

Evidence that high potency foot-and-mouth disease vaccine inhibits local virus replication and prevents the ‘carrier’ state in sheep

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Abstract

The ability of a single administration of a high, medium and low potency foot-and-mouth disease (FMD) vaccine to decrease or inhibit local virus replication and excretion in the oropharynx of sheep following aerosol challenge with homologous live virus 14 days later was examined. Unvaccinated sheep showed signs of clinical FMD, whereas all of the vaccinated sheep, regardless of antigen payload, were protected against clinical disease and development of viraemia. Virological and serological results confirmed that there had been no local virus replication in the oropharynx of sheep from the high potency vaccine group in contrast to moderate or substantial virus replication in the oropharynx of the low potency vaccinated or unvaccinated sheep respectively. The vaccines showed no evidence of promoting a local mucosal antibody response at the time of virus challenge, but were capable of stimulating a systemic gamma interferon response, the level of which was related to the antigen payload. This suggests that the systemic gamma interferon response could be a useful indicator of the ability of a FMD vaccine to elicit a sterile immunity and indicates that further work is warranted to investigate the role of systemic gamma interferon in this immunity.

This is the first experiment to clearly show that high potency, high payload, FMD vaccines are capable of inhibiting local virus replication and consequently persistence and the carrier state in this target species.

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1. Introduction

Foot-and-mouth disease virus (FMDV) produces an acute, systemic vesicular disease in cloven-hoofed animals. The natural route of infection is via the upper respiratory tract or through ingestion of the virus. Initial virus replication usually occurs in the pharyngeal epithelium resulting in primary vesicles. Fever and viraemia can occur within 1–2 days resulting in virus excretion from the respiratory tract, faeces, urine, saliva, milk and semen. Virus entering the blood disseminates to various predilection sites such as the mouth and nose, hooves and also sometimes teats and udder, in which secondary vesicles occur, and from which further virus is released. FMDV is thus extremely efficiently disseminated.

The ability of FMDV to spread very rapidly in susceptible populations necessitates strict control of live animal and animal product imports to countries that are disease free.

Foot-and-mouth disease (FMD) is thus a major constraint to international trade and its presence or introduction into a previously free country can dramatically reduce export potential and foreign earnings of the agricultural sector. It can also have major impact in other sectors, as exemplified by the loss of tourism during the United Kingdom epidemic in 2001.

Current policy for outbreaks of FMD in the United Kingdom and other developed countries is based upon total slaughter, disposal, movement restrictions, and disinfection, i.e. stamping out. However, there is provision to resort to emergency vaccination under certain circumstances, such as the threat of an outbreak becoming extensive, or if the logistics of slaughtering a high number of animals to contain the disease is unattainable. As a consequence, a number of FMD vaccine banks have been established to support this need and the development of such strategic reserves has recently been reviewed [1].

Emergency vaccination options were continuously evaluated during the 2001 UK epidemic, and a supply of vaccine was formulated by the International Vaccine Bank (IVB), based at IAH Pirbright in the UK. However, there was a

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wide diversity of opinion on the potential benefits of introducing vaccination and ultimately vaccine was not deployed. Circumstances could not be identified where it was seen as providing clear additional advantages in the control of the outbreak when weighed against the additional economic disadvantages imposed by contemporary international legislation. One of the major concerns was the possibility that following a subsequent contact with disease, vaccinated ruminants could maintain replicating virus in the upper respiratory tract and become persistently infected 'carriers' [2].

The concern regarding persistently infected vaccinated animals has had impact on control policies and vaccination for many years, even though the epidemiological significance of such carriers is by no means clear. Despite several, mainly anecdotal, reports of carriers being responsible for field outbreaks of FMD [2], conclusive evidence of disease spread from known carrier cattle to known susceptible cattle has not been realised under controlled, experimental conditions. Moreover, although there is evidence to suggest that FMD vaccination of cattle can reduce post-infection virus excretion [3,4] and transmission to susceptible, in-contact cattle [5], there has been no unequivocal experimental evidence that routine FMD vaccination reduces the establishment or the duration of persistent infection.

More recent studies have shown that emergency FMD vaccines, formulated to higher potency than conventional FMD vaccines, besides protecting cattle, pigs and sheep within 4 days of immunisation [3,4,6,7], have some inhibitory influence on local virus replication and excretion in the oropharynx [8], thereby limiting transmission of disease to other susceptible animals. However, these conclusions have been drawn from experiments designed primarily to investigate the rapidity of protective immunity following emergency vaccination, and thus the level of virus excretion was not always examined quantitatively. The information therefore, is far from comprehensive on the ability of FMD vaccines, particularly those of higher potency and greater antigen payload, to reduce the establishment of persistence following challenge.

As recognised by the report of the Royal Society enquiry [9] after the UK epidemic, there is now a growing demand for vaccination to be more readily considered as an alternative to large-scale pre-emptive culling. Various strategies could be employed including suppressive vaccination in which vaccination close to, or even within, an infected premises to reduce virus excretion is applied, a method the Netherlands implemented as a supplementary measure to control their FMD outbreak in 2001 [10]. Consequently, there is an urgent need to not only develop better vaccines but to establish the efficaciousness and limitations of those currently available.

In this study, we examine the ability of a high potency O₁ Lausanne FMD vaccine, and two similarly formulated vaccines, representing a medium and low potency, to decrease or inhibit local virus replication and excretion in the oropharynx of sheep following homologous aerosol challenge.

2. Materials and methods

2.1. Animals

Twenty-eight Polled Dorset Sheep aged between 6 and 12 months and two Large White × Landrace pigs weighing 20–30 kg were used in this study. All animals were housed in disease-secure accommodation at IAH Pirbright. The sheep were divided in to four groups of seven animals.

2.2. Preparation of vaccine and vaccination

The first group of seven sheep were each immunised with the equivalent of half bovine dose of O₁ Lausanne antigen formulated as an aqueous aluminium hydroxide/saponin adjuvanted vaccine. A 1 ml volume was administered subcutaneously over the left shoulder. This vaccine, with an antigen payload of 5.96 µg per bovine dose, had previously been shown to have a PD₅₀ value of 41 in cattle during its acceptance into the IVB [11] and therefore represented a high potency vaccine. The second and third group of sheep were vaccinated with the same volume of vaccine and amount of adjuvant, but with the antigen payload reduced to 1/10 and 1/40 of that of the first group respectively. Hence, the three vaccination groups represented a gradation of vaccine potencies of high, medium and low. The fourth group of sheep remained unvaccinated.

2.3. Infection of virus generator pigs

Two pigs were used as the source of virus for an indirect aerosol challenge of the vaccinated and unvaccinated sheep. These pigs were inoculated intradermally into the heel bulb with 10^{5.0} TCID₅₀ live O₁ Lausanne in 0.2 ml M25 phosphate buffer. At 72 h post-inoculation, they were displaying clinical signs of FMD and were used to simultaneously challenge all the sheep.

