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General Articles

A new modified live equine influenza virus vaccine: phenotypic stability, restricted spread and efficacy against heterologous virus challenge

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Summary

Flu Avert IN vaccine is a new, live attenuated virus vaccine for equine influenza. We tested this vaccine *in vivo* to ascertain 1) its safety and stability when subjected to serial horse to horse passage, 2) whether it spread spontaneously from horse to horse and 3) its ability to protect against heterologous equine influenza challenge viruses of epidemiological relevance. For the stability study, the vaccine was administered to 5 ponies. Nasal swabs were collected and pooled fluids administered directly to 4 successive groups of naïve ponies by intranasal inoculation. Viruses isolated from the last group retained the vaccine's full attenuation phenotype, with no reversion to the wild-type virus phenotype or production of clinical influenza disease. The vaccine virus spread spontaneously to only 1 of 13 nonvaccinated horses/ponies when these were comingled with 39 vaccinates in the same field. For the heterologous protection study, a challenge model system was utilised in which vaccinated or naïve control horses and ponies were exposed to the challenge virus by inhalation of virus-containing aerosols. Challenge viruses included influenza A/equine-2/Kentucky/98, a recent representative of the 'American' lineage of equine-2 influenza viruses; and A/equine-2/Saskatoon/90, representative of the 'Eurasian' lineage. Clinical signs among challenged animals were recorded daily using a standardised scoring protocol. With both challenge viruses, control animals reliably contracted clinical signs of influenza, whereas vaccinated animals were reliably protected from clinical disease. These results demonstrate that Flu Avert IN vaccine is safe and phenotypically stable, has low spontaneous transmissibility and is effective in protecting horses against challenge viruses representative of those in circulation worldwide.

Introduction

A new and unconventional modified live equine influenza, Flu Avert IN (Youngner *et al.* 2001), licensed for use in the USA in 1999, is administered through an intranasal applicator. A single dose, administered to naïve horses, provides complete protection against challenge from the homologous wild-type virus for up to 3 months and significant reduction of clinical signs for 6 months or longer (Townsend *et al.* 1999b; Lunn *et al.* 2001). The virus in

TABLE 1: Clinical signs scoring index

Clinical sign	Description	Score
Coughing	No cough during daily observation period	0
	Coughing once during daily observation	1
	Coughing twice or more during observation	2
Nasal discharge	No discharge	0
	Serous discharge	1
	Mucopurulent discharge	2
	Profuse mucopurulent discharge	3
Respiration	Normal (<36/min)	0
	Abnormal (dyspnoea, tachypnoea >36/min)	1
Depression	No depression	0
	Depression present (lethargy, inappetence)	1

TABLE 2: Serial pony-to-pony passage; virus detection from nasal samples. Titres are $TCID_{50}$/ml of nasal swab fluid, detected using MDCK cell cultures. In bold are samples selected for use in inoculum pools for the subsequent group. For *Group 1* only, the 'Day -1' sample was actually collected on Day 0 due to procedural error, and is suspected to have been contaminated with Master Seed virus used for inoculation on that day

Group No.	ID No.	Day postinoculation								
		-1	1	2	3	4	5	6	7	8
1	32	5.0×10^{2}	4.0×10^{2}	8.0×10^{1}	Neg**	4.2×10^{2}	Neg	Neg	Neg	Neg
	50	Neg	5.6×10^{3}	**>3.2×10^{5}**	**>3.2×10^{5}**	3.2×10^{4}	2.3×10^{4}	Neg	Neg	Neg
	84	Neg	1.0×10^{4}	1.8×10^{5}	**>3.2×10^{5}**	3.2×10^{4}	1.4×10^{4}	1.8×10^{3}	2.1×10^{2}	Neg
	85	Neg	4.2×10^{2}	3.2×10^{2}	1.7×10^{4}	5.8×10^{3}	3.5×10^{4}	1.8×10^{2}	Neg	Neg
	86	Neg	5.6×10^{2}	5.6×10^{4}	**>3.2×10^{5}**	4.0×10^{4}	5.6×10^{4}	4.2×10^{2}	Neg	Neg
2	81	Neg	1.7×10^{3}	2.5×10^{5}	**>3.2×10^{5}**	3.8×10^{2}	4.0×10^{3}	4.0×10^{4}	5.6×10^{3}	Neg
	105	Neg	1.7×10^{2}	1.7×10^{2}	2.3×10^{3}	4.0×10^{2}	2.0×10^{2}	Neg	Neg	Neg
3	49	Neg	Neg	2.5×10^{3}	**3.2×10^{4}**	4.0×10^{2}	3.2×10^{3}	3.2×10^{3}	Neg	Neg
	53	Neg	4.0×10^{1}	**2.5×10^{4}**	1.0×10^{3}	1.0×10^{4}	4.0×10^{3}	4.0×10^{2}	1.0×10^{2}	Neg
4	77	Neg	Neg	Neg	Neg	5.6×10^{1}	Neg	Neg	Neg	Neg
	90	Neg	Neg	Neg	4.0×10^{3}	**3.2×10^{4}**	**1.7×10^{4}**	**4.0×10^{4}**	1.7×10^{4}	2.3×10^{4}
5	70	Neg	Neg	Neg	Neg	5.6×10^{1}	2.5×10^{4}	5.0×10^{2}	1.7×10^{2}	4.3×10^{2}
	80	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
	92	Neg	Neg	Neg	Neg	5.0×10^{2}	5.6×10^{2}	2.3×10^{2}	Neg	4.0×10^{3}
	106	Neg	Neg	1.4×10^{2}	4.0×10^{3}	4.0×10^{4}	1.0×10^{3}	1.0×10^{2}	1.0×10^{3}	2.5×10^{2}
	107	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg

Neg = <10 $TCID_{50}$/ml (lower limit of detection).

Flu Avert IN vaccine was attenuated by cold-adaptation, rendering it incapable of replication at the normal body temperature of the horse.

A modified-live virus vaccine raises safety issues avoided by conventional killed virus vaccines. The attenuated virus must be stable and must not revert to virulence. To further minimise the possibility of reversion, the live vaccine organism should be self-limiting and incapable of spreading spontaneously from vaccinated to nonvaccinated animals.

Furthermore, an effective equine influenza vaccine should protect horses against the most recent circulating virus strains. Equine-2 (subtype H3N8) influenza viruses circulate in most parts of the world, including the USA, causing outbreaks of respiratory disease featuring high fever, coughing, mucopurulent nasal discharge and risk of secondary bacterial pneumonia. Influenza viruses regularly undergo antigenic drift through accumulation of point mutations, leading to changes in antigenicity which eventually compromise the effectiveness of vaccines unless the vaccine virus strains are periodically updated. Recent studies of viral antigenic drift have identified 2 diverging lineages of equine-2 influenza: the 'American' lineage which, despite its name, is now widespread elsewhere; and the 'Eurasian' lineage which remains common in the UK but has been isolated once in the western hemisphere (Daly *et al.* 1996; Lai *et al.* 2001). Since 1995, equine influenza experts from the Office International des Epizooties (OIE) and World Health Organization (WHO) have recommended that vaccines should protect against viruses of both lineages. In the case of conventional, killed virus vaccines, this requires incorporation of antigens from representative viruses of both lineages. In addition, conventional equine influenza virus vaccines typically contain a virus representative of the equine-1 (H7N7) subtype. However, this subtype is now believed to be extinct and, in 1999, OIE/WHO experts recommended that it can be eliminated from vaccines.

The present objectives were to assess: 1) the stability of attenuation of the Flu Avert IN vaccine following serial pony-to-pony passage; 2) the ability of the vaccine virus to spread spontaneously from vaccinates to comingled horses; and 3) the clinical effectiveness of the vaccine by experimental challenge with heterologous virus strains from both the Eurasian and the recent American lineage.

Materials and methods

Flu Avert IN vaccine is a cold-adapted variant of the Kentucky/91 strain of A/equine-2 influenza virus (Youngner *et al.* 2001). The master seed virus (serial passage study) and actual product (spontaneous spread and heterologous challenge studies) were obtained from Heska Corporation and administered by intranasal inoculation, using a custom applicator according to the manufacturer's directions.

Stability of attenuation

Seronegative weanling ponies (n = 16) of mixed sex, age 4–11 months, were used. They were individually housed in closed, environmentally controlled rooms. Initially, 5 ponies were vaccinated intranasally with approximately 10^8 $TCID_{50}$ units of vaccine master seed virus (equivalent to approximately 3 doses of product). Nasal swab fluids were collected daily for 8 days thereafter and their virus titres determined by $TCID_{50}$ assay as described previously (Youngner *et al.* 1994). Fluids with the highest titres (excluding the Day 1 postvaccination samples to avoid any residual vaccine) were pooled and administered intranasally to 2 other seronegative ponies. This process was repeated through 2 additional groups of 2 ponies and a final group of 5 ponies, age 5 months. Viruses isolated from the nasal swabs of the final passage were characterised by their ability to replicate in MDCK cells at 34°C but not at 39.5°C, with a detection limit of 10 $TCID_{50}$ units/ml. In no case was the virus in these samples amplified by laboratory passage before subsequent inoculation or testing. Clinical signs were recorded by the same observer, from 3 days prior to

