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# Antigenicity and immunogenicity of equine influenza vaccines containing a Carbomer adjuvant

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## SUMMARY

Equine influenza vaccines containing inactivated whole virus and Carbomer adjuvant stimulated higher levels and longer lasting antibody to haemagglutinin in ponies than vaccines of equivalent antigenic content containing aluminium phosphate adjuvants. Five months after the third dose of vaccine containing Carbomer adjuvant, ponies were protected against clinical disease induced by an aerosol of virulent influenza virus (A/equine/Newmarket/79, H3N8). In contrast ponies which received vaccine containing aluminium phosphate adjuvant were susceptible to infection and disease. There was an inverse correlation between prechallenge levels of antibody detected by single radial haemolysis (SRH) and duration of virus excretion, pyrexia and coughing. All ponies with antibody levels equivalent to SRH zones of $\geqslant 154$ mm$^2$ were protected against infection and all those with levels $\geqslant 85$ mm$^2$ were protected from disease.

## INTRODUCTION

Conventional equine influenza vaccines contain inactivated whole virus either as an aqueous suspension or combined with adjuvants such as aluminium hydroxide or aluminium phosphate. Representatives of the two equine virus subtypes A/equine 1 (H7N7) and A/equine 2 (H3N8) are included in all vaccines and many products contain two H3N8 virus strains, such as the original prototype (A/Miami/63) and a variant represented by the prototypes A/Fontainebleau/79 or A/Kentucky/81 [1].

The shortlived antibody responses stimulated in horses after a primary course of two doses of vaccine [2–4] are thought to contribute to the poor protection provided by these vaccines. Experimental infections with H3N8 strains of equine influenza virus in the horse have shown that the degree of protection against intranasal challenge correlates with levels of circulating antibody to haemagglutinin as measured by single radial haemolysis (SRH). The levels of antibody consistent with complete protection against intranasal challenge persist for 4–14 weeks [3, 5] following two doses of vaccine, depending on the potency of the vaccine. Field observations also support the view that protection against natural infection is shortlived in vaccinated horses [6].

The adjuvant Carbomer (Code name PD – Solvay Duphar, Weesp, Holland [7])

has been shown to enhance the strength and duration of antibody responses stimulated by inactivated equine influenza vaccines as compared with aqueous vaccines of equivalent antigenic content [4]. The present study was undertaken to compare the antigenicity and immunogenicity of a series of vaccines containing the adjuvant Carbomer with a vaccine containing a conventional adjuvant, aluminium phosphate. The duration of antibody following a course of three doses of vaccine was monitored and the protection provided against challenge infection was examined 5 months after the third dose of vaccine. Additionally, the effect of differences in the composition of the Carbomer vaccines with respect to the adjuvanted tetanus toxoid component and H3N8 strains was examined.

## MATERIALS AND METHODS

### *Viruses*

Influenza A/Prague/56 (H7N7), influenza A/Miami/63 (H3N8) and influenza A/Kentucky/81 (H3N8) were obtained from the WHO Influenza Reference Laboratory, Mill Hill, London. Influenza A/Newmarket/79 (H3N8) was isolated from a vaccinated horse during an outbreak of influenza in 1979. All viruses were propagated in allantoic sacs of fertile hens' eggs.

### *Vaccines*

Viruses were inactivated with $\beta$-propriolactone and antigenic content of haemagglutinin (HA) standardized by single radial diffusion (SRD) [4]. The vaccines, prepared by Solvay Duphar (Weesp, Holland), are described in detail in Table 1. Full details of Carbomer adjuvant are given in the US Pharmacopeial Convention Official Monograph [7]. Vaccine A, which contained three influenza virus strains and tetanus toxoid adsorbed to aluminium phosphate adjuvant, is representative of most commercial products widely available during the last 10 years. Vaccines B, C and E contained the Carbomer adjuvant in conjunction with 15 $\mu$g HA of each of the three strains of virus described above. Vaccines B and C were identical but Vaccine C was administered concurrently with a tetanus vaccine containing aluminium phosphate, the two antigens being injected at different sites. Vaccine E was the same as Vaccine C except that the influenza antigens with Carbomer adjuvant and the tetanus toxoid with aluminium phosphate were mixed immediately prior to administration. Vaccine D contained Carbomer adjuvant but did not contain the A/Miami/63 component or tetanus toxoid. Vaccine B is currently marketed under the name of Duvaxyn IE Plus.