2.4. Challenge of sheep

Fourteen days after vaccination all groups of sheep were herded together in to a single isolation box and simultaneously challenged by indirect aerosol using the two infected pigs. The pigs were housed in a side-ventilated crate placed under the air 'inlet' vent in a cordoned section of the room in order to prevent direct contact with the sheep. The sheep were allowed to circulate freely around the isolation box for 4 h. Twice during the challenge period the air 'exit' vent was temporarily switched off for 30 min in order to increase the concentration of virus in the air of the isolation box. Following challenge the sheep were removed from the challenge isolation box and re-housed in their individual vaccination groups and the pigs removed from the experiment. All the sheep were examined regularly for signs of clinical disease up to 42 days post-challenge and various samples

were taken. Rectal temperatures were recorded for up to 10 days post-challenge.

2.5. Sample collection

Clotted blood samples for serology and heparinised blood samples for virus isolation (whole blood) were collected pre-vaccination and/or at challenge and at regular intervals post-vaccination and challenge up to 42 days post-challenge. Samples of plasma were also prepared from heparinised blood for interferon gamma (IFN- γ) analyses. Additionally, oesophageal–pharyngeal fluid (probang) samples were collected at similar intervals for virus isolation by inoculation of cell culture and for detection of viral RNA by automated fluorogenic (5' nuclease probe-based) reverse transcription polymerase chain reaction (RT-PCR). Probang samples were routinely diluted in 2 ml transport medium (used to wash the probang cup) and stored at -70°C until required for testing.

2.6. Virus isolation

Heparinised blood samples and probang samples were examined for the presence of virus by inoculation of monolayers of primary calf thyroid (BTY) cells [12]. Four BTY tubes were inoculated with 250 μl sample/tube and incubated at 37°C on roller drums. At 24, 48 and 72 h post-inoculation cell monolayers were examined for cytopathic effect (cpe). ELISA was used to confirm the presence of FMD virus in cultures showing cpe. BTY cell culture supernatants from samples showing no sign of cpe after 72 h were collected and re-passaged.

2.7. Detection of FMD viral RNA by real-time RT-PCR

The probang samples were tested by automated real-time (fluorogenic or 5' nuclease probe-based) RT-PCR using procedures similar to those previously described by Reid et al. [13]. Probang samples were added to an equal volume of Lysis/Binding Buffer (Roche, UK) and total nucleic acid extracted from 350 to 500 μl of sample (in buffer) to a final volume of 50 μl in a MagNA Pure LC (Roche) using the programme 'Total NA External_lysis'. Automated programmes were then used to add 6 μl of nucleic acid from three consecutive panels of 32 samples (and controls) to 9 μl of reverse transcription (RT) reaction mix and the RT process completed as previously described [13]. Another automated programme on the MagNA Pure LC followed this to transfer 7 μl of the RT product into 18 μl of PCR mix in a 96-well optical reaction plate. PCR amplification was carried out in a GeneAmp[®] 5700 Sequence Detection System (Applied Biosystems) and a threshold cycle (C_T) value was assigned to each PCR reaction as previously described but 60 cycles of replication were used instead of 50 [13]. All samples with a C_T value less or equal to 45 were considered positive.

2.8. Anti-non-structural protein antibody analyses

Serum samples were also examined for the presence of antibodies against non-structural FMDV polyprotein 3ABC (indicative of virus replication having occurred) using a commercially available kit (Chekit, Bommeli Diagnostics, Intervet) [14].

2.9. Anti-FMDV antibody analyses

Systemic anti-FMDV neutralising antibody levels were measured by the microneutralisation assay as described in the OIE Manual of Standards [15] in which antibody end-point titres are calculated as the reciprocal of the last serum dilution to neutralise 100 TCID₅₀ of homologous FMDV in 50% of the wells.

Isotype-specific (IgM, IgG and IgA) antibody responses were investigated in both serum and probang samples at 0 and 10 days post-vaccination and 2 days post-challenge using a modification of the IDAS ELISA [16]. Samples of either serum diluted 1/50, or probang (used without further dilution) were spot-tested in duplicate (50 μl per well) before the incorporation of anti-ovine isotype reagents (Serotec, UK). Samples which gave an A492 of >0.2 after subtraction of the pre-vaccination absorbance value were considered positive.

2.10. Detection of interferon gamma (IFN- γ)

The cytokine IFN- γ was measured in plasma samples from all sheep at various time points before and following vaccination and challenge using a double antibody sandwich ELISA. Paired antibody reagents raised against bovine IFN- γ (Serotec, UK) and cross-reactive for ovine IFN- γ were used in assays optimised according to the recommendations on the data sheets supplied. Plates (Maxisorp, Nunc, Denmark) were coated with 2 $\mu\text{g}/\text{ml}$ mouse anti bovine IFN- γ and incubated overnight at room temperature. The plates were washed with PBS containing 0.01% Tween 20 before blocking with PBS containing 1% bovine serum albumin for 30 min. Following a further washing step recombinant bovine IFN- γ standards or samples were added in duplicate and the plates incubated at 37°C for 1 h. Dilutions of recombinant bovine IFN- γ (in blocking buffer) were added to cover the range 6000–75 pg/ml. Following washing, biotinylated mouse anti bovine IFN- γ detection antibody (2 $\mu\text{g}/\text{ml}$) was added for 1 h at 37°C followed by a further wash step and addition of 0.25 $\mu\text{g}/\text{ml}$ Streptavidin-HRP (Sigma) for 1 h at 37°C . After a final washing step substrate consisting of 0.04% *O*-phenylenediamine dihydrochloride and 0.005% hydrogen peroxide was added and left for 15 min to allow colour development before the reaction was stopped by addition of 1.25 M H₂SO₄. Absorbance values were read at 492 nm. Samples and reagents were applied in 100 μl volumes throughout and the washing steps comprised of five changes of 300 μl wash buffer.

