

## Studies with inactivated equine influenza vaccine

### 1. Serological responses of ponies to graded doses of vaccine

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#### SUMMARY

Serological responses to three bivalent aqueous equine influenza vaccines of different potency and an adjuvanted bivalent vaccine containing inactivated A/equine/Prague/56 (H7N7) and A/equine/Miami/63 (H3N8) viruses, were examined in seronegative ponies. Potencies of the vaccines, measured by single-radial-diffusion tests, ranged from 4 to 56  $\mu\text{g}$  of haemagglutinin (HA) antigen activity/virus strain per dose. Serological responses to vaccination were examined by haemagglutination-inhibition (HI) and single-radial-haemolysis (SRH) tests.

Four weeks after a primary dose, HI responses to both vaccine viruses were barely detectable; after a second dose the HI responses to A/Miami/63 virus were low or undetectable but HI responses to A/Prague/56 virus were higher (17/20 ponies with titres  $\geq 1:16$ ). In contrast SRH tests revealed dose-related antibody responses to both virus strains after one and two vaccine doses; levels after the second dose were 2- to 5-fold higher than after the primary dose. Highest post-vaccination antibody titres were obtained with the adjuvanted vaccine which contained 2- to 4-fold less antigen (13–23  $\mu\text{g}$  HA) than the most potent aqueous vaccine. Post-vaccination antibody reacted well in SRH tests with recent antigenic variants of equine influenza virus. A remarkable finding was the high rate of decline in antibody, detected by HI or SRH tests, following one or two doses of vaccine. Even in animals with the highest post-vaccine antibody levels 2–4 weeks after a booster dose, antibody levels had declined to low or undetectable levels 14 weeks later. The low antibody titres detected at 14–32 weeks after vaccination were nevertheless vaccine dose-related.

#### INTRODUCTION

The potency of equine influenza vaccines is conventionally judged by haemagglutination assays and by their ability to stimulate antibody to haemagglutinin (HA) in experimental animals. However, both the haemagglutination test for

standardizing vaccine and the haemagglutination inhibition (HI) test for measuring serological responses, have serious drawbacks which limit their usefulness (Schild, Wood & Newman, 1975; Frerichs, Frerichs & Burrows, 1973; Burrows, Spooner & Goodridge, 1973). Single-radial-immunodiffusion (SRD) techniques have now been established internationally for the standardization of human influenza vaccines (Wood *et al.* 1977), potency being described in terms of micrograms of HA activity. An additional immunodiffusion technique, single-radial-haemolysis (SRH) (Schild, Pereira & Chakraverty, 1975; Russel, McCahon & Beare, 1975) has been used to detect antibodies to influenza HA in equine sera (Bockmann, 1977; Fontaine *et al.* 1981; Yamagishi *et al.* 1982). SRH was found to be convenient for testing large numbers of sera, was particularly suitable for measuring small amounts of anti-HA antibody and was specific for virus subtype.

In the studies presented here, ponies were vaccinated with graded doses of three aqueous bivalent equine influenza vaccines and one adjuvanted vaccine and the serological responses were measured by both SRH and HI techniques. Vaccine HA antigen activity had been previously standardized by single-radial-diffusion (SRD) techniques, as described by Wood *et al.* (1983), and in further studies (Mumford *et al.* 1983), the protective efficacy of vaccines was examined in experimental ponies.

#### MATERIALS AND METHODS

##### *Viruses*

Prototype A/equine/Prague/56 and A/equine/Miami/63 viruses were as described in the accompanying paper (Mumford *et al.* 1983). A/equine/Newmarket/77 (H7N7) and A/equine/Newmarket/79 (H3N8) virus strains were from the Animal Health Trust, Newmarket.

##### *Vaccines*

Beta-propiolactone-inactivated A/equine/Prague/56 (H7N7) and A/equine/Miami/63 (H3N8) whole virus aqueous vaccine batches A, B and C and a newly developed adjuvanted, bivalent vaccine batch D (adjuvant code name PD), were standardized by SRD as described by Wood *et al.* (1983). The HA antigen concentrations of the vaccines are shown in Table 1. The adjuvant present in vaccine D did not interfere with diffusion of HA in the SRD test and the test was virus subtype specific (data not shown).

##### *Study design*

The study was carried out in 40 Welsh mountain pony yearlings which were seronegative for A/Prague/56 and A/Miami/63 as determined by SRH tests for antibody to HA. Ponies were divided into two groups of 20, one receiving only one dose of vaccine, and a second group receiving a primary dose followed by a second dose of the same vaccine 4 weeks later. Within each group the ponies were divided into four sub-groups of five, each group receiving a different vaccine (A, B, C or D). Vaccination was by intramuscular injection. Pony sera were collected for antibody studies before vaccination and 2, 3, 4, 5, 6, 8, 10, 12, 17 and 22 weeks after the first vaccination. Sera were tested for antibody by means of HI on potassium periodate-treated sera and SRH on heat-treated sera (56° for 30 min).

Table 1. SRD-detectable HA antigen activity of inactivated equine influenza vaccine

Vaccine batch	Vaccine type	HA antigen activity ( $\mu\text{g}/\text{dose}$ )	
		A/Prague/56	A/Miami/63
A	aqu. vv*	5	4
B	aqu. vv	18	12
C	aqu. vv	49	56
D	adj. vv	23	13

\* aqu. vv = aqueous whole virus vaccine; adj. vv = adjuvanted whole virus vaccine.

### HI test

The HI test was performed using conventional techniques in microtitre plates with Tween 80/ether-treated A/Miami/63 virus (Berlin *et al.* 1963; John & Fulginiti, 1966) and untreated A/Prague/56 virus.

