



Final Report for Study 5791: Efficacy of Medi-Immune Inhaled Air Sterilisation Device in the Influenza Challenge Ferret Model

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This study has been conducted in accordance with the PHE Porton Quality Management System that is compliant with BS EN ISO9001-2008. All data have been checked by Dr Anthony Marriott and verified by Professor Nigel Silman.



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Glossary

BIG	Biological Investigations Group
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle Medium
GMT	Geometric mean titre
HAI	Haemagglutination inhibition
HAU	Haemagglutinating unit
IASD	Inhaled Air Sterilisation Device
MDCK	Madin-Darby Canine Kidney
NW	Nasal wash
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PFU	Plaque Forming Units
PHE	Public Health England
RDE	Receptor-destroying enzyme
RT-PCR	Reverse-transcription Polymerase Chain Reaction

1.0 Executive summary

The aim of the study was to assess the protective effects of aerosolised, UV-inactivated virus against subsequent influenza A infection in ferrets, which are the standard animal model for human influenza infection. Control groups of ferrets were exposed to infectious virus aerosol challenge, formalin-inactivated virus, or mock exposure.

The results were as follows:

- The Inhaled Air Sterilisation Device, when used with UV light on, inactivated virus infectivity in an aerosol such that none of 6 exposed ferrets showed any signs of infection.
- Three doses (500 PFU each) of UV-treated aerosol through the IASD, given 9-10 days apart, protected against disease in terms of significantly reduced weight loss compared to the mock-treated group, although the ferrets were not protected against infection.
- Three doses (500 PFU each; measured prior to inactivation) of formalin-inactivated aerosol, given 9-10 days apart, protected against disease in terms of significantly reduced weight loss compared to the mock-treated group, although the ferrets were not protected against infection.
- Of the immune responses measured (nasal wash cell counts, HAI activity in nasal wash and serum), none showed a correlation with the protection seen in the UV-treated aerosol-challenged ferrets.

1.1 Introduction

This final report outlines study 5791 performed at PHE Porton from 10-JAN-17 to 24-MAR-17. The aim of the study was to assess the protective effect of aerosolised, UV-inactivated virus against a subsequent influenza A virus infection in ferrets. Control groups of ferrets were exposed to infectious virus aerosol challenge, formalin-inactivated virus, or mock exposure. All ferrets had been previously exposed to influenza A virus of a different subtype.

2.0 Methods and Materials

All procedures were carried out as detailed in the study plan approved by PHE and the Sponsor. The following section is an overview of the key procedures. A list of protocols used in this study can be found in Appendix 1. Protocols will be supplied to the Sponsor upon request.

2.1 Preparation of virus

Influenza A/Perth/16/09 (H3N2) was used for priming of ferrets, at passage P+3A. Influenza A/California/04/09 (H1N1) was used for aerosol sprays and post-aerosol challenges, at passage P+3H. A/California/04/09 was propagated in MDCK cells. A/Perth/16/09 was propagated by 2 passages in MDCK cells, followed by expansion in embryonated chicken eggs. Both viruses were titrated by plaque assay on MDCK cells, giving titres of 1.34×10^7 PFU/ml and 1.30×10^7 PFU/ml for H1N1 and H3N2. A stock of formalin-inactivated virus was prepared by treating a batch of A/California/04/09-MA1 virus with 0.02 % methanol-free formaldehyde for 22 hours at 37°C. Excess formaldehyde was removed by repeated washing with PBS by centrifugal filtration on Vivaspin 100K MWCO columns. The final formaldehyde concentration was estimated to be ≤ 0.0004 %. Final concentration of antigen was determined as 640 HAU/ml. The preparation contained no detectable infectivity by plaque assay.

2.2 Animals

The study was conducted in accordance with the PHE Porton Down Quality Management System that is compliant with BS EN ISO9001-2008. 24 female ferrets (6

per group) were obtained from Highgate Farm, with starting weights (T=0) between 0.753-1.068 kg (mean 913.5 g).

2.3 Experimental design

All ferrets were primed by intranasal infection with H3N2 virus (100 PFU) at T=0. After 28 days, ferrets were divided into 4 groups for aerosol spray:

Group	Aerosol virus	Days of aerosol spray
1	H1N1 untreated	T+28 only
2	H1N1, UV-inactivated	T+28, 38, 47
3	H1N1, formalin-inactivated	T+28, 38, 47
4	Mock (PBS)	T+28 only

Table 1. Study groups.

On day T+56, all ferrets were challenged with a low intranasal dose (100 PFU) of H1N1 virus.

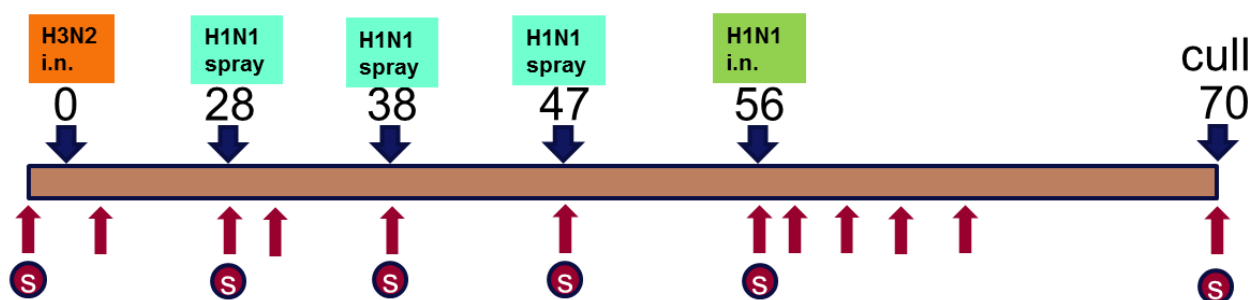


Fig. 1. Study schematic. Red arrows show days of nasal wash sampling; (S) show days of serum sampling. Days are numbered relative to the H3N2 priming infection on T=0.

2.4 Aerosol infection of ferrets

Two 5 minute sprays were conducted for each group, 3 ferrets per spray, using a 6-jet Collison nebuliser, in the order groups 4, 3, 2, 1. On days T+38 and T+47, only groups 2 and 3 were sprayed. The nebuliser delivered particles of count median aerodynamic diameter 0.72 – 0.78 μm , mass median aerodynamic diameter 1.3 – 1.5 μm .

A previous study conducted at PHE Porton using the Collison nebuliser and Henderson apparatus showed that the presence of the IASD (with UV light set to off) had no measurable effect on the spray factor of the H1N1 virus. All aerosol sprays were through the IASD to ensure an identical geometry of the apparatus; the UV light was

only switched on for group 2. Using the known mean spray factor of 1.53×10^{-6} , and a mean weight (measured on T+26) for the ferrets in groups 1-3 of 0.9066 kg, it was calculated that a nebuliser concentration of 1.88×10^5 PFU/ml was required to give a presented dose of 500 PFU to each ferret. For formalin-fixed virus, a dilution was used based on the recovery in HAU, and a ratio of 7.8×10^4 PFU/HAU for the starting material, which had a plaque titre of 1.0×10^8 PFU/ml and an HA titre of 1280 HAU/ml.

3.0 Results

3.1 Priming of ferrets with H3N2 virus

Ferrets in all groups were infected intra-nasally with 100 PFU H3N2 virus on T=0. Back-titration of the virus inoculum gave a titre of 375 PFU/ml, which is within 2-fold of the target titre of 500 PFU/ml. Successful infection of all ferrets was confirmed by a rise in nasal wash cell counts on T+4 (see Fig. 4 below), and rise in HAI titre on T+28 (Fig. 2). For the 6 ferrets showing the lowest rises in nasal wash cell count, plaque assays were performed on the nasal wash fluid, and confirmed all 6 ferrets were actively shedding virus (Appendix 2).

