantify viral RNA by real-time RT-PCR in the excretions of vaccinated challenged pigs and to analyse the excretion of virus in exhaled air after use of purified high potency emergency vaccine.

Some controversy exists over whether pigs can be FMDV carriers [12,13,34]. Due to the prior application of vaccine in this study, we were able to keep at least 16 vaccinated pigs up to 33–47 days after virus challenge. Alexandersen et al. [20] reported that pharynx, tonsil and soft palate are the major sites for FMD virus replication in pigs during the early phase of infection from where they were able to detect 10^4 to 10^6 $TCID_{50}$ equiv./g. However, their study only lasted 4 days after infection of unvaccinated pigs. To find out whether pigs are carriers of FMD virus or not beyond 28 days after infection, oro-pharyngeal fluids and tissues were collected from pigs after 28 days post-challenge. Though live virus could not be recovered in these samples, viral RNA was recovered in some tissues from four vaccinated challenged pigs by real-time RT-PCR using a probe and primers from the IRES region of the virus. Two of the four animals were from the vaccinated and clinically affected group of pigs (VO26 and 27) and two were from vaccinated and sub-clinically infected animals (VO4 and 25). A saliva sample of the pig VO26 was also positive for viral RNA at 28 days after challenge. Viral RNA was also obtained at 13 days post-challenge from pig VO4 (its latest detection in Gr-1 pigs) and in VO25 at 21 days after challenge. However, as live virus was never recovered either from oro-pharyngeal fluid (probang) or from tissues after 28 days post-challenge, these animals do not meet the definition of FMD carrier animals [7], which supports the non-carrier status of pigs [16]. RNA recovery could not be confirmed by a 3D real-time PCR that is at least as sensitive as the IRES real-time PCR [26]. Possibly, the IRES region RNA might be more resistant to degradation than certain other parts of the genome due to its high degree of structure.

NSP tests have been widely evaluated in cattle but not pigs. In unpublished studies on FMD in four vaccinated Hong Kong pig herds [37] we observed that serological evidence of infection was only found in groups of animals with healing lesions, whereas neighbouring groups without signs of disease were seronegative for NSP antibody. This led us to conclude that either infection following vaccination was rarely sub-clinical or else, if it occurred, did not lead to detectable NSP seroconversion. However, this study clearly demonstrated that, sub-clinical infections can occur in pigs. Furthermore, seroconversion in NSP tests was related to the degree of virus replication and excretion and the severity of clinical signs. As seen earlier in cattle [38], no single NSP test could detect infection in all pigs, but combining two or more tests could increase specificity and sensitivity. The failure to detect NSP antibody in the study of [36] may be attributed to the low level of viral replication in the vaccinated and subsequently FMDV inoculated animals that could not efficiently transmit infection to vaccinated in-contact pigs.

In our study, the rise in neutralising antibody after challenge of both the groups could be attributed to an anamnestic response to virus replication or a maturing primary adaptive response to vaccination since vaccinated and unchallenged pigs were not available for comparison. Comparing the two groups, the mean VNT titre of the Gr-2 pigs (vaccinated for 29 days) had significantly more neutralising antibody titre than the Gr-1 pigs (vaccinated for 10 days) on the day of challenge and therefore, there was a correlation between VN titre and both clinical and virological protection. Although vaccination could not provide complete clinical or virological

protection, it reduced the severity of the disease, virus excretion and production of non-structural FMDV antibodies in vaccinated and subsequently infected pigs. As hypothesised, vaccine-induced reduction of virus replication and excretion was found to be correlated to the severity of clinical disease.

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