### SRH test

The SRH technique was essentially as described by Oxford *et al.* (1979). Briefly, influenza virus was coupled to sheep erythrocytes using chromium chloride ( $4 \mu\text{g}$  virus protein/ml of 10% erythrocytes) and agarose gels were prepared containing sensitized erythrocytes and guinea-pig complement. Heat-treated ( $56^\circ\text{C}$  for 30 min) pony sera were introduced into wells in the gels and zones of haemolysis were measured using a calibrating viewer (Transidyne General Corp. Ann Arbor, USA) coupled to digital recording apparatus (Autodata Ltd, Hitchen, UK).

### SRH antibody adsorption studies

Analysis of strain specific (SS) and cross reactive (CR) anti-HA antibodies to the vaccine virus strains and to recently isolated antigenic variants, A/equine/Newmarket/77 (H7N7) and A/equine/Newmarket/79 (H3N8) was carried out by cross-adsorption experiments as described previously (Schild *et al.* 1977; Oxford *et al.* 1979).

Interpretation of results was performed according to the following schedule. SS antibody to A/Prague/56 virus was detected by adsorbing with A/Newmarket/77 virus (to remove all CR antibodies) and testing on an SRH plate containing A/Prague/56 virus. Similarly, SS antibody to A/Newmarket/77 virus was detected by adsorbing with A/Prague/56 virus and testing on A/Newmarket/77 SRH plates. SS antibody to A/Miami/63 and A/Newmarket/79 viruses were detected in a similar manner. CR antibody was detectable in unadsorbed sera when SRD plates did not reveal any SS antibody (only CR antibody was available to react). Sera adsorbed with a virus homologous to the SRH plate served as adsorption controls. Complete removal of SRH activity indicated that the experiment was valid.

## RESULTS

### SRH test

Both the A/Prague/56 and A/Miami/63 SRH tests gave very clearly defined haemolysis zones with post-vaccination pony sera (Plate 1).

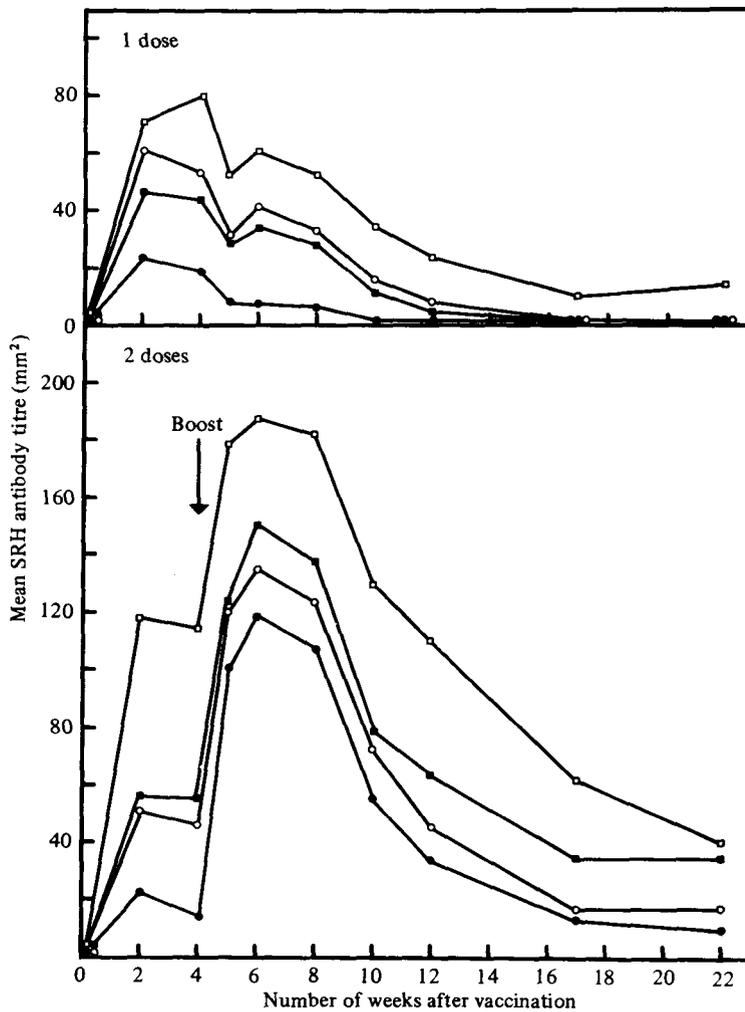


Fig. 1. Post-vaccination SRH responses to A/equine/Prague/56 virus. Responses to one and two doses of aqueous vaccine A ( $5 \mu\text{g}$  HA/ml, ●—●), B ( $15 \mu\text{g}$  HA/ml, ○—○) and C ( $50 \mu\text{g}$  HA/ml, ■—■) and adjuvanted vaccine D ( $15 \mu\text{g}$  HA/ml, □—□) are illustrated.

#### *Pre-existing antibody*

Both SRH and HI tests indicated that all of the ponies used in this study were seronegative to A/Prague/56 and A/Miami/63 HA's (Table 2, Pl. 1, figs 1-4).

#### *SRH antibody response to one dose of vaccine*

Figs 1 and 2 illustrate the A/Prague/56 and A/Miami/63 SRH responses obtained after one dose of vaccine. Two weeks after immunization, significant antibody titres (SRH zone area  $\geq 4 \text{ mm}^2$ ) were detectable to A/Prague/56 virus in all ponies and antibody to A/Miami/63 virus was detectable in four out of five ponies in vaccine A group and in all ponies in the remaining vaccine groups (Table 2). There were clear dose-related responses in antibody production to both virus strains used in the aqueous vaccines. The adjuvanted vaccine D which