3.2 Serum HAI titres

All sera were treated with 3 volumes of receptor-destroying enzyme (RDE) prior to titration to remove any non-specific inhibitors of haemagglutination. Sera from days T=-3 (pre-bleed), T+28 and T+70 were titrated against H3N2 (A/Perth/16/09) and H1N1 (A/California/07/09, antigenically indistinguishable from A/California/04/09) viruses using chicken red blood cells. In addition sera taken at T+38, T+47 and T+56 were titrated against H1N1 virus.

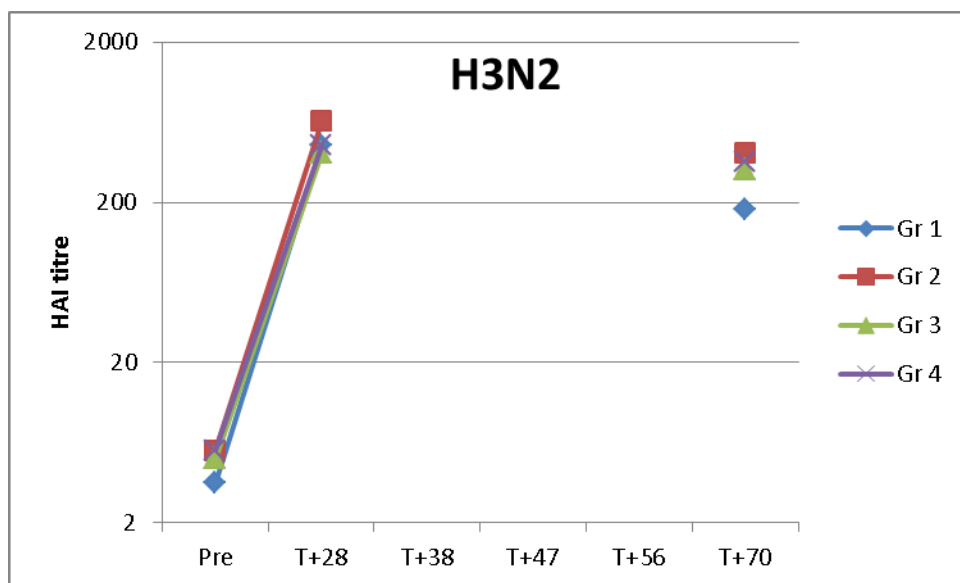


Fig. 2. Serum HAI titres against H3N2 virus for Groups 1-4. Points represent geometric mean titre for each group. Data used to plot this figure are in Appendix 3.

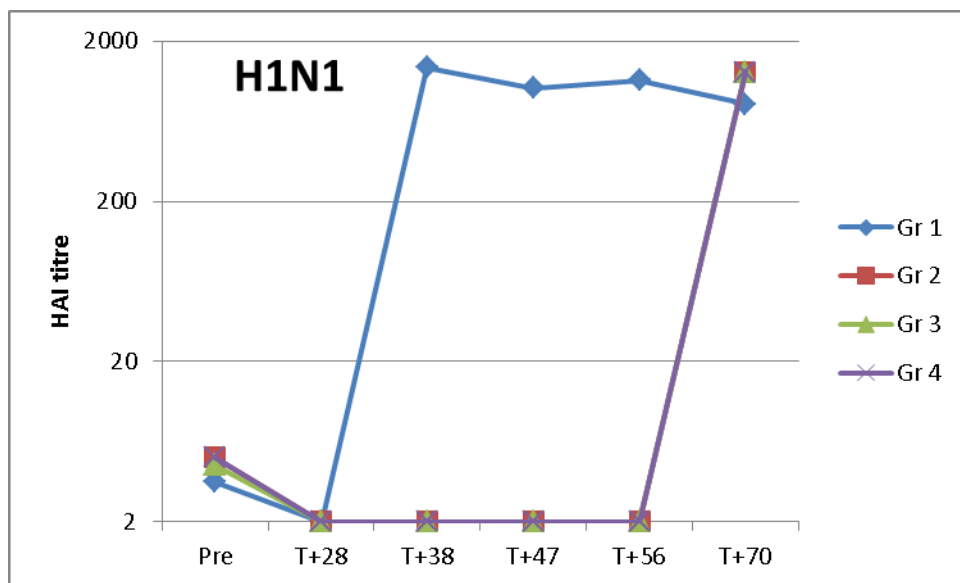


Fig. 3. Serum HAI titres against H1N1 virus for Groups 1-4. Points represent geometric mean titre for each group. Titres of <4 are plotted as 2 to allow visualisation. Data used to plot this figure are in Appendix 4.

All titres against both viruses on T=-3 were ≤ 8 , and so considered to be sero-negative. All ferrets showed sero-conversion to H3N2, but not H1N1, virus by T+28 (titres ≥ 320). H3N2 titres remained high (≥ 160) until the end of the study at T+70 (Fig. 2).

Only group 1 sero-converted to H1N1 virus by 10 days after the aerosol spray on T+28. Groups 2, 3 and 4 sero-converted to H1N1 virus by 14 days after the intra-nasal H1N1 challenge on T+56.

3.3 Nasal wash HAI titres

Nasal wash fluids taken on T+38 and T+56 were titrated by HAI without prior RDE-treatment, starting from a 2-fold dilution. All titres were ≤ 2 on both days. As these days were presumed to be the most likely to show a mucosal immune response, it was agreed with the sponsor not to titrate nasal wash fluids from additional days.

3.4 Nasal wash cell counts

Counts of viable cells in nasal wash fluid typically rise from $\leq 10^5$ cells/ml to 10^6 - 10^7 cells/ml a few days after virus infection. This rise is a consequence of the innate immune response to infection.

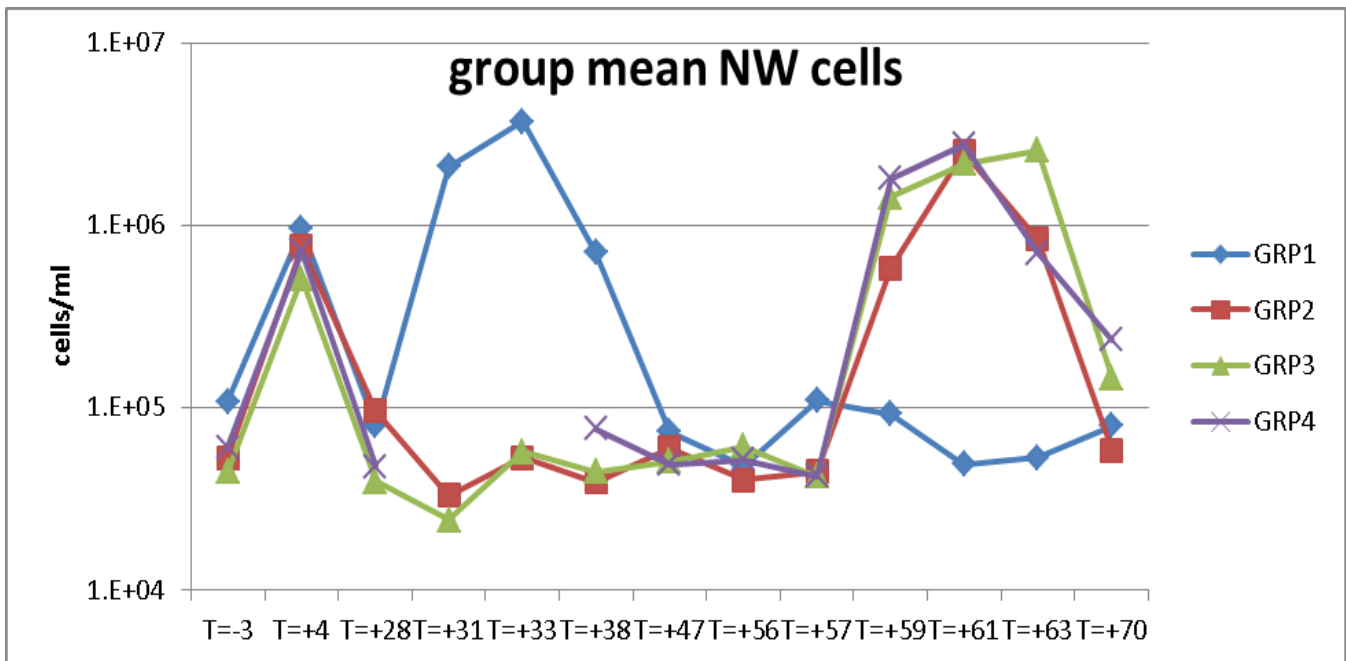


Fig. 4. Group mean nasal wash cell counts for Groups 1-4. Points represent the geometric mean titre for each group. The data used to plot this figure are in Appendix 5.