### *Experimental design*

#### *Animals*

Pony foals, 4–6 months of age, which had no detectable antibody to H3N8 and H7N7 viruses as measured by the SRH test, were used in this study.

#### *Vaccination protocol*

Each vaccine group and the control group contained 6 ponies with the exception of Group D which contained 5 ponies. In Groups A–D, ponies received three doses of vaccine on weeks 0, 4 and 31 of the study (Table 2). Influenza antigens and

Table 1. *Vaccine formulations*

| Group | Vaccine: Antigen | Vaccine: Dose μgHA/LF | Adjuvant | Volume | Formulation and administration |
|---|---|---|---|---|---|
| A | A/Prague/56 | 15 | | | |
| | A/Miami/63 | 15 | | | Combined; single |
| | A/Kentucky/81 | 15 | Aluminium phosphate | 1 × 1 ml | site of injection |
| | Tetanus toxoid* | 15 | | | |
| B | A/Prague/56 | 15 | | | |
| | A/Miami/63 | 15 | Carbomer | 1 × 1 ml | Single site |
| | A/Kentucky/81 | 15 | | | |
| C(a) | A/Prague/56 | 15 | | | |
| | A/Miami/63 | 15 | Carbomer | 1 × 1 ml | 1st site |
| | A/Kentucky/81 | 15 | | | |
| (b) | Tetanus toxoid* | 15 | Aluminium phosphate | | 2nd site |
| D | A/Prague/56 | 15 | | | |
| | A/Kentucky/81 | 15 | Carbomer | 1 × 1 ml | Single site |
| E | A/Prague/56 | 15 | | | Mixed immediately |
| | A/Miami/63 | 15 | Carbomer | 1 × 2 ml | prior to injection |
| | A/Kentucky/81 | 15 | | | at single site |
| | Tetanus toxoid* | 15 | Aluminium phosphate | | |

* Adsorbed to aluminium phosphate.

tetanus toxoid were each formulated in 1 ml aliquots. Thus, Vaccines A–D were administered in 1 ml doses and Vaccine E was administered in 2 ml doses. All doses were administered by deep intramuscular injection. Ponies in Group E received two doses of vaccine on weeks 27·5 and 32 of the trial which extended for a period of 52 weeks in total. The six unvaccinated ponies were held with the vaccinated groups as controls. Blood samples were collected at 2–4-week intervals for the duration of the experiment. Ponies were observed for any local reactions at the sites of inoculation.

*Challenge study*

Ponies were infected with an H3N8 virus (A/Newmarket/79) by exposure to an aerosol of virus produced using a DeVilbiss nebulizer (Model 65) [8]. This method of challenge was chosen in preference to intranasal instillation [3, 5] used in previous studies because it elicited more reproducible clinical signs of influenza. All ponies were challenged by exposure to an aerosol of influenza A/Newmarket/79 (H3N8) 50 weeks after the beginning of the trial, i.e. 19 weeks after the third dose received by ponies in groups A–D and 18 weeks after the second dose received by ponies in group E.

Over a period of 30 min, 20 ml of undiluted egg allantoic fluid containing $10^{7·6}EID_{50}$/ml were nebulized into an unventilated stable (56 $m^3$). The ponies were held in the aerosol for a further 60 min before being transferred in vaccine groups to ventilated loose boxes.

Protection provided by vaccination was assessed by the measurement of

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Table 2. *Vaccination and challenge timetable*

| Vaccine | No. of ponies | Vaccination and challenge schedule during weeks indicated* | | | |
|---|---|---|---|---|---|
| | | V1 | V2 | V3 | Ch |
| A | 6 | 0 | 4 | 31 | 50 |
| B | 6 | 0 | 4 | 31 | 50 |
| C | 6 | 0 | 4 | 31 | 50 |
| D | 5 | 0 | 4 | 31 | 50 |
| E | 6 | 27·5 | 32 | — | 50 |
| Control | 6 | — | — | — | 50 |

* V1, 1st dose of vaccine. V2, 2nd dose of vaccine. V3, 3rd dose of vaccine. Ch, infection by exposure to nebulised aerosol of A/equine/Newmarket/79 (H3N8) virus.

antibody responses, virus excretion and clinical signs following challenge. Blood samples were collected on the day of challenge and 14 days later. Rectal temperatures were taken daily for 10 days. Nasopharyngeal swabs were collected on a daily basis for 10 days and were processed as described below.