Table 1
Outcome of indirect aerosol challenge of sheep vaccinated with FMDV O₁ Lausanne at different antigen payloads

Animal	Vaccine dose	Protected ^a	Pyrexia ^b	Viraemia	O–P virus isolation ^c (days post-challenge)									
					2	4	7	9	11	14	22	28	35	42
UO73	1	P ^d	N ^e	N	– ^f	–	–	–	–	–	–	–	–	–
UO74	1	P	Y ^g	N	–	–	–	–	–	–	–	–	–	–
UO75	1	P	N	N	–	–	–	–	–	–	–	–	–	–
UO76	1	P	N	N	–	–	–	–	–	–	–	–	–	–
UO77	1	P	N	N	–	–	–	–	–	–	–	–	–	–
UO78	1	P	N	N	–	–	–	–	–	–	–	–	–	–
UO79	1	P	N	N	–	–	–	–	–	–	–	–	–	–
UO80	1/10	P	N	N	–	–	–	–	–	–	–	–	–	–
UO81	1/10	P	Y	N	–	–	–	–	–	–	–	–	–	–
UO82	1/10	P	N	N	–	–	–	–	–	–	–	–	–	–
UO83	1/10	P	N	N	–	–	–	–	–	–	–	–	–	–
UO84	1/10	P	N	N	–	–	–	–	–	–	–	–	–	–
UO85	1/10	P	N	N	–	–	–	–	–	–	–	–	–	–
UO86	1/10	P	N	N	–	–	–	–	–	–	–	–	–	–
UO87	1/40	P	N	N	–	+ ^h	+	–	–	–	–	–	–	–
UO88	1/40	P	N	N	–	–	+	+	+	+	+	+	–	–
UO89	1/40	P	N	N	–	–	+	–	–	–	–	–	–	–
UO90	1/40	P	N	N	–	–	–	–	–	–	–	–	–	–
UO91	1/40	P	N	N	–	+	–	–	+	–	+	+	+	+
UO92	1/40	P	N	N	–	–	–	–	–	–	–	–	–	+
UO93	1/40	P	N	N	–	–	–	–	–	–	+	–	–	–
UO94	0	N	Y	Y	–	+	+	+	–	+	+	+	+	–
UO95	0	N	Y	Y	+	+	–	+	–	–	+	–	–	–
UO96	0	N	Y	Y	+	+	+	–	+	+	+	+	–	–
UO97	0	N	Y	Y	+	+	+	–	+	+	+	+	+	–
UO98	0	N	Y	Y	+	+	–	–	+	+	+	–	–	–
UO99	0	N	Y	Y	+	+	+	–	–	+	+	+	+	+
UP0	0	N	Y	Y	+	+	+	–	–	+	+	–	–	–

^a Absence of all clinical signs of FMD.

^b At 40 °C.

^c Oesophageal–pharyngeal fluid sample.

^d Protected.

^e No.

^f No virus detected.

^g Yes.

^h Virus detected.

3. Results

3.1. Development of clinical FMD and viraemia

All of the vaccinated sheep regardless of antigen payload were protected against clinical disease and development of viraemia following indirect aerosol FMDV challenge (Table 1). The seven sheep in the non-vaccinated group, however, showed signs of clinical FMD including inappetance, panting, pyrexia, lameness and foot lesions from 4 to 10 days post-challenge. Viraemia was also detected in all these unvaccinated animals at 4 days post-challenge.

3.2. Local virus replication and development of antibodies against non-structural FMDV proteins

Probang samples were investigated for virus by both traditional bovine thyroid cell culture isolation and real-time

RT-PCR. No virus was detected in any sheep from vaccine groups 1 and 2 by either method (Tables 1 and 2). Local virus was detected by both methods in all unvaccinated sheep and some animals in vaccine group 3 (Tables 1 and 2) although the same samples were not necessarily positive for virus by both techniques, and more animals were identified virus positive by cell culture isolation than RT-PCR.

To further substantiate whether virus replication had occurred in individual animals, sera were assayed for non-structural protein antibodies, the results of which are presented in Table 3. Unequivocal non-structural antibody responses were detected in all unvaccinated sheep. Two animals in group 3 were inconclusive or just positive based on recommended cut-offs. Two further animals in group 3 from which virus was isolated and an animal (UO84) from group 2 from which virus was not isolated, also showed an increase in response (when compared to their pre challenge/early post-challenge response) towards the end of

Table 2

Real-time RT-PCR results obtained from oesophageal–pharyngeal samples at various days post-challenge in sheep vaccinated with differing antigen payloads

Animal	Vaccine dose	Days post-challenge											
		–4	2	4	7	9	11	14	22	28	35	42	
UO73	1	– ^a	–	–	–	–	–	–	–	–	–	–	–
UO74	1	–	–	–	–	–	–	–	–	–	–	–	–
UO75	1	–	–	–	–	–	–	–	–	–	–	–	–
UO76	1	–	–	–	–	–	–	–	–	–	–	–	–
UO77	1	–	–	–	–	–	–	–	–	–	–	–	–
UO78	1	–	–	–	–	–	–	–	–	–	–	–	–
UO79	1	–	–	–	–	–	–	–	–	–	–	–	–
UO80	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO81	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO82	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO83	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO84	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO85	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO86	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO87	1/40	–	–	+ ^b	+	–	–	–	–	–	–	–	–
UO88	1/40	–	–	–	+	+	–	+	+	–	–	–	–
UO89	1/40	–	–	–	–	–	–	–	–	–	–	–	–
UO90	1/40	–	–	–	–	–	–	–	–	–	–	–	–
UO91	1/40	–	–	+	+	–	+	–	+	–	–	–	–
UO92	1/40	–	–	–	–	–	–	–	–	–	–	–	–
UO93	1/40	–	–	–	–	–	–	–	–	–	–	–	–
UO94	0	–	–	+	+	+	–	–	+	+	+	+	+
UO95	0	–	+	+	–	–	–	–	+	–	–	–	–
UO96	0	–	+	+	+	+	–	–	+	+	+	+	+
UO97	0	–	+	+	+	–	–	–	+	+	–	–	+
UO98	0	–	+	+	+	–	–	+	+	+	–	–	+
UO99	0	–	+	+	–	–	–	+	+	+	+	+	+
UP0	0	–	–	+	+	–	–	–	–	+	–	–	–

^a No target sequence detected.

^b Target sequence detected.

the monitoring period, which may have been indicative of low-level non-structural antibody development.

3.3. Virus neutralising antibody induction

Fig. 1 shows serum neutralising antibody responses in sheep following vaccination with differing antigen payloads and subsequent challenge at 14 days post-vaccination. All sheep in group 1 (Fig. 1i) demonstrated a rapid development of antibody as did sheep in group 2 except UO81 (Fig. 1ii) such that all were antibody positive at time of challenge. Development of neutralising antibody in group 3, the lowest antigen payload group, was less rapid and at day of challenge some animals still did not have detectable levels of antibody (Fig. 1iii). No antibody was detected in the unvaccinated sheep prior to challenge, as would be expected (Fig. 1iv). The mean level of antibody at each time point, for each group is shown in Fig. 1v. The magnitude of the response at 7 and 10 days post-vaccination was investigated between the different groups using an analysis of variance and the level of antibody at both time points in group 1 was shown to be significantly higher than that of group 2 and 3 ($P <$

0.05). At 10 dpv, the level of neutralising antibody in group 2 was also shown to be significantly higher than that of group 3 ($P < 0.05$). Comparing the neutralising antibody responses post-challenge, it is evident that in contrast to the other groups, all of the sheep in group 1 and four out of seven of those in group 2 showed no boosting effect.