The rise in counts of all groups at T+4 represents the immune response to the H3N2 infection at T=0. Cell counts then fell to baseline levels prior to the aerosol infection on T+28. Following aerosol sprays, only group 1 show a rise in cell counts 3-5 days later, suggesting that the UV-treated and formalin-treated viruses were unable to initiate infection of the ferrets. Groups 2-4 show a rise in cell counts 3-7 days after H1N1 virus challenge on T+56, whereas group 1 did not show a rise.

3.5 Titration of aerosol-sprayed virus by plaque assay

In order to estimate presented doses of aerosolised virus, remaining nebuliser fluid and collected impinger fluid were titrated by plaque assay. As formalin-fixed virus and virus which had passed through the IASD with the UV light on, were expected to show no infectivity, samples of nebuliser and impinger fluids were extracted for RNA and titrated by real-time RT-PCR.

group	T+28		T+38		T+47	
	nebuliser	impinger	nebuliser	impinger	nebuliser	impinger
1	n/c	n/c				
2	n/c	0	2.63x10 ⁵	0	1.03x10 ⁵	2.5*
3	0	0	0	0	0	0
4	0	0				

Table 2. Plaque assay titres of nebuliser and impinger fluids. n/c, plaques not countable. Titres in PFU/ml, mean of 2 replicates. *Two plaques in one well of one replicate. The limit of detection of the assay was 1.25 PFU/ml (1 plaque averaged over 2 replicate samples).

Due to technical problems with the MDCK cells it was not possible to obtain accurate titres for the T+28 samples. The group 2 nebuliser titres on T+38 and T+47 were within 2-fold of the target titre of 1.88 x 10⁵ PFU/ml. The same dilution of the same virus stock

was used in the nebuliser for groups 1 and 2 on T+28, as for group 2 on T+38 and T+47. No virus was detected in the formalin-fixed virus group 3. No infectivity was detected in the impinger following UV-treatment of the virus for group 2 at T+28 and T+38. The low titre of 2.5 PFU/ml (calculated from 2 plaques in a single well) seen in the impinger for group 2 at T+47 was only detected in one impinger replicate. As none of the group 2 ferrets showed any signs of infection between days T+47 and T+56, it is assumed that the plaques were the result of contamination, either during collection of the impinger fluids or during set-up of the plaque assays.

3.6 Titration of aerosol-sprayed virus by RT-PCR

For RT-PCR, 0.56 ml of each fluid was extracted using the QIAamp Viral RNA Mini kit (Qiagen) and RNA was eluted in 60 µl AVE buffer. Aliquots of each RNA were quantified relative to a synthetic A/California/04/09 M gene T7 transcript by RT-PCR in the ABI 7500 FAST thermal cycler, using the Superscript III Platinum One-step Quantitative RT-PCR system and M-gene specific primers and probe. Each sample was assayed in duplicate, and compared to a standard curve constructed from triplicate samples of a 10-fold dilution series of the synthetic RNA standard. Data were analysed using SDS v1.4 software (Applied Biosystems).

		T+28	T+38	T+47
group 1	neb 1	3.39E+06		
	neb 2			
	imp 1	1.22E+04		
	imp 2	1.54E+04		
group 2	neb 1	3.04E+06	2.36E+06	2.54E+06
	neb 2		2.72E+06	2.19E+06
	imp 1	9.10E+02	1.77E+03	1.05E+03
	imp 2	1.01E+03	1.55E+03	1.06E+03
group 3	neb 1	1.23E+02	9.58E+02	1.86E+02
	neb 2	1.05E+02	8.64E+02	2.06E+02
	imp 1	3.90E+00	2.89E+00	4.15E+01
	imp 2	3.19E+01	7.14E+00	3.20E+01

Table 3. RT-PCR quantification of nebuliser (neb) and impinger (imp) fluids. Values are shown as RNA copies per µl of extracted RNA. Only 1 nebuliser replicate each was assayed for groups 1 and 2 on T+28. Values shaded in purple were below the limit of detection.

The group 2 nebuliser titres on T+38 and T+47 are all in the range $2.2-2.7 \times 10^6$ copies/ μ l, and were plaque assayed as $1.0-2.6 \times 10^5$ PFU/ml (Table 2). This suggests that group 1 and 2 nebulisers on T+28 contained at least this amount of infectivity. For group 1 the spray factor can be calculated as:

$$\text{Spray factor} = [C_{\text{imp}} * V_{\text{imp}}] / [Q_{\text{imp}} * t * C_{\text{neb}}],$$

where C_{imp} = mean impinger concentration = 1.38×10^4 ; V_{imp} = impinger volume = 10 ml; Q_{imp} = flow rate at impinger = 6000 ml/min; t = spray time = 5 min; C_{neb} = nebuliser concentration = 3.39×10^6 . Then spray factor = 1.32×10^{-6} , which agrees well with the spray factor previously calculated from virus infectivity (section 2.4).

For group 2, the impinger titres are 8.9 % of the group 1 impinger titres, suggesting that the UV treatment has reduced the measurable RNA copy number.

For group 3, nebuliser titres are greatly reduced ($< 10^3$ copies/ μ l) compared to groups 1 and 2 ($> 2 \times 10^6$ copies/ μ l), indicating that formalin-fixation is not compatible with quantitation by RT-PCR.

3.7 Virus titrations

Nasal washes from groups 1-3 for T+31 (3 days after first aerosol sprays) were titrated to confirm infection of ferrets by the aerosol route (Table 4).

Animal ID	Plaques per ml	Animal ID	Plaques per ml	Animal ID	Plaques per ml
GRP1		GRP2		GRP3	
25004	3.50E+03	24989	0	24779	0
25005	6.75E+04	24993	0	24784	0
25002	3.50E+05	24992	0	24777	0
24998	3.75E+03	24995	0	24778	0
24996	2.50E+04	24782	0	24781	0
25000	3.50E+03	24783	0	24994	0

Table 4. Plaque titration of T+31 nasal washes.

All 6 ferrets in group 1 (infected with UV light off) were infected and shedding virus. No virus was detected in nasal wash from ferrets of groups 2 (UV light on) or 3 (formalin-fixed).

H1N1 challenge virus used on T+56 was back-titrated and determined to be 150 PFU/ml. This is lower than the target of 500 PFU/ml, however the positive control virus

used on this titration also gave a value lower than expected, indicating a sub-optimal plaque assay.