## *Laboratory procedures*

### *Serological tests*

The SRH test has been described previously [9]. Briefly, after inactivation at 56 °C for 30 min, 10 $\mu$l aliquots of serum were placed in wells 3 mm diameter cut in agarose (Seakem M.E.M., Miles Laboratories) into which had been incorporated sheep erythrocytes sensitized with virus in the presence of chromium chloride and fresh undiluted guinea-pig complement. Diffusion of sera was permitted for 20 h in a humidified chamber at 34 °C. Diameters of zones of haemolysis were measured in two directions at right angles using an automatic reader (Autodata, Hitchin, England). Zone areas were calculated and a 50% increase was taken as significant [9]. Positive control antisera were included on each plate and the test rejected if there was more than a $\pm 10$% difference in control zone areas on different days of testing.

### *Virus isolation*

Nasopharyngeal swabs were kept in virus transport medium on ice during transfer from the isolation facilities to the laboratory. All swabs were frozen at −70 °C prior to virus isolation attempts. Exudate from each swab was diluted by 10-fold dilutions in phosphate buffered saline pH 7·2 and 0·1 ml from each dilution inoculated into the allantoic cavity of 10-day-old embryonated eggs (three eggs/dilution). The virus titre ($EID_{50}$/ml) in swab extracts was calculated from HA activity in allantoic fluids after 48 h incubation at 35 °C.

## *Analysis of results*

### *Serological responses to vaccination*

Mean SRH antibody levels against A/Prague/56 (H7N7), A/Miami/63 (H3N8) and A/Kentucky/81 (H3N8), expressed as $mm^2$ haemolysis zone area were calculated for each group. A/Miami/63 and A/Kentucky/81 are variants of the

H3N8 subtype and some antibodies stimulated by one strain cross-react with the other. Thus, the overall antibody response measured against one H3N8 virus is contributed to by both strains in the vaccine.

*Serological responses to infection*

A 50% or more increase in the area of haemolysis by antibody to A/Kentucky/81 virus was taken to indicate infection with the challenge virus [8], from which it is antigenically indistinguishable.

*Temperature responses*

Temperatures $> 38{\cdot}9$ °C were regarded as abnormal for the purpose of calculating the number of significant temperatures and duration of febrile responses. For each group the following expressions were calculated:

$$\text{mean significant temperature responses} = \frac{\text{the sum of the means of significant temperatures for each pony}}{\text{no. of ponies with significant temperatures}}$$

$$\text{mean duration of temperature response} = \frac{\text{no. of days with significant temperature for each pony}}{\text{no. of ponies with significant temperatures}}$$

*Virus excretion*

Duration of virus excretion was calculated from virus recovered after a single passage of swab extract in the allantoic cavity of 10-day-old fertile hens' eggs. Geometric mean virus titres in nasopharyngeal swab extracts were calculated for the positive swabs recovered from each vaccine group after titration of swab extracts in eggs. Virus titres were expressed as $EID_{50}$/1 ml swab extract.

*Statistical analyses*

Group (arithmetic) mean levels of antibody at various times post vaccination were calculated and Student's $t$ test was used to examine differences between groups. Differences between febrile responses, antibody responses and virus excretions in the vaccine and control groups were investigated using Wilcoxon rank sum tests. The relationship between post-challenge responses in individual ponies and prechallenge antibody were examined by calculation of correlation coefficients.

## RESULTS

No significant local or systemic reactions were noted following vaccinations.

### *Antibody responses to vaccination*

Figs 1, 2 and 3 illustrate the SRH antibody responses to A/Prague/56, A/Miami/63 and A/Kentucky/81 stimulated by three doses of Vaccines A, B, C and D, administered at weeks 0, 4 and 31, and two doses of Vaccine E administered on weeks 27·5 and 32.