TABLE 3: Temperature sensitivity of recovered virus from Passage *Group 5*. Values are in $TCID_{50}$ units/ml of sample. The wild-type control used wild-type equine-2 influenza virus (Kentucky/91 strain). Titrations were repeated 2 additional times with similar results

ID No.	Day postinoculation	Titre 34°C	Titre 39.5°C
70	4	9.4×10^7	Neg
	5	7.0×10^7	Neg
	6	7.0×10^7	Neg
	7	1.1×10^7	Neg
	8	9.8×10^7	Neg
92	4	1.0×10^8	Neg
	5	1.3×10^8	Neg
	6	1.1×10^8	Neg
	8	1.0×10^8	Neg
106	2	1.1×10^8	Neg
	3	1.3×10^8	Neg
	4	1.0×10^8	Neg
	5	1.6×10^8	Neg
	6	1.0×10^8	Neg
	7	5.0×10^7	Neg
	8	1.0×10^8	Neg
Wild-type control		2.4×10^7	7.2×10^6

Neg = negative for growth: less than 1.0×10^2 (assay detection limit).

14–21 days postinoculation, and converted to clinical incidence scores using a standardised system (Table 1). Each pony was observed for 10–15 min daily. In addition to clinical incidence scoring, daily examinations also included rectal temperatures, heart rate, capillary refill time, visual examination of gums, palpation of submandibular and parotid lymph nodes, and gastrointestinal sounds. Sera were collected pre-inoculation and at 14 and 21 days postinoculation, and tested for equine influenza antibodies by haemagglutination-inhibition (HI) assay (Anon 1996).

Spontaneous spread

Horses/ponies (n = 39) were vaccinated with Flu Avert IN vaccine. As sentinels for spontaneous spread of the vaccine, 13 other horses/ponies were left unvaccinated. Both groups included both seropositive and seronegative animals in proportion of approximately 2:1. The 52 animals were intermixed and maintained in 2 adjacent fields totalling approximately 6 hectares in area, each with a single mechanical watering fixture. All animals were observed daily for 14 days postvaccination for evidence of adverse reactions. Serum was collected from all animals on the day of vaccination and again 14 days later for comparison of antibody titres against Kentucky/91 virus by the HI test. Nasopharyngeal swabs were taken from all animals on Day 2 postvaccination and assayed for virus shedding by inoculation into embryonated eggs and incubation at 33°C.

Heterologous virus challenge

Separate heterologous challenge experiments were performed for the challenge viruses of the American lineage (Kentucky/98 strain) and Eurasian lineage (Saskatoon/90 strain) (Bogdan *et al.* 1992; Lai *et al.* 2001). Vaccine was administered by a custom applicator, in a single dose in one

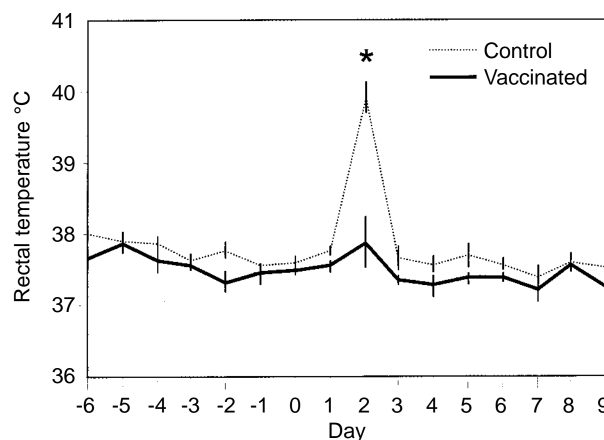

*Fig 1: Rectal temperatures of control and vaccinated ponies experimentally challenged with influenza A/equine/Kentucky/98 (H3N8) virus (American lineage); n = 4 for both groups. Vaccinated ponies received a single dose of Flu Avert IN vaccine one month prior to challenge (Day 0). Shown are means ± s.e. *Days in which differences between groups were statistically significant (P<0.05).*