Following H1N1 intra-nasal challenge on T+56, nasal washes were collected and titrated 1, 3, 5 and 7 days later:

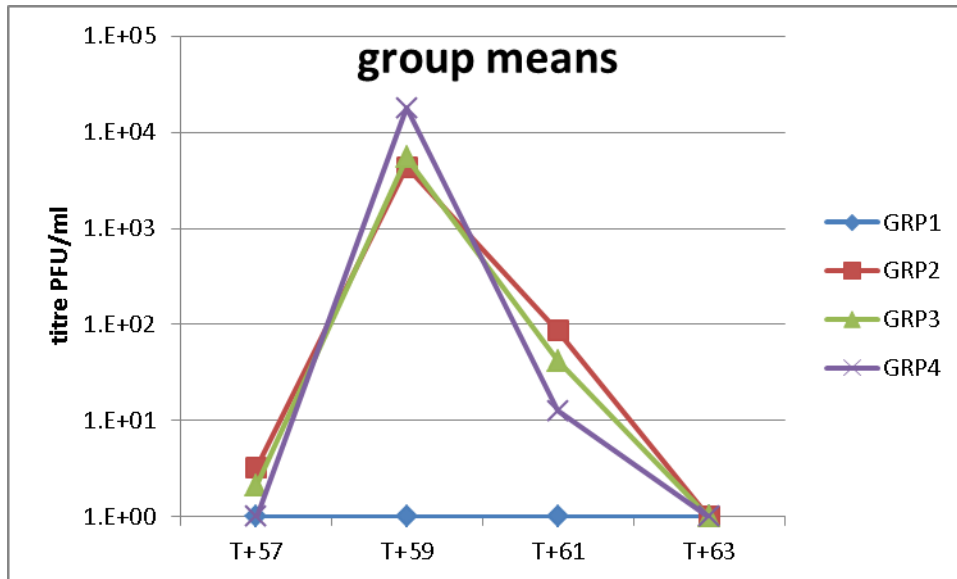


Fig. 5. Group geometric mean nasal wash titres following H1N1 challenge on day 56. Samples with no plaques detected were plotted as 1 PFU/ml. The data used to plot this figure are in Appendix 6.

No virus was detected in nasal washes from group 1 at any time-point. All 18 ferrets in groups 2-4 showed virus in nasal washes, peaking 3 days post-challenge. Titres were not significantly different between groups 2-4 on days T+57, T+59 or T+61 (1-way ANOVA). Area under the curve was calculated for each ferret, there was no significant difference between groups 2-4 (1-way ANOVA, $p = 0.20$).

3.8 Clinical signs of infection

Bodyweights were measured daily, and clinical signs were observed twice daily throughout the study, except for days T+43-55 inclusive.

The H3N2 virus challenge on T=0 resulted in minor weight loss, with each group showing a dip (1.7 % drop in 1 day) in mean weight on T+3 (Fig. 6).

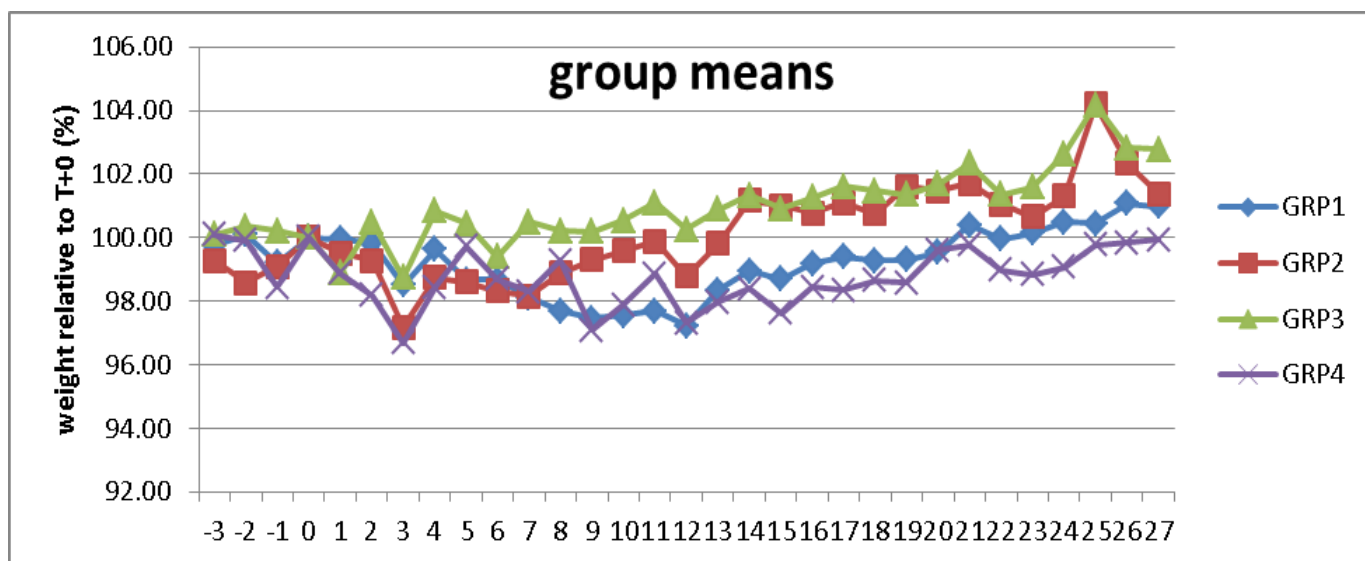


Fig. 6. Weight change following H3N2 virus challenge. Points represent group means.

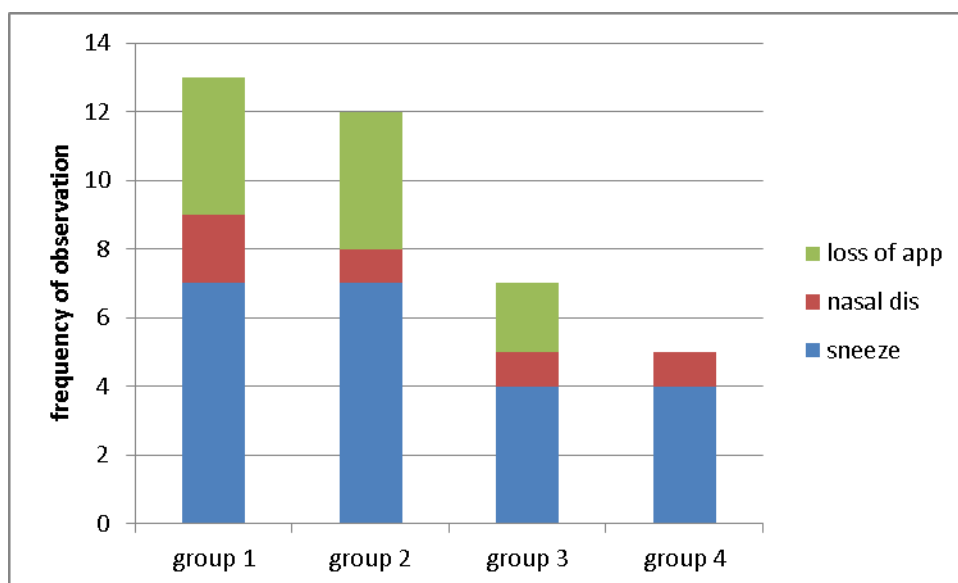


Fig. 7. Frequency of observation of clinical signs following H3N2 intra-nasal challenge. Observations of sneezing, nasal discharge and loss of appetite were summed for each group, for days T=0 to T+27 inclusive. The data used to plot this figure are in Appendix 8.

Clinical signs of infection were observed in all groups following the H3N2 challenge (Fig. 7), mostly between days 5 and 11 post-infection. No instances of inactivity or diarrhoea were observed in the 28 days post-infection.