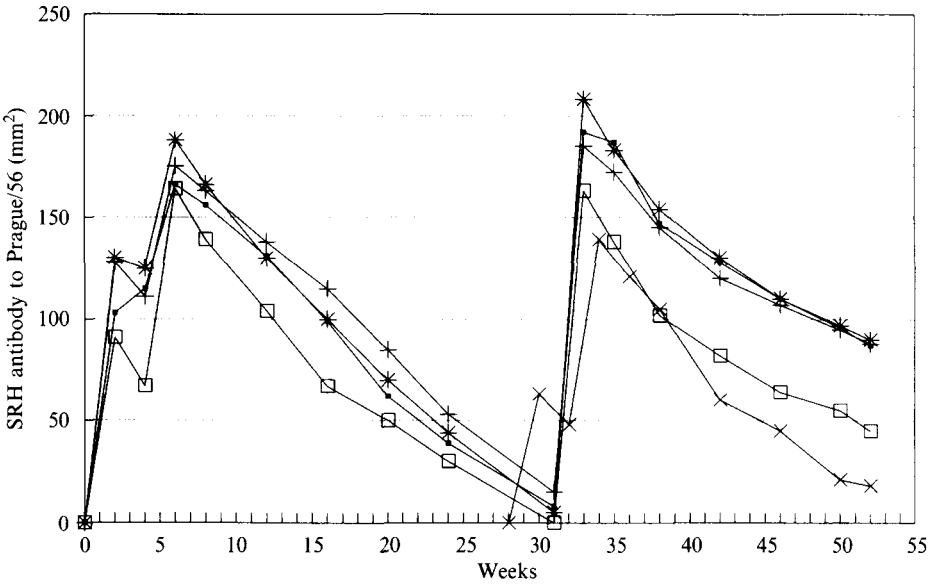


Fig. 1. Mean SRH antibody responses to A/Prague/56 (H7N7) virus in vaccinated ponies. Symbols —□— = vaccine group A. —■— = vaccine group B. —+— = vaccine group C. —*— = vaccine group D. —×— = vaccine group E.

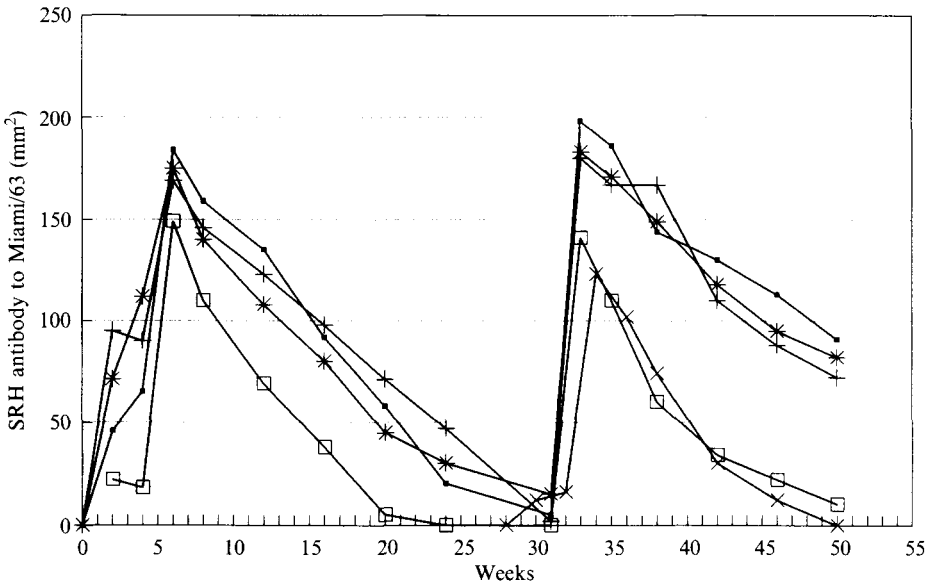


Fig. 2. Mean SRH antibody responses to A/Miami/63 (H3N8) virus in vaccinated ponies. Symbols as for Fig. 1.

*A/Prague/56 (H7N7) (Fig. 1)*

Measurable ($> 4$ mm$^2$) antibody responses to A/Prague/56 were detectable 2 weeks after the first dose of vaccine in all vaccinated ponies in groups A, B, C and D (Fig. 1). Two weeks after the second dose of vaccine these ponies developed

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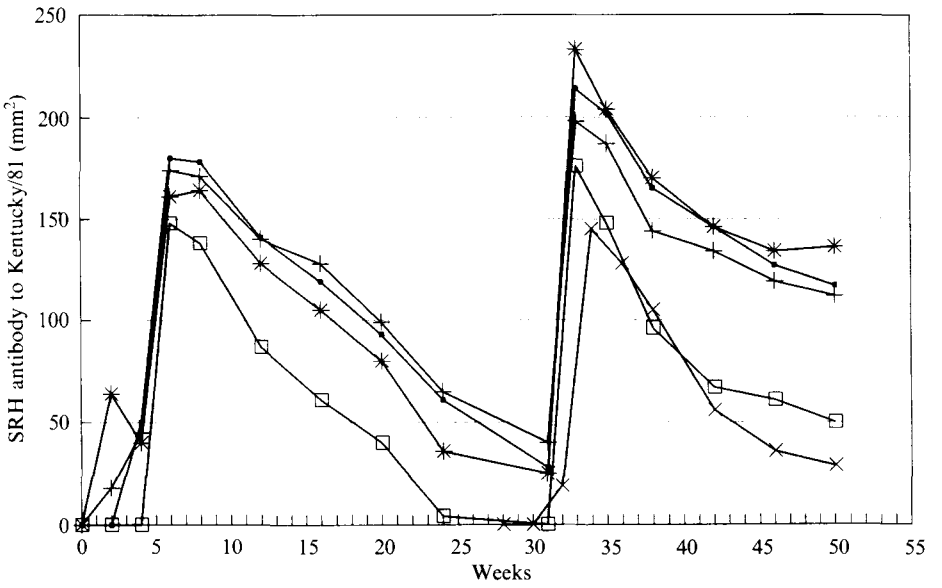