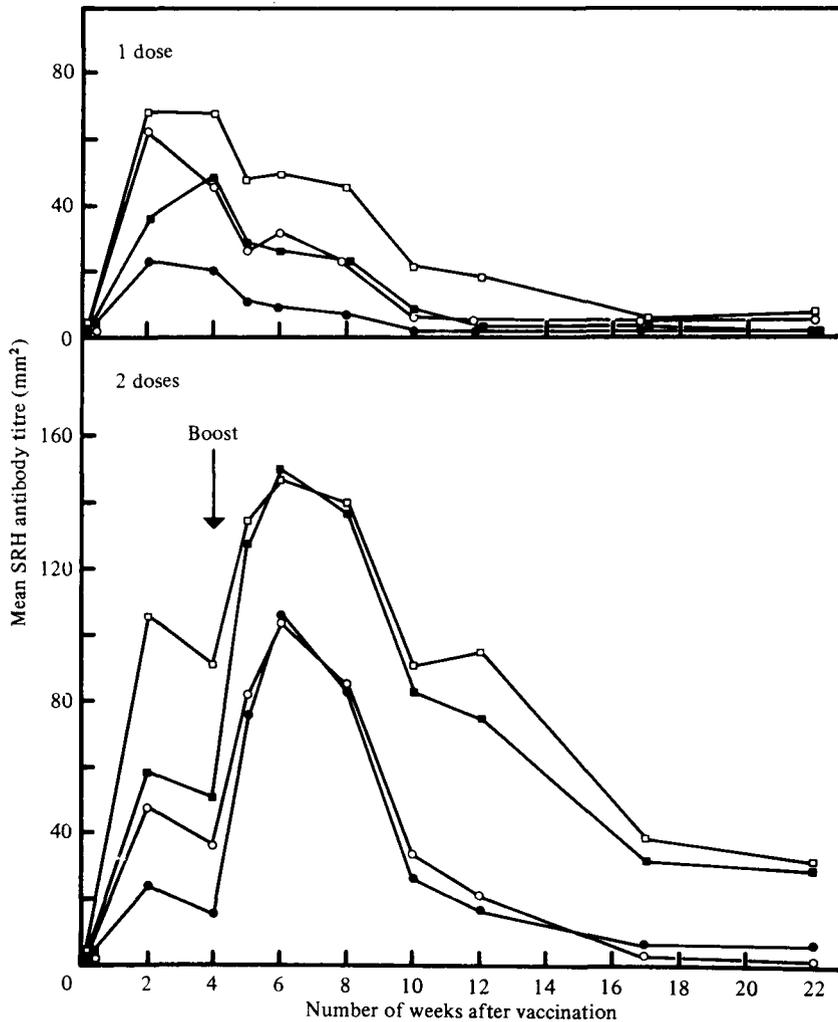


Fig. 2. Post-vaccination SRH responses to A/equine/Miami/63 virus. (Symbols are shown in Fig. 1.)

contained approximately the same quantity of antigen as vaccine B and 2- to 4-fold less antigen than vaccine C, produced the highest antibody responses to one dose of vaccine. Four weeks after immunization, antibody levels in all ponies had begun to fall and by 22 weeks there was no detectable antibody to either virus strain in ponies receiving the least potent vaccine A (vaccine group A) and barely detectable amounts in groups B and C (2/5 with significant antibody to A/Prague/56 or A/Miami/63 in both groups B and C). Antibody levels at 22 weeks were highest in the adjuvanted vaccine group D (3/5 with significant antibody to A/Miami/63; 5/5 with significant antibody to A/Prague/56).

#### *HI antibody response to one dose of vaccine*

After one dose of vaccine there were no ponies with significant levels ( $HI \geq 1:16$ ) to either virus strain (Table 2, figs 3 and 4).

Table 2. Serum SRH and HI antibody responses of ponies following one and two doses of bivalent A/equine/Prague/56 and A/equine/Miami/63 influenza virus

Vaccine batch	Potency ( $\mu\text{g}$ HA/dose)	Number of ponies showing significant antibody titres* by					
		SRH			HI		
		Pre-vacc.	Post 1 dose	Post 2 doses	Pre-vacc.	Post 1 dose	Post 2 doses
A/equine/Prague/56							
A	5, †, 4 ‡	0	5	5	0	0	4
B	18, 12	0	5	5	0	0	5
C	49, 56	0	5	5	0	0	4
D	23, 13	0	5	5	0	0	4
A/equine/Miami/63							
A	5, †, 4 ‡	0	4	4	0	0	1
B	18, 12	0	5	5	0	0	1
C	49, 56	0	5	5	0	0	2
D	23, 13	0	5	5	0	0	2

\* Antibody titres (one dose group at week 2; two dose group at week 6) were significant when they could easily be distinguished from negative results (SRH zone areas  $\geq 4 \text{ mm}^2$ ; HI  $\geq 16$ ).

† A/equine/Prague/56.

‡ A/equine/Miami/63.

#### SRH antibody response to two doses of vaccine

There were good SRH-detectable antibody responses to both virus strains after the second vaccine dose. Antibody levels were approximately 2-fold higher than those following one dose (Pl. 1, figs 1 and 2). At week 6, 39/40 horses showed significant SRH antibody levels to both virus strains (Table 2). Antibody levels after a second dose of aqueous vaccine were vaccine-dose-related. Four weeks after the second immunization (week 8), antibody levels began to fall, until by 18 weeks after the second dose (week 22), antibody levels had fallen by 5- to 50-fold. At week 22, 18/20 ponies possessed significant SRH antibody levels to A/Prague/56 (3/5 ponies for vaccine group A; 5/5 for groups B-D) and 12/20 ponies had significant SRH antibody levels to A/Miami/63 (2/5 for groups A and B; 3/5 for group C; 5/5 for group D).