nostril. For the Kentucky/98 trial, the vaccination and control groups each consisted of 4 seronegative weanling ponies age 7 months. For the Saskatoon/90 trial, vaccination and control groups each consisted of 10 seronegative yearling horses. In both trials, challenge was performed 1 month following vaccination, and controls were not comingled with vaccinates until approximately 3 weeks following vaccination. Challenge viruses were administered by aerosol inhalation (Mumford *et al.* 1990). Clinical signs were observed for periods of about 20 min each day and scored using the standardised system (Table 1). Depression was defined as failure to approach food rapidly, general lethargy, or inappetence. Observations also included measurement of rectal temperature and monitoring of the submandibular lymph nodes. In the Saskatoon/90 trial, the investigator evaluating clinical signs was blinded to the identities of the vaccinates; this was not possible in the Kentucky/98 trial. Virus shedding was assessed by daily collection of nasal swabs, continuing through Day 8 postchallenge. Swabs were tested either in embryonated eggs as described previously (Chambers *et al.* 1995), or by $TCID_{50}$ assay as above. All egg assays were done in the same laboratory by a blinded investigator. Serum was collected

TABLE 4: Heterologous challenge with Kentucky/98 virus-ranked sums of clinical scores. Clinical signs were scored daily (Days 1–9 postchallenge) as in Table 1, and cumulatively ranked from least to most severe by a blinded statistician. Cumulative differences in clinical scores between the control and vaccination groups were statistically significant (P<0.05)

Group	Total score	Rank by total scores (least to most severe)
Vaccinate	0	1
Vaccinate	0	2
Vaccinate	1	3
Vaccinate	2	4
Control	21	5
Control	25	6
Control	26	7
Control	26	8

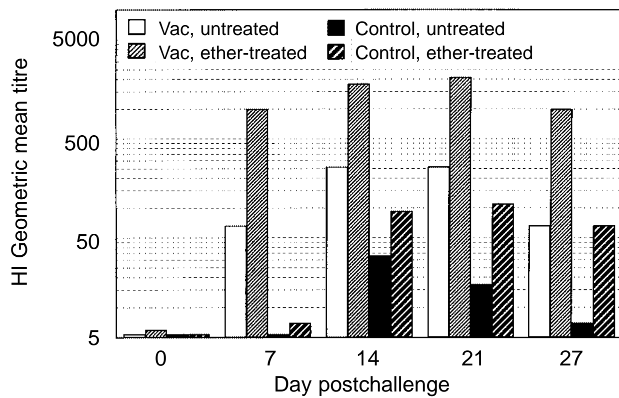

Fig 2: Serological responses of ponies following challenge with influenza A/equine/Kentucky/98 (H3N8) virus. Bars represent geometric mean titres of vaccinated (n = 4) and control (n = 4) ponies. Titres <10 are below the limit of detection and are arbitrarily equated to 5, shown here as baseline. HI assays were performed using chicken red blood cells as described previously (Anon 1996), and used both untreated Kentucky/98 virus as antigen (solid bars), and also ether-treated Kentucky/98 virus as antigen (hatched bars).

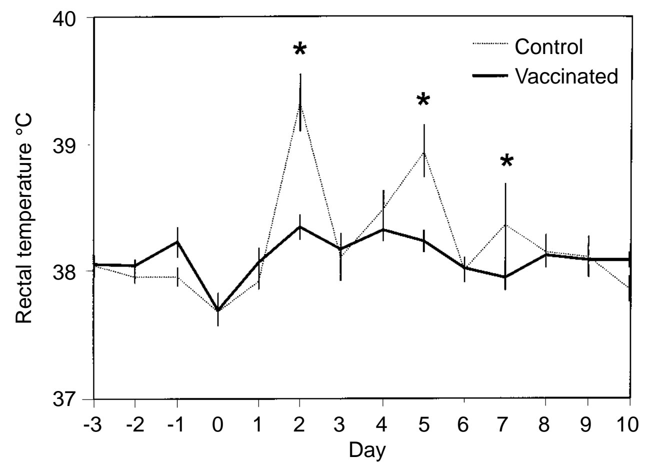

*Fig 3: Rectal temperatures of control and vaccinated horses experimentally challenged with influenza A/equine/Saskatoon/90 (H3N8) virus (Eurasian lineage); n = 10 for both groups. Vaccinated ponies received a single dose of Flu Avert IN vaccine one month prior to challenge (Day 0). Shown are means ± s.e. *Days in which differences between groups were statistically significant (P<0.05).*