Fig. 3. Mean SRH antibody responses to A/Kentucky/81 (H3N8) virus in vaccinated ponies. Symbols as for Fig. 1.

strong secondary antibody responses with mean peak responses of 169, 176, 172 and 191 $mm^2$ for Vaccines A–D respectively. During the next 25 weeks, antibody declined at a similar rate in all these four groups and antibody levels in group A were consistently lower. The third dose of Vaccines A–D produced higher antibody levels than the second dose. The peak responses to Vaccines C and D, following the third dose, were significantly higher ($P < 0{\cdot}05$) than those to Vaccine A and remained so at week 50.

Group E, which received two doses of vaccine on weeks 27·5 and 32, developed antibody in 6/6 ponies following the first dose. However, the peak antibody level 2 weeks after the second dose was lower than that stimulated by the second dose of Vaccine B (Fig. 1).

*A/Miami/63 (H3N8) (Fig. 2)*

Following the first dose of Vaccines A–D, antibody was detectable in all but one vaccinee in group A.

Following the second dose, peak responses (184, 167, 172 $mm^2$) in groups B, C and D respectively were significantly higher than in group A (144 $mm^2$) ($P < 0{\cdot}01$, $< 0{\cdot}05$, $< 0{\cdot}05$ respectively). Antibody declined at a similar rate in all these four groups, and at 16 weeks antibody levels in groups B, C and D had remained significantly ($< 0{\cdot}05$) higher than in group A. At 20 weeks, while antibody was still present in groups B, C and D, it was undetectable in group A. Following the third dose of vaccine peak responses were similar (197, 183, 181 $mm^2$) in group B, C and D and significantly higher ($< 0{\cdot}01$) than in group A (142 $mm^2$). The peak responses in all four groups were higher than those stimulated following the second dose. Antibody declined more rapidly in group A than in groups B, C and D and at week 49 (18 weeks after the third dose) antibody was barely detectable in group A (16 $mm^2$) while high levels ($> 70$ $mm^2$) were still apparent in groups B, C and D.

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In group E, vaccinated on weeks 27·5 and 32, a weak response was detected in 4/6 ponies after the first dose; a much higher response (124 $mm^2$) developed after the second dose. The peak response was significantly lower ($< 0·05$) than those stimulated by Vaccines B and D after two doses, but was not significantly lower than that stimulated by two doses of Vaccines A and C. Antibody to A/Miami/63 was barely detectable in group E 22 weeks after the first dose of vaccine.

*A/Kentucky/81 (H3N8) (Fig. 3)*

Two weeks after the first dose of vaccine, 11/23 vaccinees developed detectable antibody to A/Kentucky/81 (H3N8) virus of which only one had received Vaccine A. Following the second dose of vaccine, all four groups (A–D) developed high levels of antibody (162, 179, 170 and 173 $mm^2$ respectively). Although the response in group A was weaker than other groups, the difference was not significant. Antibody declined at a similar rate in all four groups but at week 16 the antibody level in group A was significantly lower than in groups B, C and D ($P < 0·05$). At week 24, no antibody was detectable in group A but was still present in groups B, C and D. Following the third dose of vaccine, peak responses were significantly higher ($P < 0·05$) than those stimulated after the second dose within each group and particularly group D ($P < 0·01$) containing A/Kentucky/81 as the only H3N8 component.

Following the third dose of vaccine, antibody declined more rapidly in group A than in groups B, C and D, such that at week 49, antibody (54 $mm^2$) in group A was significantly lower ($< 0·01$) than in groups B, C and D (117, 114 and 137 $mm^2$ respectively). In group E the responses to Kentucky/81 after 2 doses were inferior to those in groups B, C and D.