#### HI antibody response to two doses of vaccine

There were sharp rises in HI antibody to A/Prague/56 virus after the second vaccine dose (Figs 3 and 4). Maximum levels were achieved 1-2 weeks after boosting (weeks 5 and 6). At week 6, 17/20 ponies showed significant HI titres to A/Prague/56 virus (Table 2) (4/5 for groups A, C and D; 5/5 for group B). There was no clear cut vaccine dose-related HI response; vaccine B (15  $\mu\text{g}$  aqueous) gave a greater antibody response than vaccine C (50  $\mu\text{g}$  aqueous) and the adjuvanted vaccine D (15  $\mu\text{g}$ ) gave responses similar to vaccine C. There were rapid decreases in A/Prague/56 HI antibody levels by week 8 until by week 22 only 9/20 ponies had significant HI antibody titres (1/4 for group A, 2/4 for groups B and C, 4/4 for group D). HI antibody responses to two doses of A/Miami/63 vaccine were very poor. Only 6/20 ponies developed significant antibody levels at 6 weeks (1/5 for groups A and B; 2/5 for groups C and D and all traces of A/Miami/63 HI antibody

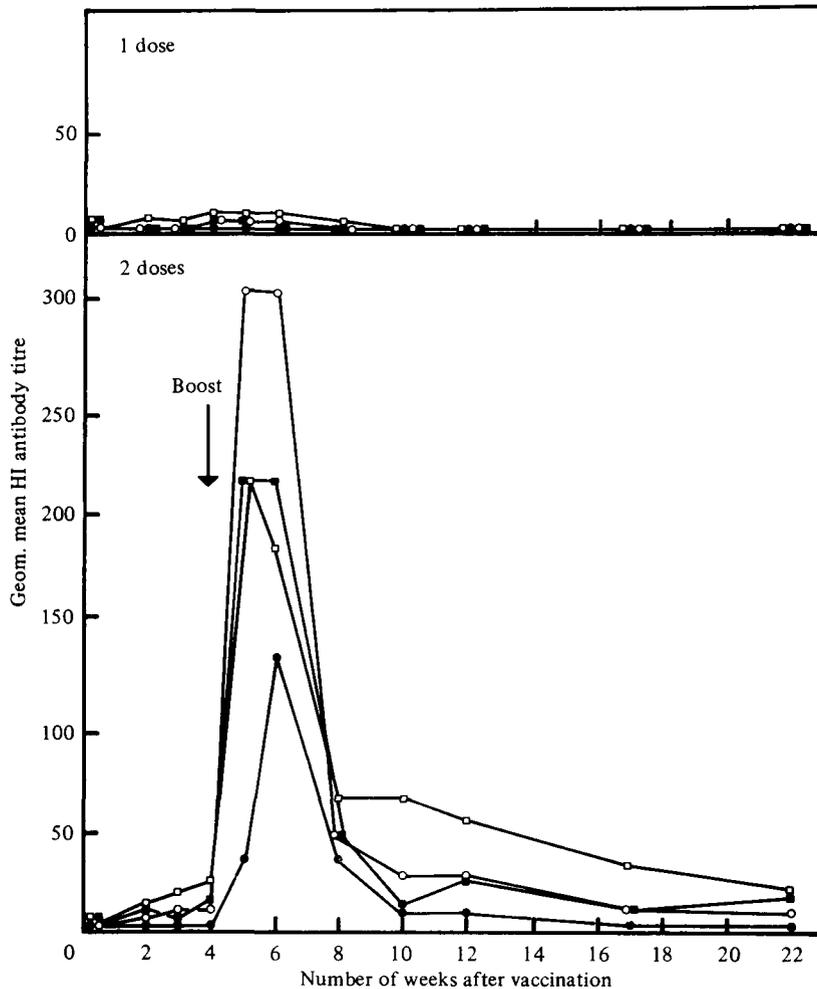


Fig. 3. Post-vaccination HI responses to A/equine/Prague/56 virus. (Symbols as shown in Fig. 1.)

had disappeared by week 22. Only one pony demonstrated HI antibody titres  $> 40$  at week 22 (two dose vaccine group C, antibody against A/Prague/56 virus).

#### *Comparison between SRH and HI titration*

The correlation between SRH and HI post-vaccination antibody levels to A/Prague/56 and A/Miami/63 HA's are illustrated in Figs 5 and 6 respectively. In general the degree of correlation was very low (correlation coefficient 0.2 for A/Prague/56 and 0.1 for A/Miami/63). The lack of agreement was largely due to the SRH test being much more sensitive than the HI test, which resulted in many sera which were negative (titre  $< 8$ ) by HI test having significant and in some cases quite high levels of SRH antibody. For A/Prague/56 titration, 17 sera which were negative by HI had significant SRH antibody (4.9% of sera) whereas 91 sera which were negative in A/Miami/63 HI tests possessed significant SRH antibody (35% of sera).

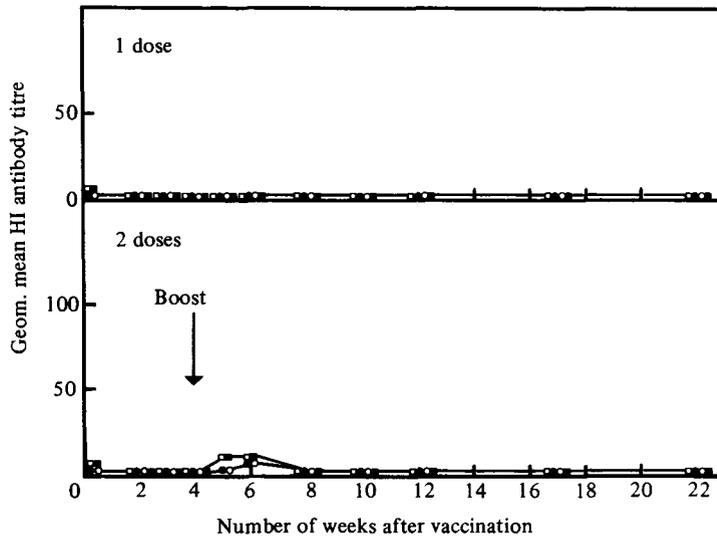


Fig. 4. Post-vaccination HI responses to A/equine/Miami/63 virus. (Symbols as shown in Fig. 1.)