Following the first aerosol sprays on T+28, only group 1 showed weight loss (Fig. 8):

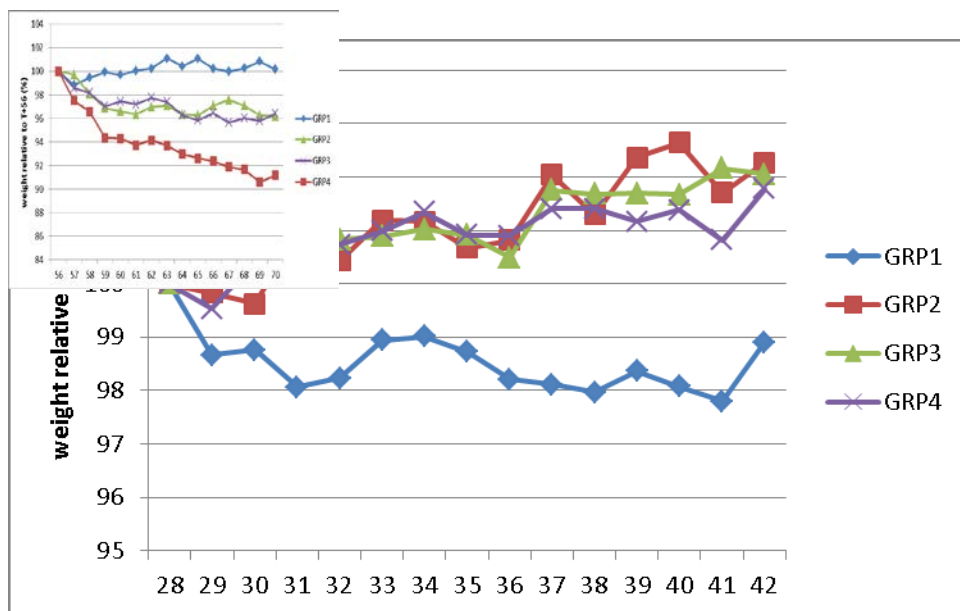


Fig. 8. Weight change following H1N1 aerosol spray. Points represent group means.

The minimum weight between T+28 and T=42 was calculated for each ferret (Appendix 7). Mean weight loss for each of groups 2-4 was between 0.7-1.1 %, whereas group 1 lost a mean of 4.0 % weight, significantly more than group 2 (t -test, $p = 0.001$).

Only group 1 showed any clinical signs following aerosol challenge, namely 5 instances of sneezing. No instances of nasal discharge, inactivity, loss of appetite or diarrhoea were observed in any of the ferrets between days T+28 and T+42 (Appendix 8).

Following the H1N1 intra-nasal challenge on T+56, significant weight loss was observed in group 4 (mock-sprayed) relative to group 1 (sprayed with infectious virus) (t -test, $p < 0.0001$ on T+59) (Fig. 9). Maximum mean weight loss of 9.7 % (group 4) was in line with previous studies using low-dose intra-nasal H1N1 challenge.

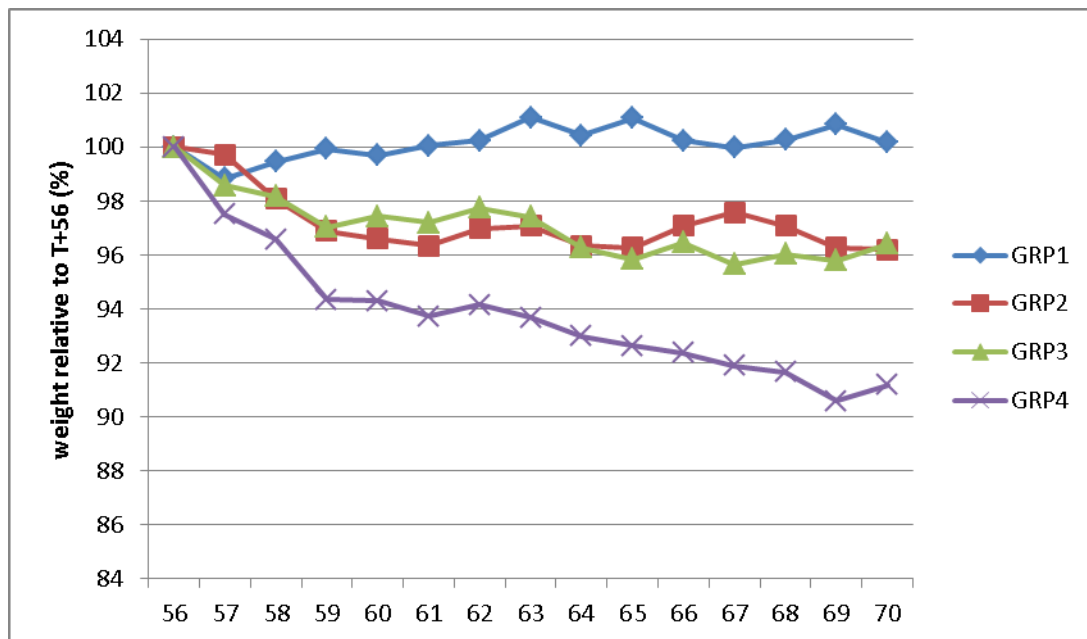


Fig. 9. Weight change following H1N1 intra-nasal challenge. Points represent group means.

Groups 2 and 3 showed an intermediate level of weight loss (mean 4.8-5.0 %) relative to groups 1 and 4. Group 2 weights were significantly less than group 1 on days T+58-66 and T+68-70 (*t*-tests, $p < 0.05$). Group 2 weights were significantly greater than group 4 on days T+57-59 and T+61-70 (*t*-tests, $p < 0.05$). In order to compare groups across days T+56 to 70 inclusive, weight gain or loss relative to T+56 (*i.e.* with T+56 set to 0 %) was plotted and area under the curve was calculated for each ferret (Appendix 7). Then groups were compared by 1-tailed *t*-test:

groups	2	3	4
1	0.007	0.002	0.001
2		0.432	0.022
3			0.024

Table 5. Comparison of areas under the curves for weight loss between groups. The *p*-values for each pair of groups are shown.

All groups showed significantly different weight loss from each other, except groups 2 and 3 were not significantly different (Table 5).

Observations of sneezing, nasal discharge and loss of appetite were summed for each group (Fig. 10). No instances of inactivity or diarrhoea were observed in any of the ferrets.

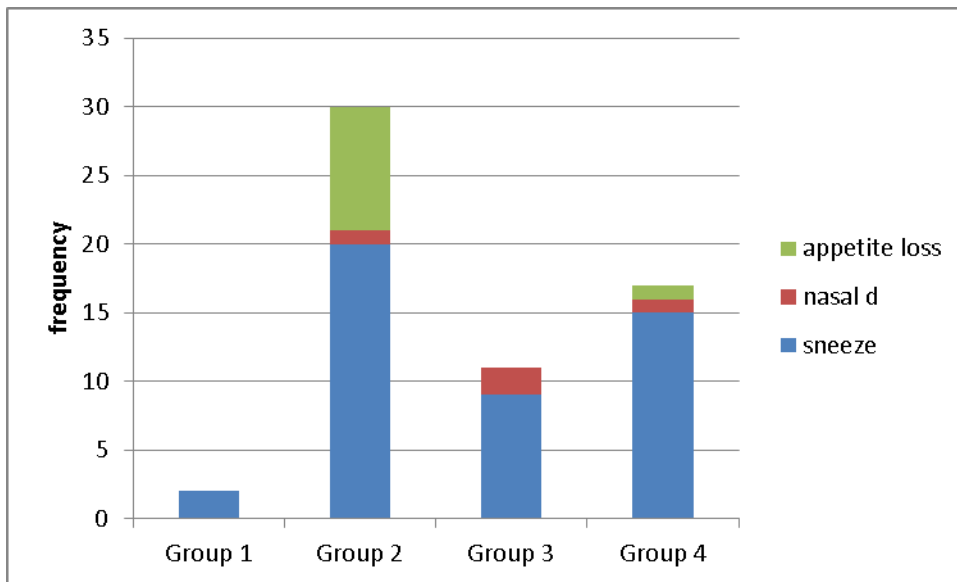


Fig. 10. Frequency of observation of clinical signs following H1N1 intra-nasal challenge. Observations of sneezing, nasal discharge and loss of appetite were summed for each group, for days T+56 to T+70 inclusive. The data used to plot this figure are in Appendix 8.