It was clear from these results that antibody responses were consistently higher in the vaccine groups containing Carbomer than those containing aluminium phosphate. These differences were more marked for the H3N8 viruses than the H7N7 virus. Further, the rate of decline in antibody was more pronounced in the group receiving vaccine containing aluminium phosphate adjuvant as compared with Carbomer adjuvant, particularly after the third dose of vaccine. Carbomer adjuvant prolonged the duration of detectable antibody to A/Miami/63 for at least 11 weeks (Fig. 2) and for A/Kentucky/81 for at least 7 weeks (Fig. 3), when compared with the aluminium phosphate adjuvant. There were no significant differences between the antibody responses stimulated by vaccines B, C and D, suggesting that omission of Miami/63 did not significantly affect the height of the antibody response, and that separate administration of tetanus did not interfere with the response to influenza. In group E, which received the Carbomer vaccine pre-mixed with tetanus toxoid, antibody responses following two doses were lower than for vaccines B, C and D, indicating that the direct mixing of the two adjuvants had an adverse effect on the antigenicity of the influenza components in group E.

*Responses following challenge*

Responses following challenge were analysed on the basis of vaccine group and were related to the mean pre-challenge antibody of the groups to A/Kentucky/81 virus which is antigenically indistinguishable from the challenge virus A/Newmarket/79 (unpublished observations). Additionally, post challenge responses

Table 3. *Antibody responses and virus excretion following challenge with Newmarket/79 virus*

| | | Antibody* responses | | Virus excretion | | |
|---|---|---|---|---|---|---|
| Group | Adjuvant (see Table 1) | Prechallenge SRH antibody to Kentucky/81 [$mm^2$] | Number responding / Number in group | Number excreting / Number in group | Mean days duration (range) | Mean titre† (range) [$EID_{50}/log_{10}ml$ swab extract] |
| A | Al. phos. | 51 | 6/6 | 5/6 | 1·6 (1–2) | 1·06 (1–1·5) |
| B | Carbomer | 117 | 5/6 | 1/6 | 1·0 (1) | 1·0 |
| C | Carbomer | 114 | 6/6 | 2/6 | 1·5 (1–2) | 2·2 (1·5–3·5) |
| D | Carbomer | 137 | 2/5 | 3/5 | 1·0 (1) | 2·5 |
| E | Carbomer | 29 | 6/6 | 6/6 | 2·2 (1–4) | 1·77 (1·0–3·5) |
| F | None | 0 | 6/6 | 6/6 | 5·5 (3–7) | 2·0 (1·0–4·0) |

* 50% increase in SRH antibody to A/Kentucky/81.

† $\frac{\text{Sum of the positive titres } (EID_{50}\log_{10}/\text{ml swab extract})}{\text{No. of positive swabs}}$

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have been analysed in relation to prechallenge antibody levels in individual ponies.

*Antibody responses*

The mean prechallenge antibody to A/Kentucky/81 of the individual vaccine groups is given in Table 3. There were no significant differences between the mean values 117, 114 and 137 $mm^2$ for groups B, C and D respectively. The mean values in group A (51 $mm^2$) and group E (29 $mm^2$) were significantly lower ($< 0{\cdot}01$) than those in B, C and D.

Following challenge, all seronegative control ponies and 25/29 vaccinated ponies seroconverted. The four ponies which did not show a 50% increase in SRH antibody had prechallenge levels $> 154$ $mm^2$ and also had the highest antibody levels among the vaccinees. One pony belonged to group B and three to group D (Fig. 5).

Antibody responses to A/Miami/63 virus were consistent with those seen for A/Kentucky/81 virus. As expected no increases in SRH antibody to A/Prague/56 (H7N7) virus were demonstrated following challenge with an H3N8 virus.

*Virus excretion*

All six unvaccinated control ponies shed virus (A/Newmarket/79) from the nasopharynx. Virus was recovered from 4/6 control ponies on day 1, from 6/6 ponies on days 2 and 4, and from 5/6 ponies on days 3 and 5. Subsequently, individual ponies shed virus between days 6 and 10. Virus shedding followed a biphasic response with peak titres detected on days 2 and 4 post-infection.

Of the 29 vaccinees, 17 shed virus after infection. Only 6 of these 17 ponies were from groups B, C and D (mean prechallenge antibody, 117, 114 and 136 $mm^2$ respectively). In group A (mean prechallenge antibody 51 $mm^2$) 5/6 ponies shed virus and in group E (mean prechallenge antibody 29 $mm^2$) 6/6 did so. Thus the number of ponies shedding virus per group reflected the mean prechallenge antibody levels of the group (Table 3).