#### *Antibody responses to recent equine virus strains*

Sera were tested by SRH using erythrocytes sensitized with recent antigenic variants of the prototype A/Prague/56 virus (A/equine/Newmarket/77) and A/Miami/63 virus (A/equine/Newmarket/79). For these studies, sera were tested from all one-dose ponies 4 weeks after immunization, and all two-dose ponies 1 week after the second dose. In order to standardize the SRH responses, similar amounts of each virus within a subtype were used to treat the erythrocytes (3  $\mu\text{g}$  protein/ml erythrocytes for H7N7 virus; 5  $\mu\text{g}$  protein/ml erythrocytes for H3N8 virus). With the exception of one serum with very low antibody levels to A/Prague/56 virus and no detectable antibody to A/Newmarket/77 virus, all sera reacting in A/Prague/56 and A/Miami/63 plates also reacted in A/Newmarket/77 and A/Newmarket/79 plates (Table 3). The magnitude of responses to the recent virus strains was significantly inferior ( $P < 0.05$ ) to the response to vaccine virus strains. In order to investigate the antigenic specificity of the antibody responses, virus adsorption studies were carried out with representative pony sera from each vaccine group and each dose schedule (Table 4). All of the sera tested contained CR antibody which produced SRH zones with both vaccine viruses and recent virus strains. After adsorption of sera with A/Newmarket/77 virus, 10/12 sera possessed SS Prague antibody; two ponies possessed only CR antibody to equine 1 virus.

#### *SRH antibody to host antigen*

Seventy-nine per cent of post-vaccination pony sera showed evidence of antibody which reacted on SRH plates containing A/PR/8/34 (H1N1) and B/Singapore/222/79 viruses. This heterotypic SRH activity was completely eliminated by adsorption of sera with homogenized chorioallantoic membranes of uninfected eggs. Similar adsorption experiments showed that on SRH plates containing A/Prague/56 or A/Miami/63 viruses, zone sizes were unaffected by the presence of anti-host antibody.

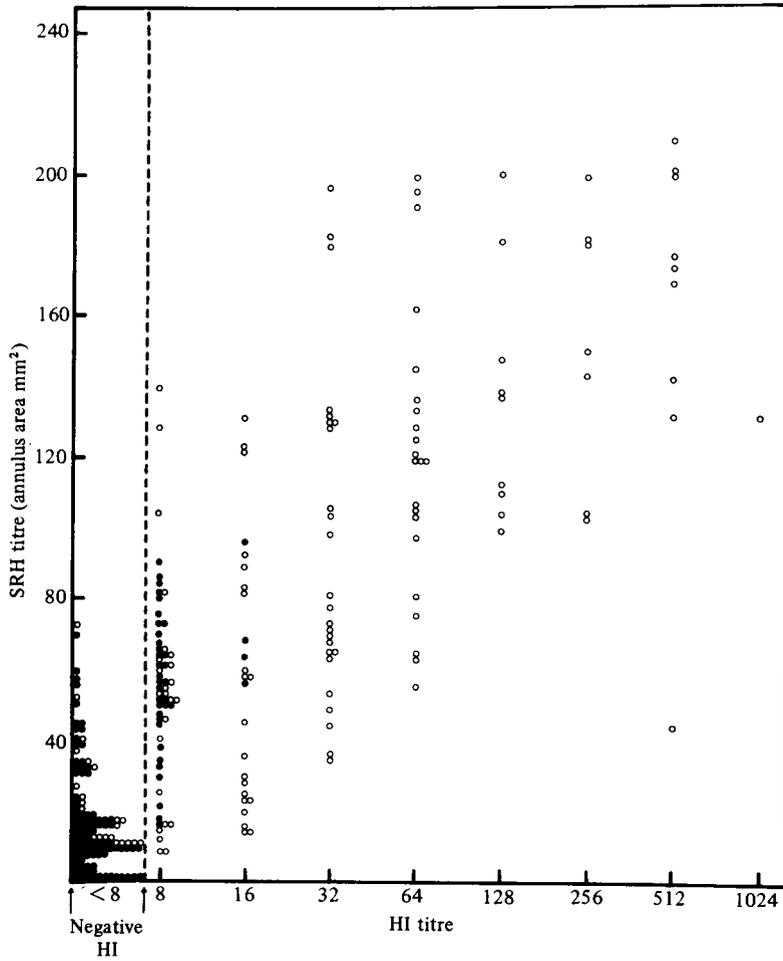


Fig. 5. Correlation between HI and SRH post-vaccination antibody titres to A/equine/Prague/56 virus (●, one vaccine dose; ○, two vaccine doses).

Table 3. SRH antibody responses of ponies to recent equine 1 and 2 virus strains following vaccination with A/equine/Prague/56 and A/equine/Miami/63 viruses

Vaccine group	Vaccine dose	Mean SRH zone areas (mm <sup>2</sup> ) to stated virus strain			
		Miami/63	Newmarket/79	Prague/56	Newmarket/77
A	1 dose	0	0	2.4	0
	2 doses	71.7	59.3	99.1	86.7
B	1 dose	16.7	7.9	22.3	21.7
	2 doses	80.4	75.7	97.9	101.1
C	1 dose	20.0	14.8	16.0	12.2
	2 doses	98.5	97.1	109.0	97.9
D	1 dose	20.7	20.0	20.5	25.3
	2 doses	96.3	89.6	135.8	145.8