prior to challenge and each week for 4 weeks after challenge, for assay of HI antibody titres against the challenge viruses and Kentucky/91 virus. Sera were collected by jugular venipuncture on the indicated day postchallenge, and pretreated with trypsin-periodate (Anon 1996) to remove nonspecific inhibitors of hemagglutination. Ether treatment was performed as described previously (John and Fulginiti 1966); it effectively magnifies HI titres, making the assay more sensitive to low levels of antibodies; however, the scale of magnification is highly variable. In the Kentucky/98 trial, nebulised challenge virus (5 x 10^7 EID_{50} units/pony) was delivered directly to a mask on the pony's nose over a period of about 20 min. The Saskatoon/90 trial was performed according to the method described by Townsend *et al.* (2001), in which nebulised virus (approximately 2 x 10^7 EID_{50} units) was delivered into the atmosphere of a closed 16 m^3 chamber inside which 5 horses (3 vaccinates and 2 controls, or vice-versa) inhaled the virus for about 40 min. This procedure was repeated until all the horses had been challenged.

Statistical methods

All the data were analysed using SAS (The SAS Institute, Cary, North Carolina, USA). Rectal temperature data were analysed using a mixed model ANOVA appropriate for a repeated measures experiment. An autoregressive covariance structure was incorporated into this model. Main effects of treatment and day and the interaction between day and treatment were tested as fixed effects. Animals were nested within treatment as a random effect. Interactions were deemed significant if $P<0.10$. If the interaction was significant, the 2 treatments were compared using a *t* test at each time point. Clinical scores were summed on each day for each animal, and the vaccinates and controls were compared using the Wilcoxon rank sums test. In addition, these scores were summed across all days for each animal and compared in the same manner. The percent of animals shedding virus during the postchallenge period was assessed using Fisher's exact test, comparing the vaccinated group to the nonvaccinated control group. The number of days that animals in each group were shedding the challenge virus was tested using the Wilcoxon rank sums test.

Results

Serial pony-to pony passage

Serology: All ponies were initially seronegative for equine-2 influenza and seroconverted by Day 14 postinoculation, except for 2 in Passage *Group 5* that shed little or no virus. This demonstrates that, except for those 2, the serial pony-to-pony passage of virus was successfully accomplished.

Virus shedding: All but 1 of the 16 ponies shed detectable virus at least once during the study, based on embryonated egg analysis. In Passage *Group 5*, virus was detected from one pony in eggs but not in MDCK cells. The shed virus concentrations varied from 4 x 10^1 to ≥ 3.2 x 10^5 $TCID_{50}$ units/ml in fluid collected from individual swabs (Table 2). The amount of virus transferred between passage groups varied from 8 x 10^5/pony (into Passage *Group 2*) to 2.2 x 10^4/pony (into Passage *Group 5*), reflecting a general trend towards reduced virus shedding in the successive pony-to-pony passages.

Clinical signs: The primary indicator used to determine whether vaccine virus reverted to virulence was the occurrence of clinical signs consistent with equine influenza subsequent to inoculation. No reversion to virulence occurred in any of the 16 ponies. There were occasional instances of nonzero clinical scores both before and after inoculation, primarily nasal discharge. However, there was no pattern of disease development except in one pony from Passage *Group 4*, and that was not associated with virus administration. This 4-month-old pony exhibited rectal temperatures of 39.0–39.3°C on Days 7, 8, and 12 postinoculation. It also exhibited coughing starting 2 days prior to inoculation and continuing to Day 11, and mucopurulent nasal discharge starting 1 day prior to inoculation. However, it shed detectable virus only on Day 4 postinoculation, detected by both MDCK and embryonated egg analysis.

TABLE 5: Heterologous challenge with Saskatoon/90 Virus (Eurasian lineage); ranked sums of clinical scores. Clinical signs were scored daily (Days 1–10 postchallenge) as in Table 1 by a blinded clinician, and cumulatively ranked from least to most severe by a blinded statistician. Cumulative differences in clinical scores between the control and vaccination groups were statistically significant (P<0.05)

Group	Total score	Rank by total scores (least to most severe)
Vac	8	1
Vac	8	2
Vac	8	3
Vac	9	4
Vac	9	5
Vac	9	6
Vac	11	7
Vac	11	8
Control	13	9
Vac	14	10
Vac	16	11
Control	18	12
Control	18	13
Control	19	14
Control	21	15
Control	23	16
Control	27	17
Control	27	18
Control	30	19
Control	35	20

Phenotypic analysis: All samples from the final group, Passage *Group 5*, that were virus-positive in MDCK cells, were tested *in vitro* for temperature sensitivity (Table 3). No virus replication occurred in any sample at the nonpermissive temperature (39.5°C), indicating stable retention of the temperature-sensitive attenuation phenotype through the course of the serial passages. A wild-type equine influenza virus control was also tested and did replicate at 39.5°C.