Virus excretion in unvaccinated controls continued for 3–7 days, with a mean duration of 5·5 days. In all vaccinated groups the mean duration of virus excretion was significantly reduced ($P < 0{\cdot}01$) (Table 3). There was a clear inverse relationship between the duration of virus shedding and the prechallenge antibody of the groups. In group D, virus was recovered from one pony which did not show a seroconversion and may, therefore, represent non-replicating challenge virus.

The peak titres of virus shed by the unvaccinated controls were greater than that shed by the vaccinees. However, there were no significant differences between the mean titres of virus recovered from ponies in the different groups because of the variability in virus titre recovered from individual ponies within a group on consecutive days (Table 3). For example, among control ponies (group F) titres of virus in swab extracts ranged from 1·0 to 4·0 $EID_{50}log_{10}$/ml and among vaccinated ponies in group C titres ranged from 1·0 to 3·5 $EID_{50}log_{10}$/ml.

## *Clinical responses*

*Pyrexia*

All six seronegative control ponies developed febrile responses ($> 38{\cdot}9$ °C) following challenge infection with peak responses occurring on day 2 post-

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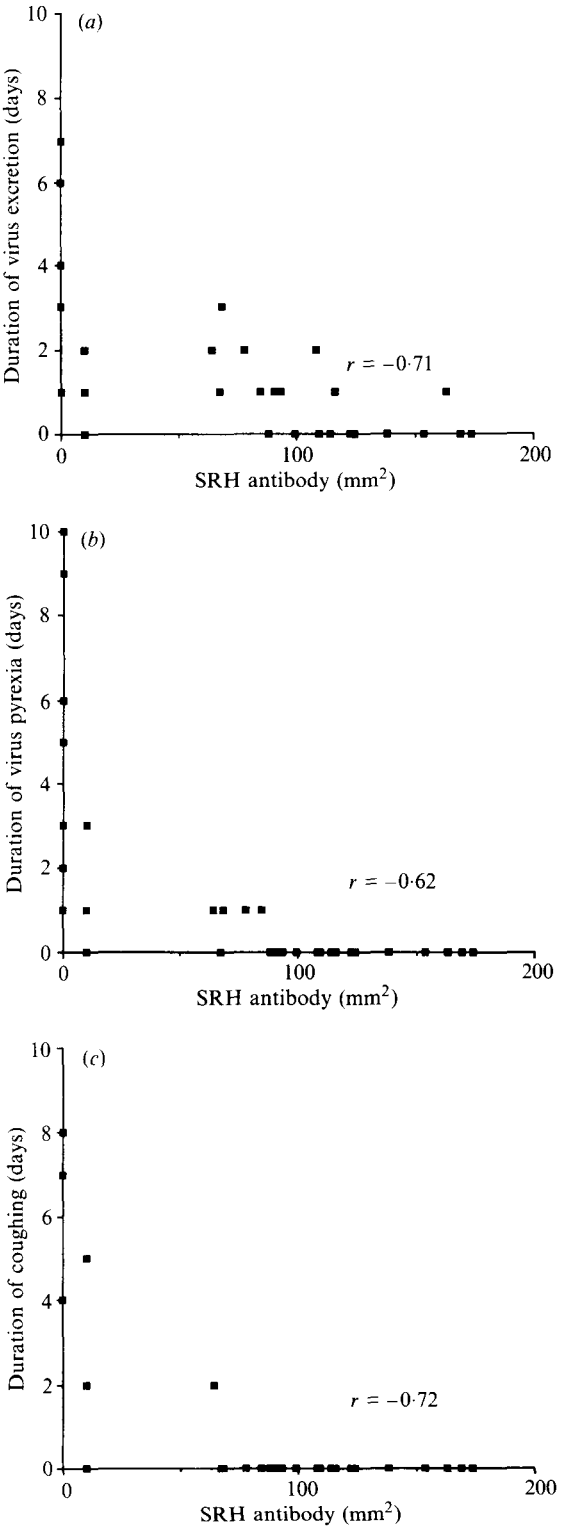


Fig. 4. Correlation between prechallenge SRH antibody to A/Kentucky/81 (H3N8) duration of virus excretion (*a*), pyrexia (*b*) and cough (*c*).