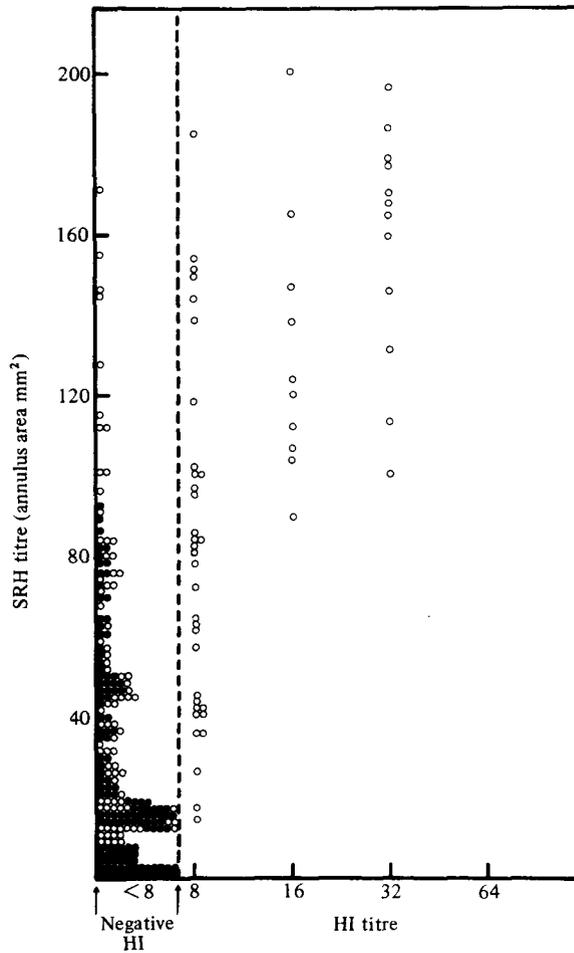


Fig. 6. Correlation between HI and SRH post-vaccination antibody titres of A/equine/Miami/63 virus (●, one vaccine dose; ○, two vaccine doses).

#### DISCUSSION

In this study, serological responses to comparable doses of A/Prague/56 and A/Miami/63 whole virus vaccine over a wide range of vaccine potencies, have been examined in seronegative ponies. The study has involved SRD techniques to standardize vaccine doses and a comparison of HI and SRH techniques for analysing serological responses.

After one vaccine dose, only trace amounts of serum antibody to A/Prague/56 HA (maximum GMT 9.5) and no antibody to A/Miami/63 HA was detectable by the HI test even though the more sensitive HI test using tween 80/ether-treated antigens was used (John & Fulginitti, 1966). However, SRH tests indicated that, by 2–4 weeks after a single dose of vaccine, significant amounts of antibody to both virus strains were detectable and the antibody titres for the aqueous vaccines were directly related to the amount of HA in the vaccines. The adjuvanted vaccine D gave better serological responses than an aqueous vaccine containing 2- to 4-fold

Table 4. *Specificity of antibody produced in vaccinated ponies*

Serum no.	Vaccine group*	SRH zone areas (mm <sup>2</sup> ) on plates containing stated virus							
		Subtype H7N7 (equine 1)				Subtype H3N8 (equine 2)			
		A/Prague/56		A/Newmarket/77		A/Miami/63		A/Newmarket/79	
		Un-adsorbed	Adsorbed New/77	Un-adsorbed	Adsorbed Prague	Un-adsorbed	Adsorbed New/79	Un-adsorbed	Adsorbed Miami
169	1 dose B	45.7	9.6	17.6	0	39.5	16.7	29.3	0
160	1 dose B	52.4	14.2	29.3	0	48.4	23.1	24.1	0
153	1 dose C	53.8	12.6	32.5	0	49.7	43.2	41.9	0
137	1 dose C	37.1	0	8.8	0	44.5	24.1	38.3	0
155	1 dose D	59.5	32.5	34.8	0	51.0	30.3	26.1	0
166	1 dose D	48.4	0	27.1	0	48.4	24.1	30.3	0
167	2 doses A	89.7	11.8	48.4	0	45.7	27.1	66.3	0
116	2 doses B	127.7	24.1	79.5	0	90.2	55.1	73.1	0
131	2 doses B	111.7	27.1	74.6	0	56.5	52.4	54.9	0
139	2 doses C	91.5	29.3	55.1	0	74.6	48.4	77.9	0
138	2 doses D	109.8	22.2	66.8	0	68.4	34.8	65.3	0
145	2 doses D	123.6	29.3	77.9	0	73.1	56.6	77.9	0

\* No data available for one-dose group A due to small SRH zones.

more antigen. In previous vaccine trials with unprimed horses, HI antibody titres after one vaccine dose have also been very low for A/Prague/56 virus, and barely detectable for A/Miami/63 virus (Bryans, 1973; Burrows, Spooner & Goodridge, 1977).

After two vaccine doses, HI antibody responses to A/Prague/56 virus were much higher (maximum titre 304 at 1–2 weeks after second dose), however, A/Miami/63 HI titres remained very low (maximum titre 19 at 1–2 weeks after boost) with only 30% of ponies developing significant antibody levels to A/Miami/63. HI antibody titres did not correlate with the amount of antigen included in the vaccines. In previous trials with two doses of aqueous equine influenza vaccine, HI titres have in general been lower than those reported here (Burrows, Spooner & Goodridge, 1977; Powell & Burrows, 1973).

These studies have demonstrated that the serological responses of ponies to equine influenza vaccines, correlate extremely well with the HA antigen concentration of the vaccine when SRD techniques are used to quantify HA antigen activity and SRH techniques are used to measure anti-HA antibody activity.

Although in HI tests, ponies appeared to respond better to A/Prague/56 vaccine than to A/Miami/63 vaccine, SRH data indicated that in fact the responses were similar for either virus strain. SRH responses after second doses were approximately 2- to 5-fold higher than primary responses and the vaccine dose-related responses, which were established after one vaccine dose, were maintained after the second dose. The adjuvanted vaccine D gave the highest SRH responses.

Our studies show a rapid rate of decline in post-vaccination antibody, detectable by both HI and SRH tests. This is consistent with results from previous studies in horses (Powell & Burrows, 1973; Pressler, 1973; Bryans, 1973; Burrows,

Spooner & Goodridge, 1977). In man, post vaccination antibody declines at a slower rate (Potter *et al.* 1980; Noble *et al.* 1977). Only one of the ponies at the end of this study possessed HI antibody titres  $\geq 40$  (antibody to A/Prague/56 HA, double dose group C). These results imply that, in order to maintain satisfactory antibody levels in horses, it may be necessary to vaccinate at frequent (4–6 month) intervals. The poor persistence of antibody after vaccination may offer some explanation of the low level of protection provided by vaccines in certain equine influenza outbreaks (Van Oirschot *et al.* 1981; Hinshaw *et al.* 1983).