3.4. Antibody isotype

Antibody isotype responses against FMDV were investigated in both serum and probang at 10 dpv and 2 dpc (Table 4). IgM and IgG were detected most often in serum of sheep in group 1. No IgA was detected in serum from any sheep. Only IgM was detected in probangs and to a very limited degree both post-vaccination and challenge.

3.5. IFN- γ responses

No systemic IFN- γ was detected either before or after challenge in plasma samples from the unvaccinated sheep (Fig. 2). However, all sheep in group 1 demonstrated increased plasma IFN- γ , some as early as 3 days

Table 3

Serum non-structural protein (NSP) antibody results from indirect aerosol challenged sheep vaccinated with differing antigen payloads

Animal	Vaccine dose	Days post-challenge											
		–11 ^a	–4 ^b	2	4	7	9	11	14	22	28	35	42
UO73	1	1.5 ^c	2.3	2.3	0.8	1.5	1.5	IS	2.3	0.8	1.5	2.3	2.3
UO74	1	1.5	1.5	3	0.8	IS	1.5	1.5	1.5	0.8	1.5	0.8	1.5
UO75	1	1.5	3	1.5	1.5	0.8	0.8	2.3	2.3	2.3	0.8	0	3.8
UO76	1	2.3	0.8	0	0.8	0.5	0	–1.5	0	–0.8	–1.5	–1.5	0
UO77	1	–0.8	–0.8	–1.5	0	–0.8	2.3	–0.8	0	0	–1.5	0	0.8
UO78	1	0	0.8	0	–0.8		0.8	0	0	–0.8	0	–0.8	–0.8
UO79	1	1.2	1.2	1.2	0.6	0.6	0	0	0.6	0	0.6	1.2	5.5
UO80	1/10	0	1.2	0	0.6	0	0.6	2.4	4.9	3.7	3.7	2.4	2.4
UO81	1/10	–0.8	IS	–0.8	0.8	0	0	0.8	–0.8	2.3	1.5	IS	2.2
UO82	1/10	0	0	0.6	0	0.6	0.6	1.2	3.7	IS	0.6	0	0
UO83	1/10	2.2	1.5	0.7	0.7	1.5	2.2	2.2	0.7	2.9	2.2	0.7	1.5
UO84	1/10	IS	2.2	2.2	2.2	2.9	2.9	3.6	10.1	9.4	5.1	8.7	6.5
UO85	1/10	0.7	0.7	0.7	1.5	2.2	2.9	0.7	0.7	–0.7	3.6	2.2	2.9
UO86	1/10	1.3	2	0.7	2.6	1.3	0	0.7	2	1.3	2	0.7	2
UO87	1/40	1.3	0.7	0.7	1.3	IS	3.3	4.6	8.6	7.9	6.6	9.9	6.6
UO88	1/40	0.7	0.7	0.7	1.3	IS	1.3	0.7	3.3	19.7	19.7	20.4	12.5
UO89	1/40	2	3.3	5.9	6.6	7	6.2	4.7	6.2	3.9	3.9	4.7	3.1
UO90	1/40	1.6	1.6	0.8	2.3	IS	3.1	3.9	3.1	3.1	2.3	1.6	2.3
UO91	1/40	–0.8	1.6	0.8	1.6	IS	0.8	0	0	3.9	5.4	IS	14
UO92	1/40	–1.6	–0.8	0	0.8	IS	0	–0.8	–0.8	5.4	0	0	0
UO93	1/40	0.6	0	–0.6	0.6	0	1.3	13.8	47.2	39	23.3	15.7	10.1
UO94	0	0.6	0.6	1.3	1.3	3.8	2.5	17.6	71.7	90	78	83.6	68.6
UO95	0	0.6	2.5	2.5	1.3	0.6	2.5	13.8	50.6	46.5	39.6	35.8	28.9
UO96	0	2.8	–1.4	–1.4	–1.4	–1.4	–0.7	7.5	IS	41.5	34.7	30	23.1
UO97	0	0.7	–0.7	–0.7	–1.4	0	–1.4	4.1	48.3	86.4	83	85.7	60.5
UO98	0	0.7	0.7	0	0	1.4	2	12.2	34.7	38.1	30.6	39.5	25.9
UO99	0	3.1	2.3	1.5	2.3	IS	2.3	6.9	19.1	29	IS	73.3	80.1
UP0	0	3.1	2.3	3.1	2.3	3.1	17.6	39.7	77.1	84.7	92.4	74.8	74

^a 7 days post-vaccination.^b 10 days post-vaccination.^c Percentage of positive control: <20% negative; 20–30% ambiguous; >30% positive.

post-vaccination and all by 10 days post-vaccination. The response was less pronounced in group 2 both in terms of magnitude and number of animals having a measurable response at time of challenge although eventually six out of the seven sheep demonstrated an IFN- γ response. Group 3 sheep showed even less of a response, IFN- γ being detected in only one animal.

4. Discussion

During and subsequent to the FMD epidemic of 2001 in the United Kingdom, one of the liveliest topics for debate has been the question of if and how vaccination could have been used to control the disease. The question of how effectively vaccination can reduce local virus replication and the development of the carrier state in subsequently challenged animals has been a key consideration in these discussions. Vaccinated ruminants that are subsequently exposed to FMD virus may be protected from disease but still support virus replication, which in some cases may continue beyond 28 days at a low level in the upper respiratory tract. The longest

recorded carriage of FMD virus in unvaccinated animals has been for 5 years in buffalo, 3.5 years in cattle, 12 months in sheep and 4 months in goats [2]. The disease would thus be “cloaked” and could conceivably be passed on to naive animals causing further spread of infection. Although there is little hard evidence of transmission from such carrier animals, the risk that it might occur has a significant impact on control strategies and was certainly one of the reasons that vaccination was not used in the United Kingdom during 2001.