Spontaneous spread

During 14 days of observation following vaccination, the only adverse effect observed was the appearance of white flecks of mucus (1 or 2 mm diameter) in either nostril. This sign disappeared after 1 or 2 days and was not correlated with vaccination. Some controls, as well as other horses at remote locations on the farm, showed the same sign around this time (late spring). Of the 39 vaccinates, 13 shed detectable virus on Day 2 postvaccination, whereas none of 13 unvaccinated sentinels shed virus. Also, 30 of 39 vaccinates but only 1 of 13 sentinels showed a rise in antibody titre by Day 14. As both groups included both seropositive and seronegative animals in similar proportions, antibody rises represent predominantly anamnestic responses in the vaccinates which largely failed to occur among the sentinels.

Heterologous virus challenge

Pilot challenge experiments using Kentucky/98 and Saskatoon/90 viruses, including 2 animals in each, demonstrated induction of typical clinical signs for influenza (data not shown). Also, vaccination of the seronegative animals with the Flu Avert IN vaccine did not induce any clinical signs or adverse reactions in any of the 14 vaccinates in the 2 challenge studies.

Kentucky/98 (American lineage) challenge: Mean rectal temperature profiles are shown in Figure 1, and ranked-sum clinical scores are shown in Table 4. Prior to challenge, only one pony (a control) exhibited any consistent clinical sign, which was a slight nasal discharge. On Day 2 postchallenge, all control ponies showed rectal temperatures of 39.3–40.5°C (Fig 1). Also on Day 2, all control ponies developed cough and mucopurulent nasal discharge which persisted through Days 7–9. Among the vaccinates, one pony developed a rectal temperature of 38.9°C on Day 2, whereas the others remained at ≤ 37.6°C. The difference in mean temperatures on Day 2 was significant (P<0.05). Throughout the study, vaccinates rarely coughed and had no nasal discharge. Differences in clinical scores were significant (P<0.05) on Days 3 through 7 and Day 9, and also cumulatively over the duration of the study.

All control and vaccinated ponies shed virus on at least one day after challenge (data not shown), and the percentage of animals shedding virus was not significantly different between groups on any day. However, incidence of shedding was significantly reduced in the vaccinates. The mean number of days of detectable virus shedding was 3.5 in vaccinates versus 7.0 in controls (P<0.05).

All control and vaccinated ponies were seronegative for Kentucky/91 and Kentucky/98 viruses at the time of challenge. They differed, however, in their serological responses following challenge (Fig 2). All vaccinates exhibited an anamnestic response with high titres evident by Day 7 and persisting at Day 27. Controls exhibited no response on Day 7; their response on Day 14 was less than that of the vaccinates on Day 7 and diminished thereafter.

Saskatoon/90 (Eurasian lineage) challenge: Individual horses in both control and vaccination groups exhibited minor clinical signs, mostly nasal discharge, prior to challenge. Following challenge, mucopurulent nasal discharge and coughing were elevated in most controls compared to most vaccinates; indeed, vaccinates were never observed to cough from Day 1 onwards. Although the difference in clinical scores was less marked than in the Kentucky/98 challenge study, it was statistically significant (P<0.05) for each day from Day 4 on, as was the cumulative rank of clinical scores over the entire test period (Table 5). Mean rectal temperatures of controls peaked at 39.3°C on Day 2, whereas means of vaccinates did not exceed 38.3°C throughout the study (Fig 3). Rectal temperatures of vaccinates were significantly lower than controls (P<0.05) on Days 2, 5 and 7.

All controls, but only half of vaccinates, shed detectable virus at least once during the study (Table 6). The percent of horses shedding challenge virus in the vaccinated group was lower (P<0.05) on Days 2–7 postchallenge. The mean duration of shedding among the control horses was 5.5 days, based on $TCID_{50}$ assay, whereas it was only 0.5 days among the vaccinates (P<0.05). No individual vaccinate shed virus longer than one day. Also when the virus content of swabs was titrated, positive swabs from vaccinates never exceeded 4.1×10^2 $TCID_{50}$/ml, whereas swabs from controls averaged 4×10^3 $TCID_{50}$/ml overall and sometimes exceeded 10^5.