Antigenic drift in equine influenza viruses also has implications with respect to the efficacy of vaccines. Since the original isolates of A/Prague/56 (Sovino *et al.* 1958) and A/Miami/63 viruses (Waddell, Teigland & Sigel, 1963) antigenic variants have periodically been isolated but the differences have been small (Pereira *et al.* 1972; Powell *et al.* 1974; Burrows *et al.* 1981). However, recent epizootics in Europe and the USA have been caused by H3N8 viruses, differing significantly from the prototype A/Miami/63 virus (Van Oirschot *et al.* 1981; Hinshaw *et al.* 1983).

Consequently, post vaccination pony sera in the present study were examined for reactivity with recent equine influenza virus isolates, A/equine/Newmarket/79 (H3N8) and A/equine/Newmarket/77 (H7N7). A/equine/Newmarket/79 viruses is antigenically similar (A. Douglas, personal communication) to the prototype representative of recent H3N8 variants A/equine/Fontainebleu/1/79 (WHO, 1982). Vaccination studies in man have demonstrated that both strain specific antibody and antibody cross-reacting with antigenically similar members of the same HA subtype are produced (Schild *et al.* 1977). All pony sera responded to vaccination by producing large amounts of antibody which cross-reacted with A/Newmarket/77 and A/Newmarket/79 viruses. Similar results have also been obtained in SRH studies in France with recent French virus variants (Fontaine *et al.* 1981). However, most of the ponies in the present study also possessed antibody which could not be removed by adsorption with recent variants, yet would react on SRH plates containing A/Prague/56 and A/Miami/63 virus (SS antibody to vaccine viruses). Therefore a component of the antibody response in ponies consisted of immunoglobulins specific for vaccine virus which did not react with recent antigenic variants.

Most of the ponies responded to vaccination by producing antibody which cross-reacted with heterotypic influenza viruses. It is likely that this antibody was directed against a host egg cell component as it could be completely removed by adsorption with chick chorioallantoic membranes. Previous studies (Harboe, 1963) have demonstrated that influenza virus reacts with antisera raised against host antigen and that the cross-reacting antigen is carbohydrate in nature (Jackson, Brown & White, 1981). The presence of anti-host antibody in pony sera in the present study did not affect the evaluation of antibody to HA by SRH tests, but these results should be considered in further serological studies with SRH.

These studies have demonstrated that SRH tests offer distinct advantages over HI tests for antibody assays of equine sera. The main advantage is one of increased sensitivity. Many sera (35% of sera in A/Miami/63 assays; 4.9% of sera in A/Prague/56 assays) which were negative by HI, had significant, and in some cases extremely high SRH titres.

In addition, SRH is simple, reproducible, allows tests of many sera on one immunoplate and is antigenically subtype specific (Wood, unpublished observations; Fontaine *et al.* 1981).

Further studies on the protective efficacy of vaccines in animals challenged with equine influenza virus demonstrating a relationship between SRH antibody and immunity are described in an accompanying paper (Mumford *et al.* 1983).

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## REFERENCES

- BERLIN, B. S., McQUEEN, J. L., MINUSE, E. & DAVENPORT, F. M. (1963). A method of increasing the sensitivity of the haemagglutination inhibition test with equine influenza virus. *Virology*, pp. 665–666.
- BÖCKMAN, J. (1977). Comparative measurement of influenza virus antibody in horse sera by the single radial haemolysis test and haemagglutination inhibition test. *Zentralblatt für Bakteriologie Parasitenkunde Infektionskrankheiten and Hygiene* (Abteilung 1, Originale Reihe) B **238**, 1–8.
- BRYANS, J. T. (1973). The antibody response of horses to two inactivated oil adjuvanted equine influenza virus vaccines. *Symposia Series in Immunobiological Standardization* **20**, 311–317.
- BURROWS, R., DENYER, M., GOODRIDGE, D. & HAMILTON, F. (1981). Field and laboratory studies of equine influenza viruses isolated in 1979. *Veterinary Record* **109**, 353–356.
- BURROWS, R., SPOONER, P. R. & GOODRIDGE, D. (1977). A three year evaluation of four commercial equine influenza vaccines in ponies maintained in isolation. *Developments in Biological Standardization* **39**, 341–346.
- FONTAINE, M. P., FONTAINE, M., AYMARD, M. & NAFTI, K. (1981). Technique d'hémolyse radiale pour la mesure des anticorps anti-influenza chez le cheval. *Bulletin de l'Académie de France* **54**, 105–112.
- FRERICHS, C. C., FRERICHS, G. N. & BURROWS, R. (1973). Some aspects of the haemagglutination inhibition test used in serological studies of equine influenza. *Symposia Series in Immunobiological Standardization* **20**, 311–317.
- HARBOE, A. (1963). The normal allantoic antigen which neutralizes the influenza virus HI-antibody to host material. *Acta Pathologica et Microbiologica Scandinavica* **57**, 488–492.
- HINSHAW, V. S., NAEVE, C. W., WEBSTER, R. G., DOUGLAS, A., SKEHEL, J. J. & BRYANS, J. (1983). Analysis of antigenic variation in equine 2 influenza viruses. *Bulletin of the World Health Organization*. (In the Press.)
- JACKSON, D. C., BROWN, L. E. & WHITE, D. O. (1981). Antigenic determinants of influenza virus haemagglutinin VI. Antigenic characterization of the oligosaccharide sidechains from HA1 of influenza virus haemagglutinins. *Journal of General Virology* **52**, 163–168.
- JOHN, T. J. & FULGINITI, V. A. (1966). Parainfluenza 2 virus. Increase in haemagglutinin titre on treatment with tween-80 and ether. *Proceedings of the Society for Experimental Biology and Medicine* **212**, 109–111.
- MUMFORD, J., WOOD, J. M., SCOTT, A. M., FOLKERS, C. & SCHILD, G. C. (1983). Studies with inactivated equine influenza vaccine 2. Protection against experimental infection with influenza virus A/equine/Newmarket/79 (H3N8). *Journal of Hygiene* **90**, 385–395.
- NOBLE, G. R., KAYE, H. S., O'BRIAN, R. J., KENDAL, A. P., BREGMAN, D. J., WRIGHT, P. F., AMLER, R. W. & DOWDLE, W. R. (1977). Persistence of influenza A/New Jersey/76 (Hsw1N1) antibody one year after vaccination. *Developments in Biological Standardization* **39**, 253–260.
- OXFORD, J. S., SCHILD, G. C., POTTER, C. W. & JENNINGS, R. (1979). The specificity of the anti-haemagglutinin antibody response induced in man by inactivated influenza vaccines and by natural infection. *Journal of Hygiene* **82**, 51–61.
- PEREIRA, H. G., TAKIMOTO, S., PIEGAS, N. S. & RIBERIRO DO VALLE, L. A. (1982). Antigenic variation of equine (Heq2Neq2) influenza virus. *Bulletin of the World Health Organization* **47**, 465–469.
- POTTER, C. W., CLARKE, A., JENNINGS, R., SCHILD, G. C., WOOD, J. M. & McWILLIAM, P. K. A. (1980). Reactogenicity and immunogenicity of inactivated influenza A (H1N1) virus vaccine