The protective effect of FMD vaccination against local virus replication and subsequent persistence is poorly documented. It is clear from the successful use of FMD vaccines in the field, that vaccination can reduce local virus replication and virus circulation. However, it is sometimes said that a similar proportion of ruminant animals exposed to FMDV have been shown to become carriers, regardless of whether or not they have been vaccinated. Whilst on occasion this has been demonstrated experimentally, there is also ample evidence to the contrary. In 1974, Anderson reported that in an endemic area of Kenya the prevalence of FMDV carriers amongst regularly vaccinated cattle was

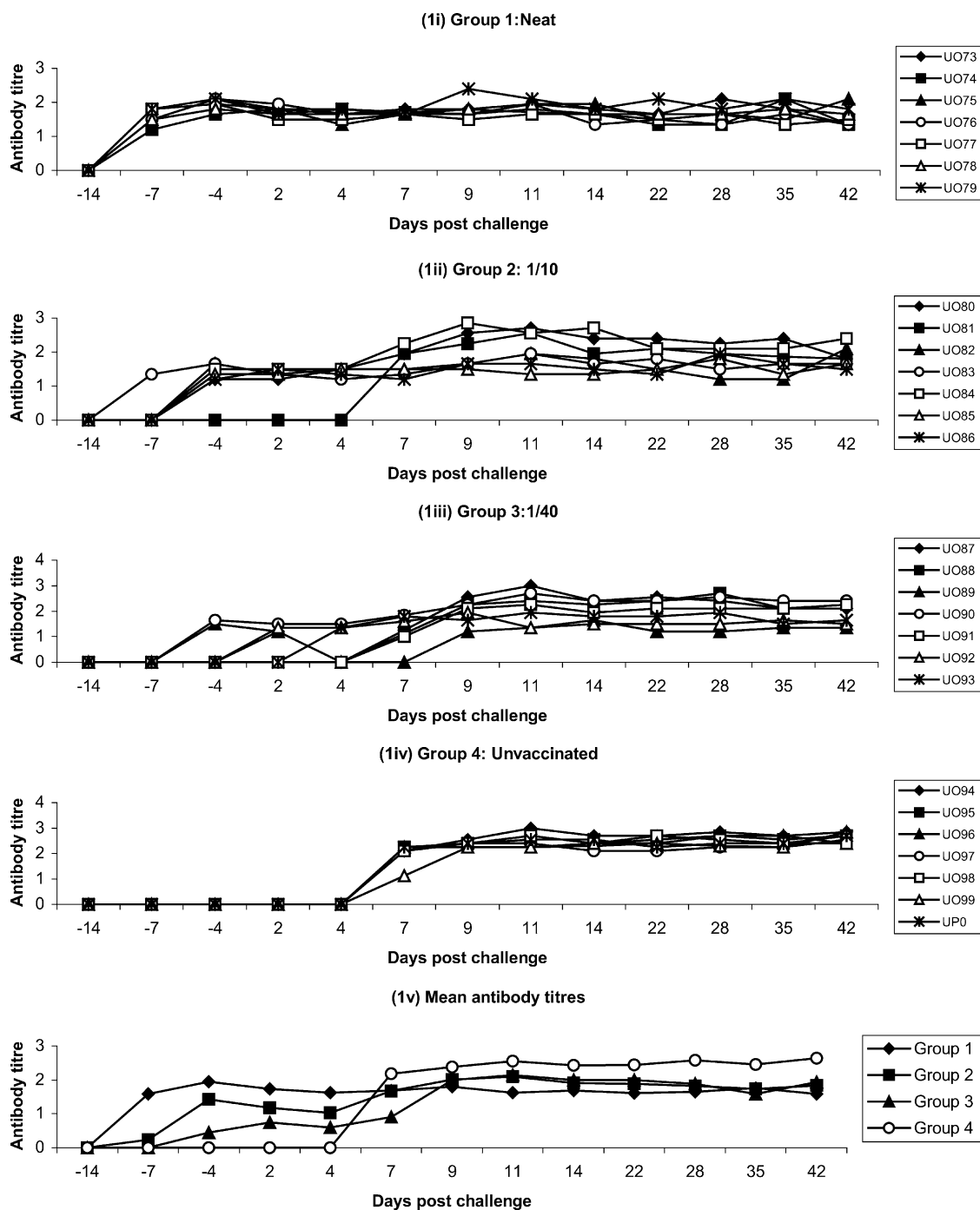


Fig. 1. Serum neutralising antibody responses in sheep following vaccination with differing antigen payloads and subsequent challenge at 14 days post vaccination.

lower than in non-vaccinated animals [17] and there has been other experimental evidence that vaccination of cattle reduces post-infection persistence and virus excretion [3,18,19]. Moreover, higher potency vaccines can result in reduction in transmission to contact susceptible cattle [5]. The experiments by Doel in 1994 [3], designed primarily to examine the rate of protective immunity, showed that virus excretion was reduced significantly by using so-called “emergency” vaccines with a higher payload than

conventional FMD vaccines, if applied with a sufficient vaccine-to-challenge interval. Further evidence has come from similar studies in sheep using three different vaccine strains, where it was again concluded that vaccines administered with longer vaccine-to-challenge intervals were more effective at reducing persistence and virus excretion [7]. However, there was also a suggestion of a relationship between potency and the incidence of virus replication in the oropharynx [20]. Perhaps this should not be that sur-

Table 4

Antibody isotype responses against FMDV in either sera or probang samples in groups of sheep vaccinated with differing antigen payloads around time of an indirect aerosol challenge with FMDV type O₁

Animal	Vaccine dose	Serum IgM		Serum IgG		Serum IgA		Probang IgM		Probang IgG		Probang IgA	
		10 dpv ^a	2 dpc ^b	10 dpv	2 dpc	10 dpv	2 dpc	10 dpv	2 dpc	10 dpv	2 dpc	10 dpv	2 dpc
UO73	1	+ ^c	+	+	+	– ^d	–	+	+	–	–	–	–
UO74	1	+	+	–	+	–	–	–	–	–	–	–	–
UO75	1	+	–	+	–	–	–	–	–	–	–	–	–
UO76	1	+	–	+	+	–	–	–	–	–	–	–	–
UO77	1	+	–	+	+	–	–	–	–	–	–	–	–
UO78	1	+	+	+	+	–	–	+	+	–	–	–	–
UO79	1	+	+	+	+	–	–	–	+	–	–	–	–
UO80	1/10	–	–	–	–	–	–	–	–	–	–	–	–
UO81	1/10	IS ^e	–	–	–	–	–	IS	–	–	–	–	–
UO82	1/10	+	+	–	–	–	–	+	+	–	–	–	–
UO83	1/10	+	+	–	–	–	–	–	+	–	–	–	–
UO84	1/10	–	–	–	+	–	–	–	–	–	–	–	–
UO85	1/10	+	–	–	+	–	–	–	–	–	–	–	–
UO86	1/10	–	–	–	+	–	–	+	–	–	–	–	–
UO87	1/40	–	–	–	+	–	–	–	–	–	–	–	–
UO88	1/40	–	–	–	–	–	–	–	–	–	–	–	–
UO89	1/40	–	–	–	–	–	–	–	–	–	–	–	–
UO90	1/40	–	+	–	–	–	–	–	–	–	–	–	–
UO91	1/40	–	–	–	–	–	–	+	–	–	–	–	–
UO92	1/40	–	+	–	+	–	–	–	–	–	–	–	–
UO93	1/40	–	+	–	–	–	–	–	–	–	–	–	–
UO94	0	–	–	–	–	–	–	–	+	–	–	–	–
UO95	0	–	–	–	–	–	–	–	–	–	–	–	–
UO96	0	–	–	–	–	–	–	–	–	–	–	–	–
UO97	0	–	–	–	–	–	–	–	–	–	–	–	–
UO98	0	–	–	–	–	–	–	–	–	–	–	–	–
UO99	0	–	–	–	–	–	–	–	–	–	–	–	–
UP0	0	–	–	–	–	–	–	–	–	–	–	–	–

^a Days post-vaccination.