Discussion

Safety and phenotypic stability are critical issues with regard to modified-live organism vaccines. A large-scale field safety study

TABLE 6: Heterologous challenge with Saskatoon/90 virus; virus shedding postchallenge as detected by MDCK cell culture. Titres are $TCID_{50}$/ml of nasal swab fluid. Below each group is shown the number of positive samples for the group on that day; on the right is the number of days each horse yielded a positive sample

	Horse ID	Days postchallenge -1	1	2	3	4	5	6	7	8	No. days positive per horse
Vaccinates	B	Neg	Neg	Neg	Neg	3.2×10^2	Neg	Neg	Neg	Neg	1
	D	Neg	Neg	Neg	Neg	Neg	Neg	2.3×10^2	Neg	Neg	1
	E	Neg	Neg	Neg	Neg	Neg	5.6×10^2	Neg	Neg	Neg	1
	S	Neg	Neg	Neg	Neg	7.4×10^1	Neg	Neg	Neg	Neg	1
	T	Neg	Neg	Neg	Neg	Neg	Neg	4.1×10^2	Neg	Neg	1
No. horses positive per day		0	0	0	0	2	1	2	0	0	
Controls	A	Neg	Neg	1.8×10^3	3.2×10^4	2.5×10^5	4.0×10^4	3.2×10^5	5.6×10^3	Neg	6
	C	Neg	Neg	4.0×10^2	4.0×10^4	1.8×10^4	1.0×10^5	3.2×10^5	5.6×10^2	1.4×10^2	7
	F	Neg	Neg	Neg	Neg	1.0×10^4	3.2×10^4	3.2×10^4	3.2×10^2	Neg	4
	I	Neg	Neg	1.0×10^3	1.0×10^2	4.0×10^2	3.2×10^2	5.0×10^2	3.0×10^3	Neg	6
	J	Neg	Neg	1.8×10^4	2.3×10^4	2.5×10^5	1.0×10^5	3.2×10^3	4.6×10^2	Neg	6
	K	Neg	Neg	2.3×10^3	5.0×10^3	5.6×10^4	1.0×10^5	1.8×10^4	3.2×10^3	5.6×10^3	7
	L	Neg	Neg	Neg	4.0×10^3	5.6×10^3	2.3×10^3	3.2×10^3	1.4×10^4	3.2×10^2	6
	N	Neg	Neg	7.4×10^1	1.8×10^4	3.2×10^4	5.6×10^3	5.6×10^4	3.2×10^4	Neg	6
	O	Neg	Neg	Neg	Neg	3.2×10^2	2.5×10^2	2.5×10^5	Neg	Neg	3
	P	Neg	Neg	3.2×10^3	Neg	3.2×10^4	2.5×10^3	3.2×10^4	Neg	Neg	4
No. horses positive per day		0	0	7	7	10	10	10	8	3	

Horses G, H, M, Q and R (vaccination group): all swabs negative. Neg = <10 $TCID_{50}$/ml (lower limit of detection).

of Flu Avert IN vaccine, involving 435 horses at 7 sites in 6 states of the USA, was published by Wilson and Robinson (2000). In that study, at 6 of the 7 sites the overall incidence of adverse effects was 0.8%, and the effect noted was a scanty and transient serous nasal discharge. At the seventh site the investigator observed similar incidence of slight, transient serous and ocular discharge in 27.5% of vaccinates but also in a similar number of nonvaccinates and concluded that this was normal seasonal incidence and not vaccine-associated. No adverse signs were observed following vaccination in any of the present studies. A second aspect of safety, regarding use in performance horses, was addressed in a different study (Lunn *et al.* 2001). Exercise stress in unconditioned horses is immunosuppressive (Horohov *et al.* 1999). In ponies subject to exercise stress, no adverse reactions or clinical signs were observed following vaccination; also the vaccination proved effective against subsequent experimental challenge.

We tested the ability of Flu Avert IN vaccine to mutate or revert into a more infectious and virulent form following its introduction into the target animal, the horse. Forced passage of the modified-live virus directly and repeatedly from pony to naïve pony (which should not occur in field use) gave the greatest chance for the multigeneration virus replication needed for mutation, and thereby the best chance for reversion. The intranasal route was most consistent with the natural route of exposure. We found, first, that the modified virus did not revert following 5 consecutive direct pony-to-pony passages. The vaccine retained its attenuation based on clinical evaluation, and retained its temperature-sensitive phenotype based on *in vitro* testing of the viruses isolated from the ponies of the final passage. Therefore, the Flu Avert IN vaccine virus is stably attenuated and stably temperature-sensitive, and highly unlikely to revert to virulence when used in the field.