4.0 Conclusions and notification of termination of study

The study was carried out as specified in Study Plan 5791. The success of the H3N2 priming step was confirmed by the rise in nasal wash cell counts in all groups, and the high H3N2-specific HAI serum titres seen at T+28. The sprays on T+28 resulted in infection of all ferrets in group 1 (as expected for infectious virus), but no ferrets in groups 2-4, confirming that UV-treatment and formalin treatment had ablated the infectivity of the virus inocula. The spray factor calculated from the RT-PCR titres for group 1 was very close to the expected value, implying that the target presented dose of 500 PFU per ferret had been achieved, although this was not confirmed from plaque assay data due to a technical problem. Reduced RT-PCR titres for UV-treated, and especially for formalin-treated, samples indicated that RT-PCR could not be used to accurately quantify viral RNA following these treatments. A single UV-induced lesion in the amplicon target RNA molecule, such as a uracil dimer, would be able to block the RT-PCR reaction.

Group 1 showed clear protection from the H1N1 virus challenge on T+56: no weight loss, minimal clinical signs, no rise in nasal wash cell count, and no detectable virus in nasal washes. This strong protection was correlated to the high H1N1-specific serum HAI titre observed at T+56. Groups 2 and 3 showed no detectable HAI titres on T+38 and T+56 in either serum or nasal wash. Groups 2 and 3 were not protected against infection (rise in nasal wash cells, virus shedding with peak on T+59, sero-conversion to H1N1; all as for control group 4), but showed protection against disease in terms of reduced weight loss relative to the control group 4.

This report forms notification of termination of the study. Samples generated during the study will be retained at PHE for internal use, or discarded, with agreement of the Sponsor.

5.0 Appendices

Appendix 1. List of protocols used in study 5791.

IF10	Influenza plaque assays with MDCK cells
IF13	Serum separation from whole blood
IF14	Influenza Haemagglutination assay protocol
IF15	Influenza Haemagglutination Inhibition assay (HAI)
IF16	Nasal wash cell count
IF22	Counting cells using the NucleoCounter NC200
IF24	Real-time PCR

Appendix 2. Plaque titres of nasal washes taken on T+4.

Group	Ferret ID	Titre, PFU/ml
1	25002	2.50×10^4
2	24995	3.75×10^4
3	24779	1.48×10^4
3	24784	1.20×10^3
3	24994	1.00×10^3
4	24990	5.00×10^4

Appendix 3. Serum HAI titres against H3N2 virus.

Ferret
ID

GRP1	T=-3	T+28	T+70
25004	4	>1280	160
25005	4	320	160
25002	4	640	320
24998	<4	320	160
24996	4	320	160
25000	4	320	160

GRP2

24989	<4	320	320
24993	4	>1280	320
24992	8	>1280	640
24995	8	320	320
24782	8	640	640
24783	8	640	320

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GRP3

24779	4	320	320
24784	4	320	160
24777	8	640	320
24778	4	320	320
24781	8	320	320
24994	4	640	640

GRP4

24991	4	320	320
24997	8	320	320
24990	4	320	320
24999	8	640	640
24776	8	640	320
25001	4	640	320

Appendix 4. Serum HAI titres against H1N1 virus.

Ferret

ID	HAI titre vs A/Cal/07/09					
GRP1	T=-3	T+28	T+38	T+47	T+56	T+70
25004	4	<4	1440	>1280	>1280	>1280
25005	4	<4	1440	>1280	>1280	>1280
25002	4	<4	1440	>1280	>1280	>1280
24998	<4	<4	1440	>1280	>1280	640
24996	4	<4	1440	>1280	1280	640
25000	4	<4	1040	320	640	320

GRP2

24989	<4	<4	<4	<4	<4	>1280
24993	4	<4	<4	<4	<4	>1280
24992	4	<4	<4	<4	<4	>1280
24995	8	<4	<4	<4	<4	>1280
24782	8	<4	<4	<4	<4	>1280
24783	8	<4	<4	<4	<4	>1280

GRP3

24779	4	<4	<4	<4	<4	>1280
24784	4	<4	<4	<4	<4	>1280
24777	4	<4	<4	<4	<4	>1280
24778	4	<4	<4	<4	<4	>1280

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24781	8	<4	<4	<4	<4	>1280
24994	4	<4	<4	<4	<4	>1280

GRP4

24991	4	<4	<4	<4	<4	>1280
24997	8	<4	<4	<4	<4	>1280
24990	4	<4	<4	<4	<4	>1280
24999	8	<4	<4	<4	<4	>1280
24776	4	<4	<4	<4	<4	>1280
25001	4	<4	<4	<4	<4	>1280

Appendix 5. Nasal wash cell counts.

		T=-3	T=+4	T=+28	T=+31	T=+33	T=+38	T=+47
GRP1	Live H1N1 spray							
	25004	2.77E+05	7.86E+05	4.79E+05	1.96E+06	2.78E+06	1.42E+06	1.18E+05
	25005	6.03E+04	1.51E+06	4.87E+04	2.39E+06	6.25E+06	8.94E+05	9.13E+04
	25002	8.17E+04	2.20E+05	2.26E+04	4.63E+06	2.47E+06	3.42E+05	9.54E+04
	24998	1.52E+05	3.74E+06	5.13E+05	3.95E+05	5.69E+06	1.30E+06	5.49E+04
	24996	1.39E+05	1.45E+06	3.36E+04	3.72E+06	4.48E+06	3.29E+05	8.85E+04
	25000	5.50E+04	5.25E+05	2.75E+04	2.58E+06	2.34E+06	6.73E+05	3.16E+04
	<i>geomean</i>	1.08E+05	9.52E+05	7.94E+04	2.09E+06	3.70E+06	7.07E+05	7.35E+04
GRP2	UV H1N1							
	24989	1.81E+05	6.27E+05	6.68E+05	6.52E+04	7.21E+04	9.20E+04	3.36E+04
	24993	4.74E+04	1.07E+06	5.97E+04	4.19E+04	4.87E+04	7.07E+04	9.95E+04
	24992	4.34E+04	9.37E+05	8.10E+04	3.36E+04	4.53E+04	2.61E+04	5.01E+04
	24995	3.01E+04	3.17E+05	1.05E+05	4.05E+04	4.87E+04	7.69E+04	1.06E+05
	24782	4.19E+04	8.63E+05	1.99E+04	8.07E+03	8.72E+04	8.92E+03	4.53E+04
	24783	4.67E+04	1.17E+06	1.10E+05	4.26E+04	3.36E+04	2.95E+04	5.63E+04
	<i>geomean</i>	5.29E+04	7.65E+05	9.52E+04	3.29E+04	5.32E+04	3.88E+04	5.97E+04
GRP3	Formalin H1N1							
	24779	4.91E+04	2.62E+05	4.05E+04	6.18E+03	4.39E+04	5.01E+04	3.04E+04
	24784	1.81E+05	3.73E+05	5.15E+04	2.81E+04	8.30E+04	5.19E+04	6.45E+04
	24777	1.74E+05	4.44E+05	4.19E+04	4.05E+04	7.60E+04	8.27E+04	1.36E+05
	24778	8.41E+03	7.17E+05	2.29E+04	3.77E+04	8.51E+04	4.60E+04	3.16E+04
	24781	6.18E+03	1.41E+06	3.84E+04	2.95E+04	3.29E+04	2.95E+04	1.06E+05
	24994	9.60E+04	3.90E+05	4.74E+04	2.47E+04	4.53E+04	2.61E+04	1.85E+04
	<i>geomean</i>	4.45E+04	5.08E+05	3.92E+04	2.40E+04	5.72E+04	4.44E+04	5.05E+04
GRP4	Mock spray							
	24991	1.19E+05	1.65E+06	7.16E+04			5.56E+04	1.48E+05