^b Days post-challenge.

^c Antibody detected.

^d No antibody detected.

^e Insufficient sample.

prising given that an old method for qualitatively measuring the potency of FMD vaccine, referred to as the *K* index method, was based on quantifying the reduction in titre of tongue inoculated virus between vaccinated and unvaccinated cattle. Moreover, experienced FMD workers involved with vaccine potency trials in cattle have observed that high quality and high potency vaccines can suppress vesicular erosions at the sites in the tongue in which the challenge virus has been administered [21]. This would seem to imply that FMD vaccination exerts not only an affect systemically but also locally. Overall, there is certainly a number of observations to support an effect by vaccination on local virus replication. As potency is directly related to antigen payload, a key question is whether any correlation exists between payload and the establishment of local viral replication and persistence. Certainly for another list A disease, Avian Influenza, the degree of clinical protection and the reduction of virus shedding are positively correlated with the antigen mass used in the vaccine [22].

The specific antibody response to FMD vaccination has been considered a major indicator of vaccine efficacy and the benefits of increasing antigen payload with respect to the humoral antibody response was examined in detail in the mid 1980s [23]. It was concluded that above a certain payload, little further benefit was gained with respect to amplifying the specific antibody response. However, little consideration was given to other aspects of the immune response, some of which had not been recognised at that time. More recent studies in pigs have shown that the innate immune system is also stimulated by FMD vaccination, leading to elevated levels of cytokines IL-6, IL-8 and IL-12 [24]. It is likely that similar and/or other immune responses are stimulated in ruminants and these might be augmented by the use of higher antigen payload vaccines.

This experiment was therefore designed to establish whether a correlation exists between antigen payload and the establishment of local viral replication and persistence, and to examine associated changes in the immune response.

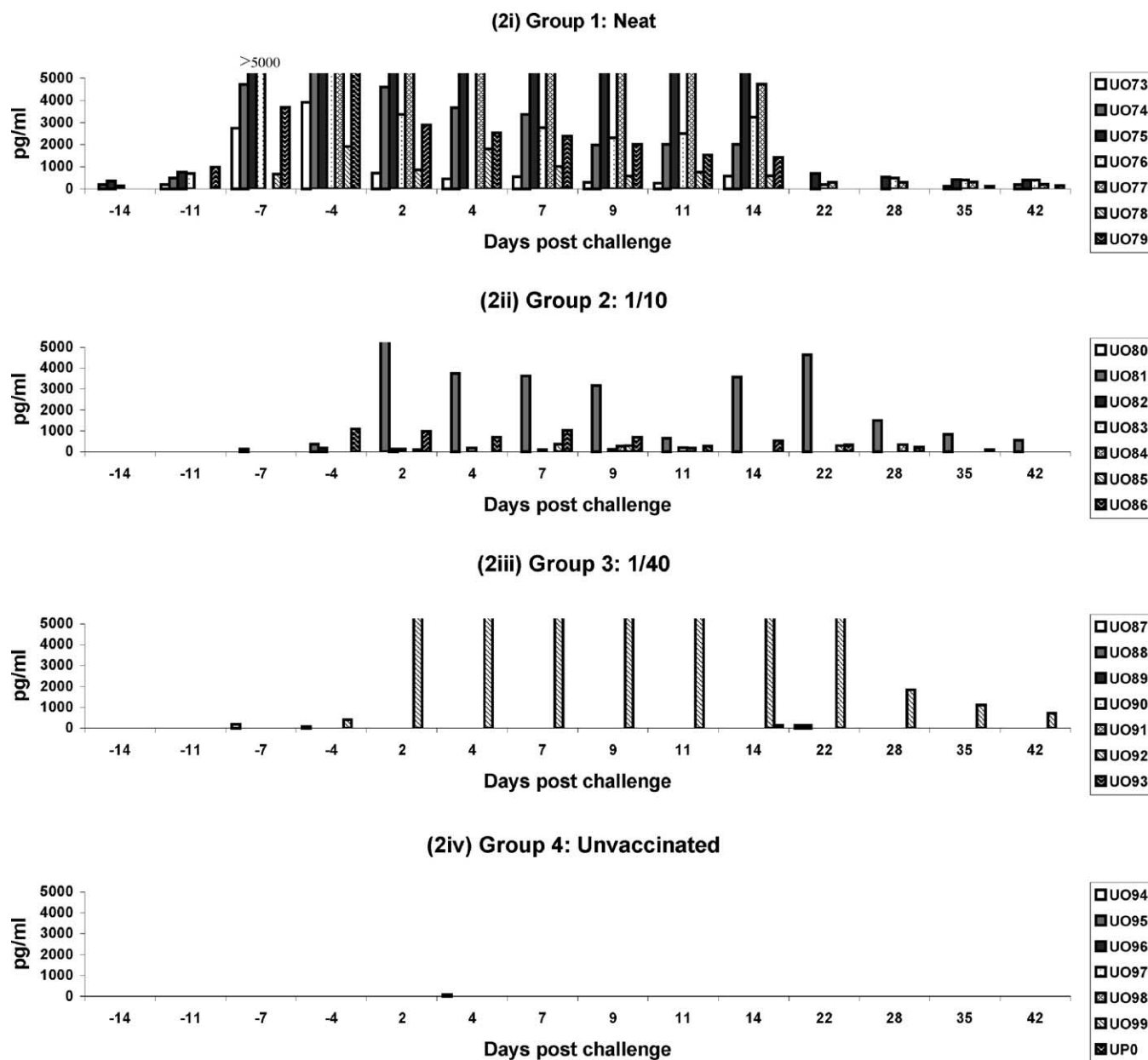


Fig. 2. IFN- γ responses in sheep following vaccination with differing antigen payloads.