Secondly, we found that the vaccine virus spread only with difficulty from the animal to which it was originally administered. In the present study, a trend of reduced shedding was evident and virus failed to replicate detectably in 2/5 ponies of the final Passage Group. Furthermore, the study reported here was actually the second attempt to complete the serial pony-to-pony passages; in an earlier trial, detectable virus replication ceased midway through the passages. Possibly the attenuation of infection was due to interference by residual maternal antibodies against equine influenza in the 5-month-old ponies of Passage *Group 5* (Holland *et al.* 1999); however, in our experience, 5-month-old ponies shed virus when infected with equine influenza even if maternal antibodies inhibit development of clinical signs (R.E. Holland and T.M. Chambers, unpublished data). We next tested the ability of the vaccine virus to spread from horse to horse by natural exposure during an outdoor field trial, by inclusion of nonvaccinated sentinel animals. Wild-type equine influenza spreads easily to contact horses via aerosol transmission. However we found that spread of the vaccine virus to sentinels occurred only rarely (1/13 animals). The probable reason for the vaccine virus's inability to spread is that highly efficient viral replication is not occurring even within seronegative animals and that vaccinates do not cough.

Our studies also demonstrate that the Flu Avert IN vaccine, administered intranasally in a single dose to young seronegative equids, was effective in protecting them from clinical disease caused by a recent equine-2 influenza virus isolate from the USA and also an isolate of the Eurasian lineage. These strains were not components of the vaccine. The Kentucky/98 strain was, at the time, the latest equine influenza strain to be isolated in the USA, and is antigenically similar to the equine/Kentucky/94 strain which the OIE expert surveillance panel for equine influenza recommended for inclusion in the conventional killed-virus equine influenza vaccines (J.A. Mumford and T.M. Chambers, unpublished data). In both American and Eurasian lineage challenges, the vaccinates exhibited significantly reduced clinical signs of disease (rectal temperatures and clinical sign

scores for coughing and nasal discharge) relative to controls. In the Kentucky/98 trial, after Day 2, rectal temperatures returned to normal in both groups, perhaps because undetectable levels of residual maternal antibodies helped attenuate the infection. Furthermore, following challenge both the numbers of horses shedding virus and the number of days of shedding were significantly reduced in vaccinates. In the Saskatoon/90 trial, where virus in nasopharyngeal swabs was also titrated, vaccinates which did shed had greatly reduced titres.

The vaccine clearly demonstrated clinical protection in 2 independent challenges using recent and relevant heterologous wild-type challenge viruses. Therefore, the Flu Avert IN vaccine is expected to be effective against the equine-2 influenza strains now in circulation. Eventually, viral antigenic drift may require updating of Flu Avert IN vaccine. It can be argued, however, that a modified-live virus vaccine may inherently induce a broader range of immune responses than a killed-virus vaccine, including cross-reactive responses (Belshe *et al.* 2000) and, consequently, may require less frequent updating than a conventional vaccine. This remains to be tested.

Historically, efficacies of conventional killed-virus equine vaccines have been judged based on their ability to induce high titres of serum antibodies after a primary course of 2 or more doses (Townsend *et al.* 1999a). High serum antibody titres are correlated with protection against natural challenge in the field (Lai *et al.* 1994), but are also generally of short duration, necessitating frequent revaccination. A single dose of Flu Avert IN vaccine reliably provides protection against experimental challenge by the homologous wild-type virus for a period of 3 to 6 months or greater, in the absence of high titres of induced antibody (Lunn *et al.* 1999, 2001; Townsend *et al.* 1999b). It seems probable that Flu Avert IN vaccine, as a modified live virus vaccine, may have the capability of producing mucosal and cell-mediated immune responses that contribute significantly to immunity yet are difficult to measure, especially locally in the respiratory tract. Therefore, the efficacy of this vaccine has been established exclusively on the basis of experimental challenge using wild-type influenza viruses which produce disease in nonimmune animals. Experimental challenge using naïve animals and blinded investigators is a more stringent and more representative demonstration of vaccine efficacy (protection from clinical disease) than is serological response. The availability of an experimental challenge model for equine influenza, used in North America for the first time to validate the Flu Avert IN vaccine, is a new standard for vaccine performance and should lead to overall improvement in equine veterinary preventive care.

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