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24997	4.62E+04	8.73E+05	2.13E+04	7.69E+04	1.78E+04
24990	2.47E+04	6.04E+04	8.85E+04	3.50E+04	4.05E+04
24999	5.33E+04	6.69E+05	1.17E+04	5.01E+04	3.16E+04
24776	3.16E+04	5.23E+06	3.98E+04	3.95E+05	1.35E+05
25001	2.13E+05	4.56E+05	1.76E+05	6.73E+04	2.95E+04
<i>geomean</i>	<i>6.04E+04</i>	<i>7.20E+05</i>	<i>4.72E+04</i>	<i>7.64E+04</i>	<i>4.88E+04</i>

T=+56	T=+57	T=+59	T=+61	T=+63	T=+70
9.13E+04	1.45E+05	6.59E+04	4.53E+04	3.77E+04	8.99E+04
1.30E+04	1.27E+05	9.53E+04	4.74E+04	5.97E+04	6.66E+04
6.59E+04	5.19E+04	1.00E+05	3.98E+04	3.71E+04	1.58E+04
4.60E+04	3.11E+05	2.05E+05	3.15E+04	1.17E+05	8.37E+04
6.86E+04	4.12E+04	1.41E+05	6.11E+04	7.14E+04	2.13E+05
4.87E+04	1.39E+05	3.29E+04	8.24E+04	3.09E+04	1.50E+05
<i>4.79E+04</i>	<i>1.09E+05</i>	<i>9.18E+04</i>	<i>4.88E+04</i>	<i>5.28E+04</i>	<i>7.95E+04</i>
1.01E+05	5.63E+04	2.98E+06	2.70E+06	2.68E+05	1.72E+04
1.99E+04	3.98E+04	6.47E+05	9.76E+05	4.72E+05	7.00E+04
4.12E+04	2.94E+04	3.16E+04	3.21E+06	3.59E+05	4.74E+04
5.19E+04	5.15E+04	7.91E+05	3.69E+06	3.33E+06	2.28E+05
1.44E+04	8.85E+04	5.63E+05	2.01E+06	1.17E+06	2.94E+04
6.52E+04	2.54E+04	1.41E+06	4.21E+06	1.98E+06	1.03E+05
<i>3.99E+04</i>	<i>4.44E+04</i>	<i>5.80E+05</i>	<i>2.53E+06</i>	<i>8.40E+05</i>	<i>5.83E+04</i>
3.01E+04	6.86E+03	4.40E+06	4.15E+06	3.00E+06	7.76E+04
1.71E+05	9.88E+04	5.68E+05	4.19E+05	2.28E+06	1.66E+05
1.27E+05	5.40E+04	2.26E+06	1.99E+06	1.56E+06	1.72E+05
8.78E+04	4.80E+04	5.89E+06	4.89E+06	5.23E+06	2.95E+05
2.06E+04	5.08E+04	3.63E+05	7.19E+06	3.49E+06	3.37E+05
4.25E+04	6.04E+04	7.03E+05	8.73E+05	1.51E+06	4.32E+04
<i>6.07E+04</i>	<i>4.19E+04</i>	<i>1.43E+06</i>	<i>2.18E+06</i>	<i>2.58E+06</i>	<i>1.46E+05</i>
1.51E+05	1.92E+04	2.62E+06	2.60E+06	1.83E+06	3.26E+05
1.99E+04	3.09E+04	2.57E+06	1.46E+06	1.94E+05	7.89E+04
2.75E+04	5.19E+04	6.06E+05	3.78E+06	2.42E+05	1.56E+05
7.55E+04	6.59E+04	2.47E+06	5.01E+06	2.64E+06	5.00E+05
2.26E+04	2.34E+05	1.87E+06	1.79E+06	5.97E+05	3.05E+05
1.35E+05	1.17E+04	1.85E+06	3.44E+06	8.81E+05	2.70E+05
<i>5.17E+04</i>	<i>4.21E+04</i>	<i>1.81E+06</i>	<i>2.76E+06</i>	<i>7.02E+05</i>	<i>2.34E+05</i>

Values highlighted in pink were deemed to be unusually low, and plaque assays were performed to confirm infection in these ferrets (section 3.1). Cells highlighted in green were deemed to be unusually high. 'Geomean' is geometric mean count for each group.

Appendix 6. Nasal wash plaque assay titres.

Plaque assay titres, PFU/ml

GRP1	T+57	T+59	T+61	T+63
25004	1.00E+00	1.00E+00	1.00E+00	1.00E+00
25005	1.00E+00	1.00E+00	1.00E+00	1.00E+00
25002	1.00E+00	1.00E+00	1.00E+00	1.00E+00
24998	1.00E+00	1.00E+00	1.00E+00	1.00E+00
24996	1.00E+00	1.00E+00	1.00E+00	1.00E+00
25000	1.00E+00	1.00E+00	1.00E+00	1.00E+00
<i>geomean</i>	1.00E+00	1.00E+00	1.00E+00	1.00E+00
GRP2				
24989	1.00E+00	1.43E+04	3.75E+01	1
24993	2.50E+00	3.75E+03	2.50E+02	1
24992	1.00E+00	5.00E+01	5.00E+00	1
24995	1.00E+00	2.50E+04	3.50E+02	1
24782	4.75E+02	1.13E+03	7.50E+00	1
24783	1.00E+00	9.25E+04	3.25E+03	1
<i>geomean</i>	3.25E+00	4.37E+03	8.58E+01	1.00E+00
GRP3				
24779	1.00E+00	3.50E+04	5.00E+00	1
24784	1.00E+00	6.25E+03	6.00E+01	1
24777	2.50E+00	2.75E+04	1.75E+02	1
24778	3.75E+01	8.25E+01	1.50E+01	1
24781	1.00E+00	1.15E+04	3.00E+02	1
24994	1.00E+00	4.75E+03	2.25E+01	1
<i>geomean</i>	2.13E+00	5.48E+03	4.18E+01	1.00E+00
GRP4				
24991	1.00E+00	7.50E+04	5.50E+01	1
24997	1.00E+00	6.75E+04	1.00E+00	1
24990	1.00E+00	2.25E+03	5.00E+00	1
24999	1.00E+00	9.50E+04	4.75E+02	1
24776	1.00E+00	4.75E+04	3.25E+01	1
25001	1.00E+00	6.25E+02	1.00E+00	1
<i>geomean</i>	1.00E+00	1.78E+04	1.27E+01	1.00E+00

Note: all zero values were counted as 1 to allow log plot. 'Geomean' is geometric mean count for each group.

Appendix 7. Ferret weights.

Raw weight data and normalised weight data will be supplied to the Sponsor electronically, as Excel files.

Weight loss from T+28 to T+42, expressed as minimum weight for each ferret, normalised to 100 % on T+28:

Max wt loss

GRP1

25004	95.79
25005	96.14
25002	96.15
24998	97.17
24996	93.03
25000	97.69

<i>mean</i>	<i>96.00</i>
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GRP2

24989	100
24993	100
24992	98.15
24995	98.16
24782	99.56
24783	98.66

<i>mean</i>	<i>99.09</i>
-------------	--------------

GRP3

24779	99.35
24784	97.66
24777	100
24778	98.63
24781	100
24994	100

<i>mean</i>	<i>99.27</i>
-------------	--------------

GRP4

24991	100.00
24997	99.79
24990	100.00
24999	95.65
24776	99.60
25001	98.08

<i>mean</i>	<i>98.85</i>
-------------	--------------

Weight loss from T+56 to T+70, expressed as change from weight on T+70 (%):

T+	GRP1	25004	25005	25002	24998	24996	25000
56.00		0.00	0.00	0.00	0.00	0.00	0.00
57.00		-2.00	-0.60	-0.92	-0.70	0.35	-3.06