Three O₁ Lausanne vaccines were formulated to contain different antigen payloads but with identical amounts of adjuvant, so as to cover the range from a high potency emergency vaccine to a conventional FMD vaccine. Using a time point of 14 days post-vaccination the simultaneous indirect air challenge of all 28 sheep with two infected pigs resulted in all the unvaccinated sheep showing clinical signs of the disease and becoming viraemic. In contrast, all the vaccinated sheep were protected from clinical signs and did not develop detectable viraemias, regardless of antigen payload. A similar finding was obtained for sheep that were challenged 1 week after vaccination with different doses of a conventional monovalent type O₁ BFS FMD vaccine [25]. In the present study, an extensive sampling regime,

including regular sampling of oropharyngeal fluids from individual sheep up to 42 days post-challenge, should have ensured the detection and isolation of replicating virus, if it had been present in the upper respiratory tract. Surprisingly, and in contrast to the previous study using three- and six-fold larger doses of conventional monovalent FMD vaccine [25], no virus was isolated in bovine thyroid cell cultures from the oropharyngeal fluid samples of the neat and 1/10 antigen payload groups throughout the trial. It is unclear why these virus isolation results should differ so greatly from the previous study, but in the previous experiment the potency value was not defined, and the vaccine, prior to its use, had been stored at +4 °C for 10 months. FMD vaccines have a shelf-life of around 12–18 months

and it is therefore likely that the period of storage would have resulted in some loss of its original potency. Nevertheless, it was still concluded from this previous work that increasing the vaccine dose did reduce the amounts of virus excreted in oropharyngeal fluids and the duration of virus excretion in the breath. Only in our 1/40 payload group was virus consistently isolated from two sheep, namely UO88 and UO91, for a period that qualified them as “carriers”. Virus was also sporadically isolated in another three animals UO87, UO89 and UO93, leaving only two sheep in this dose level group free of virus in their oropharyngeal fluid samples throughout the trial. As expected, virus was consistently isolated in the oropharyngeal fluid samples of all the unvaccinated sheep.

Other methodologies were also employed to establish if any local viral replication had occurred in the sheep following challenge. A real time RT-PCR method was used to detect viral RNA in oropharyngeal fluid samples and results generally mirrored the virus isolation results. For example, the three sheep in the 1/40 payload group (UO87, UO88 and UO91) from which virus was isolated on multiple occasions were also scored positive more than once by RT-PCR. The PCR method also provided evidence that local viral replication and persistence had occurred in all of the unvaccinated animals. However, fewer samples were found positive by RT-PCR compared to the results obtained from virus isolation and none of the “carrier” animals identified in the 1/40 payload group were scored RT-PCR positive beyond 22 days post infection. This apparent lower sensitivity of the RT-PCR method could have been simply due to the greater volume of sample used in virus isolation by tissue culture. Additionally, Allerton and co-workers at the Pirbright Laboratory have recently demonstrated that probang samples may interfere with the sensitivity of the RT-PCR assay (unpublished results).

Serological responses also gave a clue to the extent of virus replication post-challenge. Antibody responses to non-structural proteins (NSPs) of FMD virus, and in particular to 3ABC [27–29] are specific to infection with replicating FMD virus and are not induced by suitably purified killed vaccines, such as those used in the current study. Using a commercially available kit (Chekit-FMD) we found no evidence of NSP antibody and by inference virus replication, in animals in the group that received the highest potency vaccine. Very slight and more marked NSP antibody responses respectively were apparent in one or more animals in each of the 1/10 and 1/40 vaccine potency groups, whereas all of the unvaccinated sheep showed convincing evidence of seroconversion to 3ABC. A similar inference could be drawn from analysis of the neutralising antibody responses of the different groups. In the group receiving the highest potency vaccine, there was no secondary antibody response to challenge, whereas a booster effect due to challenge was evident in some sheep in the 1/10 vaccine group and all sheep in the 1/40 vaccine group suggestive of a low level virus replication.

Collectively these results from this single experiment provide strong evidence that a correlation exists between antigen payload and the establishment of local virus replication, and shows that provided the antigen payload is sufficient, an FMD vaccine can inhibit local virus replication, persistence and the carrier state in sheep following indirect aerosol challenge. Indirect aerosol challenge of vaccinated animals probably depicts the likeliest field scenario following an outbreak and the implementation of movement restrictions and vaccination. However, other routes of challenge, such as direct contact, use of alternative donor infected species, or use of other virus strains would undoubtedly influence, to a greater or lesser extent, the severity of challenge and therefore the possible outcome. Further studies would be needed to evaluate the impact of different challenge conditions.

If antigen payload has such a significant effect, by what means is it impeding local virus replication? Antibody mediated protection is one possibility and the current study provided evidence that increasing the antigen payload promoted a more rapid and greater systemic antibody response. However, previous studies by the authors (unpublished results) on local, oropharyngeal antibody responses in emergency vaccinated sheep provided little evidence to suggest that IgG_{1/2} or IgA were consistently induced in the oropharynx following vaccination. Such oropharyngeal fluid antibody as could be detected was also slow to appear and IgG_{1/2} predominated. Neither IgG nor IgA isotypes were detected in probang samples from any of the animals in the current experiment at 10 days post-vaccination, or shortly after challenge, and only sporadic detection of IgM was observed, suggesting that vaccination and increased antigen payload had little effect at promoting a local antibody response. Moreover, since the vaccine is applied parenterally, the local antibody that was detected may be due to extravasation of serum through the mucosa rather than through local production. In the absence of any evidence of local antibody production, attention was turned to other factors.

We have recently shown that high potency FMD vaccines are capable of eliciting systemically detectable cytokine responses in pigs [24], which can be measured for up to 6 months after vaccination [26]. The pattern of cytokine responses detected in pigs, IL-6, IL-8 and IL-12, have suggested that monocytic cell activity may be an important part of an innate immune defence that contributes to the early protection of this host. Bovine and ovine cytokine responses following FMD vaccination have yet to be similarly examined. However, recent evidence has shown another cytokine, gamma interferon, to be a potent inhibitor of FMD virus in persistently infected bovine epithelial cells *in vitro* [27]. Other studies have shown that alpha and beta interferons inhibit FMDV replication [28]. Whilst the interferon system is considered a first line of defence against viral infection in mammals, it also has pleiotropic effects on cell growth [29], motility and functions including immunomodulatory activity [30]. IFN- γ is produced primarily by natural killer cells and sub-populations of T-cells during the innate and adaptive

phases of a response against viral infection [31]. Agents that promote T-cell activation should induce IFN- γ synthesis, and it is known that double-stranded RNA, gram-positive bacterial components and endotoxins stimulate IFN- γ , often following the release of IL-12, another cytokine already shown to be stimulated by emergency FMD vaccination. Double-stranded RNA would be a likely by-product of FMD antigen production, which, if not removed by further purification processes, could theoretically act as a stimulus for IFN- γ . Using cross-reactive anti-bovine reagents it was shown that FMD vaccine was capable of stimulating a IFN- γ response that was amplified by the increase in antigen payload. The significance of this finding needs further study and it will be important to find out where the IFN- γ is coming from and whether it has any direct role in local protection.

High potency vaccine, following a single administration, has previously been shown to confer early and rapid protective immunity against aerosol challenge in cattle, sheep and pigs within 4 days. Reducing the antigen payload and thereby the potency of such a vaccine as in this study, and subjecting animals to a similar aerosol challenge 14 days later has additionally shown the potential of high potency vaccine to inhibit local virus replication and prevent the carrier status in sheep. The absence of any low level virus replication in the highest payload/potency group lead to the lack of any specific secondary antibody response or non-structural antibody response. An advantage of this is that during a surveillance programme, following implementation of emergency vaccination, it would be easier to assess whether animals had become infected.