- in unprimed children. Report to the Medical Research Council Committee on influenza and other respiratory virus vaccines. *Journal of Biological Standardization* **8**, 35–48.
- POWELL, D. G. & BURROWS, R. (1973). Field observations on influenza vaccination of thoroughbred horses. *Symposia Series in Immunobiological Standardization* **20**, 332–337.
- POWELL, D. G., THOMSON, G. R., SPOONER, P., PLOWRIGHT, W., BURROWS, R. & SCHILD, G. C. (1974). The outbreak of equine influenza in England April/May 1973. *Veterinary Record* **94**, 282–287.
- PRESSLER, K. (1973). Vaccination against horse influenza virus. Experimental and serological results. *Symposia Series in Immunobiological Standardization* **20**, 311–317.
- RUSSELL, S. M., MCCAHERN, D. & BEARE, A. S. (1975). A single radial haemolysis technique for the measurement of influenza antibody. *Journal of General Virology* **27**, 1–10.
- SCHILD, G. C., PEREIRA, M. S. & CHAKRAVERTY, P. (1975). Single-radial-haemolysis: a new method for the assay of antibody to influenza haemagglutinin. *Bulletin of the World Health Organization* **52**, 43–50.
- SCHILD, G. C., SMITH, J. W. G., CRETESCU, L., NEWMAN, R. W. & WOOD, J. M. (1977). Strain-specificity of antibody to haemagglutinin following inactivated A/Port Chalmers/1/73 vaccine in Man; evidence for a paradoxical strain-specific antibody response. *Developments in Biological Standardization* **39**, 273–281.
- SCHILD, G. C., WOOD, J. M. & NEWMAN, R. W. (1975). A single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen. *Bulletin of the World Health Organization* **52**, 223–230.
- SOVINOVA, O., TUMOVA, B., POUŠKA, F. & NEMEC, J. (1958). Isolation of a virus causing respiratory disease in horses. *Acta Virologica, Praha* **2**, 52–61.
- VAN OIRSCHOT, J. T., MASUREL, N., HUFFELS, A. D. N. H. J. & ANKER, W. J. J. (1981). Equine influenza in the Netherlands during the winter of 1978–79; antigenic drift of the A-equi 2 virus. *Veterinary Quarterly* **3**, 80–84.
- WADDELL, G. H., TEIGLAND, M. B. & SIGEL, M. M. (1963). A new influenza virus associated with equine respiratory disease. *Journal of the American Veterinary Medical Association* **143**, 587–590.
- WHO (1982). Equine influenza surveillance. *World Health Organization Weekly Epidemiological Record* **15**, 119.
- WOOD, J. M., SCHILD, G. C., FOLKERS, C., MUMFORD, J. & NEWMAN, R. W. (1983). Standardization of inactivated equine influenza vaccines by single-radial-immunodiffusion. *Journal of Biological Standardization*. (In the Press.)
- WOOD, J. M., SCHILD, G. C., NEWMAN, R. W. & SEAGROATT, V. (1977). An improved single-radial-immunodiffusion technique for the assay of influenza haemagglutinin antigen. Application for potency determinations of inactivated whole virus and subunit vaccines. *Journal of Biological Standardization* **5**, 237–247.
- YAMAGISHI, H., NAGAMINE, T., SHIMODA, K., IDE, S., IGARASHI, Y., YOSHIOKA, I. & MATUMOTO, M. (1982). Comparative measurement of equine influenza virus antibodies in horse sera by single radial haemolysis, neutralization, and haemagglutination inhibition tests. *Journal of Clinical Microbiology* **15**, 660–662.

#### EXPLANATION OF PLATE

SRH responses of ponies to A/equine/Prague/56 vaccination. 5  $\mu$ l volumes of heat-treated (56° for 30 min) sera were added to wells in SRH plates containing sheep erythrocytes treated with A/equine/Prague/56 virus (4  $\mu$ g virus protein/1 ml erythrocytes). Responses before vaccination, after one dose of vaccine and three doses of vaccine are illustrated for representative sera from each vaccine group. Arrows indicate the first and second vaccinations. Dose-related antibody responses were established after one aqueous vaccine dose and maintained after the second dose. Responses after two doses were much higher than those after one dose, they reached a peak at week 6 and then began to fall. Responses to the adjuvanted vaccine (15  $\mu$ g HA/ml) were as good as the most potent aqueous vaccine (50  $\mu$ g HA/ml).