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58.00	-2.30	0.97	-1.13	-0.30	0.81	-1.36
59.00	-1.50	1.21	-1.23	0.89	1.62	-1.36
60.00	-1.60	0.85	0.00	0.20	0.92	-2.15
61.00	-1.40	1.57	-0.62	-0.10	3.35	-2.49
62.00	-2.40	1.69	0.72	1.09	1.62	-1.25
63.00	-1.90	2.18	1.23	1.39	3.58	0.11
64.00	-1.90	0.48	0.51	1.29	2.42	-0.23
65.00	1.40	1.45	-0.31	1.09	2.77	0.00
66.00	-2.90	0.00	0.72	1.29	2.89	-0.57
67.00	-2.90	0.24	1.33	0.30	1.85	-0.91
68.00	-3.10	0.73	1.23	1.19	2.19	-0.68
69.00	-2.90	1.33	1.54	1.39	4.50	-0.91
70.00	-1.70	0.36	1.64	0.40	2.31	-2.04

GRP2	24989	24993	24992	24995	24782	24783
	0.00	0.00	0.00	0.00	0.00	0.00
	0.39	-0.73	-1.28	2.21	-1.29	-1.05
	-1.75	-0.52	-1.60	-1.33	-4.42	-1.87
	-0.68	-1.98	-2.66	-3.43	-7.87	-2.10
	-0.58	-2.29	-2.66	-3.20	-8.52	-3.15
	-1.07	-2.71	-3.30	-2.98	-7.55	-4.32
	-1.07	-2.50	-2.66	-2.54	-7.12	-2.22
	-2.04	-2.71	-2.77	0.33	-7.23	-3.15
	-3.01	-3.85	-4.15	-0.44	-6.47	-3.97
	-3.78	-4.48	-2.66	-0.99	-6.90	-3.62
	-2.62	-3.85	-3.51	1.33	-6.90	-1.98
	-1.94	-3.85	-4.26	2.54	-6.26	-0.82
	-3.30	-4.38	-3.51	2.10	-7.12	-1.28
	-4.66	-4.58	-3.83	1.10	-7.34	-3.15
	-4.66	-4.69	-4.04	0.44	-7.55	-2.33

GRP3	24779	24784	24777	24778	24781	24994
	0.00	0.00	0.00	0.00	0.00	0.00
	-1.55	-0.87	-3.32	-0.60	-0.95	-1.32
	-0.41	-1.65	-3.77	-2.74	-1.04	-1.44
	-1.45	-1.16	-4.21	-3.33	-3.60	-4.07
	0.21	-1.36	-4.98	-2.50	-2.65	-4.07
	-1.04	-1.65	-4.43	-3.33	-2.75	-3.59
	0.72	-0.68	-4.98	-2.26	-3.60	-2.75
	0.52	-1.55	-5.54	-1.67	-3.89	-3.35
	0.00	-2.42	-6.76	-3.21	-5.78	-4.19
	-1.24	-3.49	-6.76	-4.52	-4.93	-4.07
	-0.72	-2.81	-6.31	-3.10	-4.93	-3.35
	-1.66	-3.29	-6.42	-5.12	-4.55	-5.03

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	-1.04	-2.13	-7.31	-4.05	-5.02	-4.19
	-1.24	-2.13	-6.20	-5.12	-4.93	-5.63
	-0.21	-1.26	-6.20	-5.12	-4.17	-4.55
GRP4	24991	24997	24990	24999	24776	25001
	0.00	0.00	0.00	0.00	0.00	0.00
	-3.08	-2.15	-2.32	-3.77	-1.54	-2.13
	-4.25	-3.03	-3.15	-4.82	-1.25	-3.97
	-7.11	-5.48	-5.94	-7.64	-3.84	-3.97
	-8.07	-4.40	-4.27	-7.96	-4.32	-5.23
	-8.28	-4.99	-4.55	-10.16	-4.32	-5.33
	-7.01	-4.21	-3.15	-11.31	-5.09	-4.26
	-7.32	-3.03	-2.32	-12.15	-7.20	-5.91
	-7.96	-3.62	-4.17	-12.46	-7.77	-6.10
	-7.32	-4.60	-3.43	-14.45	-6.91	-7.46
	-7.86	-4.60	-3.99	-16.34	-7.20	-5.81
	-8.60	-4.50	-4.27	-17.80	-7.29	-6.10
	-8.60	-5.19	-4.73	-18.74	-6.91	-5.91
	-9.66	-5.19	-6.68	-19.58	-8.06	-7.36
	-8.17	-4.79	-6.86	-20.42	-7.20	-5.43

Area under the curve from the above data (T+56 to T+70) for each ferret:

GRP1	25004.00	25005.00	25002.00	24998.00	24996.00	25000.00
	-26.2763	12.2733	3.8974	9.2354	30.0231	-15.855
GRP2	24989.00	24993.00	24992.00	24995.00	24782.00	24783.00
	-28.419	-40.7813	-40.8511	-5.0829	-88.781	-33.839
GRP3	24779.00	24784.00	24777.00	24778.00	24781.00	24994.00
	-9.0062	-25.8236	-74.0864	-44.1071	-50.7109	-49.3413
GRP4	24991.00	24997.00	24990.00	24999.00	24776.00	25001.00
	-99.2038	-57.3875	-56.4007	-167.382	-75.2879	-72.2868

Appendix 8. Clinical observations.

Summary of clinical observations between T=0 and T+27.

Ferret ID	sneezing		loss of appetite		nasal discharge	
	sum	group sum	sum	group sum	sum	group sum
GRP1						
25004	0	7	0	4	0	2
25005	1		2		0	
25002	0		0		2	
24998	4		2		0	
24996	0		0		0	

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25000	2		0		0	
GRP2						
24989	0	7	0	4	0	1
24993	3		2		0	
24992	1		2		0	
24995	0		0		0	
24782	3		0		0	
24783	0		0		1	
GRP3						
24779	0	4	0	2	0	1
24784	0		0		1	
24777	2		0		0	
24778	1		2		0	
24781	0		0		0	
24994	1		0		0	
GRP4						
24991	1	4	0	0	0	1
24997	0		0		1	
24990	0		0		0	
24999	1		0		0	
24776	0		0		0	
25001	2		0		0	

	Diarrhea	inactivity
group 1	none	none
group 2	none	none
group 3	none	none
group 4	none	none

Summary of clinical observations between T+28 and T+42

GRP1	sneezing	
	sum	total
25004	3	5
25005	0	
25002	0	
24998	2	
24996	0	
25000	0	
GRP2		
24989	0	0
24993	0	

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24992 0
 24995 0
 24782 0
 24783 0

GRP3

24779 0 0
 24784 0
 24777 0
 24778 0
 24781 0
 24994 0

GRP4

24991 0 0
 24997 0
 24990 0
 24999 0
 24776 0
 25001 0

	Diarrhea	inactivity	nasal discharge	appetite loss
group 1	none	none	none	none
group 2	none	none	none	none
group 3	none	none	none	none
group 4	none	none	none	none

Summary of clinical observations between T+56 and T+70

GRP1	sneezing		nasal discharge		loss of appetite	
	sum	group sum	sum	group sum	sum	group sum
25004	0	2	0	0	0	0
25005	0		0		0	
25002	1		0		0	
24998	1		0		0	
24996	0		0		0	
25000	0		0		0	
GRP2						
24989	2	20	0	1	1	9
24993	7		1		2	
24992	2		0		1	
24995	3		0		1	
24782	4		0		2	
24783	2		0		2	

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GRP3

24779	2	9	0	2	0	0
24784	1		0		0	
24777	0		0		0	
24778	1		2		0	
24781	2		0		0	
24994	3		0		0	

GRP4

24991	2	15	0	1	0	1
24997	2		0		0	
24990	2		0		0	
24999	2		0		0	
24776	5		0		1	
25001	2		1		0	

Inactivity

Group 1	None recorded
Group 2	None recorded
Group 3	None recorded
Group 4	None recorded

Diarrhea

Group 1	None recorded
Group 2	None recorded
Group 3	None recorded
Group 4	None recorded