Supplementary data

2. Material and Methods

2.3 Vaccine and Viruses - Vaccine production.

Egg derived MDV's [3] were kindly provided by Institute of Experimental Medicine and cloned (prior to co-infection) over two rounds of plaque purification in MDCK cells according to standard techniques from original ampoules from 1963 and 1973 respectively. The resulting clones were amplified in MDCK cells under Good Manufacturing Practice (GMP) conditions prior to use (further referred to as MDV seed virus). The MDV seed viruses were characterized for absence of extraneous agents [31] and their full length sequence was confirmed to be identical to the original [32]. After co-infection, 6:2 re-assortant viruses were selected by plaque purification in MDCK cells using anti MDV rabbit antiserum as negative selection. Single plaque clones obtained over two rounds, were amplified in MDCK cells. The *ts* and *ca* phenotypes of the re-assortant vaccine viruses were confirmed once more by titration of the virus at various temperatures and their genetic conformation was confirmed by PCR using strain specific primers and sequence analysis to show the identity of the HA and NA inserts with the used reference strains and to acertain the presence of the *ca/ts* mutations [9, 31-34]. Vaccine seed virus stocks of each of the re-assortants were propagated in MDCK cells at 32°C under GMP and sequenced to confirm the genotype.

Monovalent vaccine virus lots were reared by amplification of seed viruses under GMP in MDCK cells grown in animal component free medium containing recombinant trypsin at 32^{o}C for three days. The viral harvest was clarified by depth filtration, concentrated 100-fold in phosphate buffer (61.4mM K_2HPO_4 and 38.2mM KH_2PO_4) by ultrafiltration, mixed 1:1 with a 2x concentrated stabilizer solution

(phosphate buffer containing 237.4mM arginine monohydrochloride, 16.0 mM glutamic acid monosodium monohydrate and 584.2mM sucrose) and stored at -70^{o}C. Aliquots were taken to determine the live virus titer of each of the monovalent vaccines. Based on these titers the various vaccines were blended and filled into 0.4 mL vials and stored at -20^{o}C. Prior to use, each vial was assembled into an intranasal applicator (Pfeiffer-Valois) validated to deliver 0.1 mL vaccine per nostril. Immediately prior to administration the strength of each of the strains in the vaccine was reconfirmed by titration (Table 1). The subtype of each of the strains in the formulation was detected in individual assay runs by Immunofluoresence using strain specific antibodies. These antibodies were provided by NIBSC and are normally used for seasonal influenza quantification in the Single Radial immunoDiffusion (SRD) test, and were biotin labeled prior to use. For each subtype reference and negative control viruses were included to control the assay performance. This cell culture derived, trivalent Live Attenuated Influenza Vaccine (code name SCH 900795), will be further referred to as LAIV.

2.5 Haemaglutination and Virus Neutralisation assays - Serology

Haemagglutination Inhibition. Briefly, serum samples were treated with receptor destroying enzyme (RDE: cholera filtrate (CF) obtained from *Vibrio cholerae* cultures) for 16 hrs at 37ºC. After preparation of two-fold dilutions of CF-treated sera in microplate wells (lowest dilution of 1:20, highest dilution of 1:20480), serum dilutions were incubated with 4 haemagglutinating units (HAU) of wildtype season matched Influenza virus for 30 minutes at 37ºC. Subsequently, Turkey Red Blood Cells were added to each well, the microtiter plates were incubated for 30 min at 4ºC and then the microtiter plates were scored visually. The highest serum dilution giving complete inhibition of haemagglutination was defined as the HI titre.

Virus Neutralisation. Briefly, two-fold serial dilutions of a serum sample starting at a dilution of 1:32 were incubated with 100 $TCID_{50}$ of wildtype season

matched influenza virus (H1N1, H3N2 or B). The mix was subsequently transferred to 96 wells plates with a confluent layer of MDCK - cells (ATCC CCL34). After incubation at 37°C for 1 hour the cells were washed and cultured for 6-7 days at in the presence of 5% CO_2. Virus propagation in the wells was read by screening for agglutination of turkey red blood cells. Virus titers were scored by taking the highest dilution of serum in which 50% of the inoculated cell cultures demonstrated agglutination.

Tables

Table 1: Geometric Mean Increase in HI Titers from Baseline to Day 29, Adjusted for Baseline HI Titer and Age Stratum, by Vaccine Strain and Vaccine Group, Per Protocol Set

Strain	Dose group ($TCID_{50}$)	GMI [a]	95%CI	
A/H1N1	10^5	1.20	0.99	1.44
	10^6	1.29	1.07	1.56
	10^7	1.08	0.89	1.29
A/H3N2	10^5	2.38	1.60	3.52
	10^6	2.44	1.64	3.64
	10^7	2.31	1.56	3.43
B	10^5	1.17	0.96	1.43
	10^6	1.11	0.91	1.36
	10^7	1.01	0.83	1.23

a: Adjusted for baseline HI titer and age stratum

CI = confidence interval; $TCID_{50}$ = tissue culture infectious dose (50%).

Table 2: Percentage of Seroprotection at Day 29, by Strain, Dose Group and Assessment, Per Protocol Set

		Dose group											
		10^5 $TCID_{50}$			10^6 $TCID_{50}$			10^7 $TCID_{50}$			Placebo		
		N=29			N=29			N=29			N=30		
Strain	Day	n	%	95% CI [a]	n	%	95% CI [a]	n	%	95% CI [a]	n	%	95% CI [a]
A/H1N1	Baseline	15	51.7	(32.5, 70.6)	9	31.0	(15.3, 50.8)	10	34.5	(17.9, 54.3)	18	60.0	(40.6, 77.3)
	Day 29	16	55.2	(35.7, 73.6)	11	37.9	(20.7, 57.7)	11	37.9	(20.7, 57.7)	15	50.0	(31.3, 68.7)
A/H3N2	Baseline	10	34.5	(17.9, 54.3)	12	41.4	(23.5, 61.1)	13	44.8	(26.4, 64.3)	12	40.0	(22.7, 59.4)
	Day 29	19	65.5	(45.7, 82.1)	18	62.1	(42.3, 79.3)	21	72.4	(52.8, 87.3)	11	36.7	(19.9, 56.1)
B	Baseline	25	86.2	(68.3, 96.1)	24	82.8	(64.2, 94.2)	20	69.0	(49.2, 84.7)	26	86.7	(69.3, 96.2)
	Day 29	26	89.7	(72.6, 97.8)	23	79.3	(60.3, 92.0)	21	72.4	(52.8, 87.3)	25	83.3	(65.3, 94.4)

a: 95% CI is calculated according to Clopper-Pearson

n = number of subjects in the group with a serum titer > 40.

CI = confidence interval; $TCID_{50}$ = tissue culture infectious dose (50%).