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References

- [1] Forman AJ, Garland AJM. Foot-and-mouth disease: the future of vaccine banks. *Rev Sci Tech Off Int Epiz* 2002;21:3 601–612.
- [2] Salt JS. Persistent infections with foot-and-mouth disease virus. *Trop Virol* 1998;1:77–128.
- [3] Doel TR, Williams L, Barnett PV. Emergency vaccination against foot-and-mouth disease: rate of development of immunity and its implications for the carrier state. *Vaccine* 1994;12:592–600.
- [4] Salt JS, Williams L, Statham R, Barnett PV. Further studies on the rate of development of protection in cattle given emergency vaccination against foot-and-mouth disease. Report of the Standing Technical Committee of the European Commission for the Control of foot-and-mouth disease and the foot-and-mouth disease subgroup of the Commission of the European Community, Moeldling. Appendix 17; 1995. p. 90–7.
- [5] Donaldson AI, Kitching RP. Transmission of foot-and-mouth disease by vaccinated cattle following natural challenge. *Res Vet Sci* 1989;46:9–14.
- [6] Salt JS, Williams L, Barnett PV. Emergency vaccination of pigs against foot-and-mouth disease: protection against disease and reduction in contact transmission. *Vaccine* 1998;16:746–54.
- [7] Cox SJ, Barnett PV, Dani P, Salt JS. Emergency vaccination of sheep against foot-and-mouth disease: protection against disease and reduction in contact transmission. *Vaccine* 1999;17:1855–68.
- [8] Barnett PV, Carabin H. A review of emergency foot-and-mouth disease (FMD) vaccines. *Vaccine* 2002;20:1505–14.
- [9] Follet B. *Infectious diseases in livestock*. London: Royal Society; 2002.
- [10] Plumiers FH, Akkerman AM, van der Wal P, Dekker A, Bianchi A. Lessons from the foot-and-mouth disease outbreak in The Netherlands in 2001. *Rev Sci Tech Off Int Epiz* 2002;21(3):711–21.
- [11] Doel TR, Pullen L. International bank for foot-and-mouth disease vaccine: stability studies with virus concentrates and vaccines prepared from them. *Vaccine* 1990;8:473–8.
- [12] Ferris NP, Dawson M. Routine allocation of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet Microbiol* 1988;8:249–56.
- [13] Reid SM, Grierson SS, Ferris NP, Hutchings GH, Alexandersen S. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J Virol Methods* 2003;107(2):129–39.
- [14] Schalch L, Rebeski DE, Samaras H, Lozano G, Thuer B, Schelp C. Recently generated data with the CHEKIT-FMD-3ABC ELISA kit and methods to monitor the operational performance of a 3ABC ELISA. In: Published in the Report of the European Commission for the Control of Foot-and-Mouth Disease, Session of the Research Group of the Standing Technical Committee. Izmir, Turkey, 17–20 September 2002, Rome 2001. p. 283–302.
- [15] Anon. Section 2.1. List A Diseases, Chapter 2.1.1. Foot-and-mouth disease. OIE manual of standards for diagnostic tests and vaccines; 2000. p. 77–92.
- [16] Salt JS, Mulcahy G, Kitching RP. Isotype-specific antibody responses to foot-and-mouth disease virus in sera and secretions of carrier and non-carrier cattle. *Epidemiol Infect* 1996;117:349–60.
- [17] Anderson EC, Doughty WJ, Anderson J. The effect of repeated vaccination in an enzootic foot-and-mouth disease area on the incidence of virus carrier cattle. *J Hyg (Camb)* 1974;73:229–35.
- [18] McVicar JW, Suttmoller P. Growth of foot-and-mouth disease virus in the upper respiratory tract of non-immunised vaccinated and recovered cattle after intranasal inoculation. *J Hyg (Camb)* 1976;76:467–81.
- [19] Sellers RF, Herniman KAJ, Gumm ID. The airborne dispersal of foot-and-mouth disease virus from vaccinated and recovered pigs, cattle and sheep after exposure to infection. *Res Vet Sci* 1977;23:70–5.
- [20] Cox SJ, Dani P, Salt JS, Barnett PV. Effect of emergency vaccines on local virus replication and virus persistence in sheep using two different adjuvant formulations. Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease and the Foot-and-Mouth Disease Subgroup of the Scientific Veterinary Committee of the Commission of the European Community, United Kingdom. Appendix 17; 1998. p. 139–43.
- [21] Doel TR. Potency assessment of inactivated viral vaccines. *Vaccine manual*. The production and quality control of veterinary vaccines for use in developing countries. In: Mowat N, Rweyemamu M, editors. FAO Animal Production and Health Series No. 35. Rome: Food and Agriculture Organization of the United Nations; 1997. p. 395–409.
- [22] Swayne DE, Beck JR, Garcia M, Stone HD. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. *Avian Pathol* 1999;28:245–55.

- [23] Rweyemamu MM, Black L, Boge A, Thorne AC, Terry GM. The relationship between the 140S antigen dose in aqueous FMD vaccines and the serum antibody response of cattle. *J Biol Standard* 1984;12:111–20.
- [24] Barnett PV, Cox SJ, Aggarwal N, Gerber H, McCullough KC. Further studies on the early protective responses of pigs following immunisation with high potency foot-and-mouth disease vaccine. *Vaccine* 2002;20:3197–208.
- [25] Gibson CF, Donaldson AI, Ferris NP. Response of sheep vaccinated with large doses of vaccine to challenge by airborne foot-and-mouth disease virus. *Vaccine* 1984;2:157–61.
- [26] Cox SJ, Aggarwal N, Statham RJ, Barnett PV. Longevity of antibody and cytokine responses following vaccination with high potency emergency FMD vaccines. *Vaccine* 2003;21:1336–47.
- [27] Zhang ZD, Hutchings G, Kitching P, Alexandersen S. The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells. *Arch Virol* 2002;147:2157–67.
- [28] Chinsangaram J, Koster M, Grubman MJ. Inhibition of L-deleted foot-and-mouth disease virus replication by alpha/beta interferon involves double-stranded RNA-dependent protein Kinase. *J Virol* 2001;75(12):5498–503.
- [29] Grander D, Sangfelt O, Erickson S. How does interferon exert its cell growth inhibitory effect? *Eur J Haematol* 1997;59:129–35.
- [30] Biron CA. Interferons- α and - β as immune regulators—a new look. *Immunity* 2001;14:661–4.
- [31] Sen GC. Viruses and interferons. *Annu Rev Microbiol* 2001;55:221–81.