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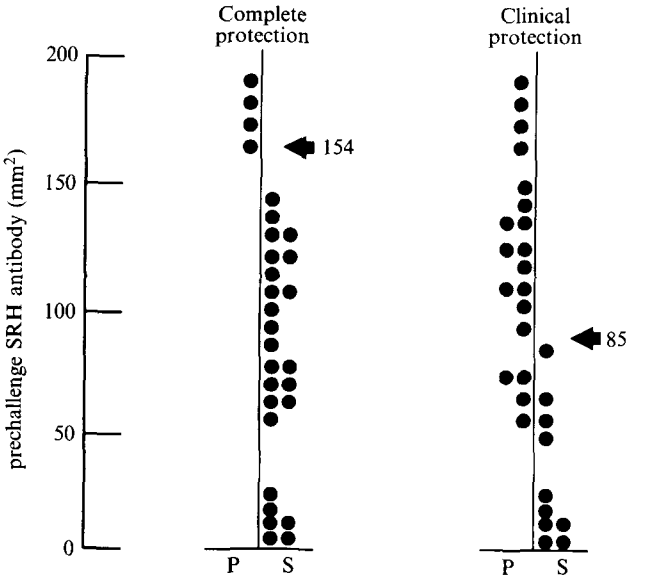


Fig. 5. Relationship between prechallenge SRH antibody to A/Kentucky/81 (H3N8) virus and protection against infection and disease.

infection. In the vaccinated groups, 9/29 ponies developed febrile responses, and of those, five occurred in group E (prechallenge antibody 29 mm²), 3 in group A (prechallenge antibody 51 mm²) and only 1 occurred in groups B, C and D, receiving 3 doses of Carbomer vaccine. Febrile responses persisted for 1–10 days (mean duration 5·7 days) among unvaccinated controls, whereas those in vaccinated ponies persisted for 1–3 days only. The mean duration of febrile responses in all vaccinated groups was significantly reduced ($< 0{\cdot}05$) as compared with the controls. The reduction in duration of pyrexia reflected the mean prechallenge antibody of the vaccine groups (Table 4). There were no significant differences in the height of the febrile responses in any of the groups.

*Cough*

Following challenge, all 6 control ponies developed the harsh, dry cough typical of natural influenza infections, whereas cough was recorded in only 5/29 vaccinees. Of these, 4 ponies were in group E (which had a mean prechallenge antibody of 29 mm²) and 1 belonged to group A (group mean prechallenge antibody 51 mm²). No cough was noted in any of the ponies which had received three doses of Carbomer vaccine. The duration of cough in all vaccine groups was significantly reduced as compared with the controls ($P < 0{\cdot}05$).

*Individual variation in serological, virological and clinical responses*

The data above describe the responses of vaccine groups. However, considerable differences in prechallenge antibody levels were noted among individuals, particularly within vaccine groups A and E. Therefore, the data have also been examined in relation to the antibody levels of individual ponies before challenge.

In group A, antibody levels ranged from trace amounts to 92 mm² and in group E from $< 4$ to 109 mm². In the groups B, C and D, there was less individual

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Table 4. *Clinical responses following challenge with Newmarket/79 virus*

| Group | Adjuvant (see Table 1) | Prechallenge SRH antibody to Kentucky/81 [mm²] | Pyrexia | | | Cough | |
|---|---|---|---|---|---|---|---|
| | | | Number responding / Number in group | Mean significant temperature (°C)* | Mean duration (range) | Number coughing / Number in group | Days mean duration (range) |
| A | Al. phos. | 51 | 3/6 | 39·6 | 1·67 (1–3) | 1/6 | 5 |
| B | Carbomer | 117 | 0/6 | — | 0 | 0/6 | 0 |
| C | Carbomer | 114 | 0/6 | — | 0 | 0/6 | 0 |
| D | Carbomer | 137 | 1/5 | 39·3 | 1 | 0/6 | 0 |
| E | Carbomer | 29 | 5/6 | 39·5 | 1·2 (1–2) | 4/6 | 4·5 (1–8) |
| F | None | 0 | 6/6 | 39·7 | 5·7 (1–10) | 6/6 | 5·8 (4–8) |

* Temperatures > 38·9 °C are regarded as significant.

variation; in group B, levels varied between 90 and 154 $mm^2$; in group C between 88 and 138 $mm^2$ and in group D between 85 and 174 $mm^2$. The wide variation in individual antibody responses within groups largely accounted for the variability of clinical and virological responses within groups following challenge.

There was an inverse correlation between duration of virus excretion and prechallenge antibody levels ($r = -0{\cdot}71$). No virus was recovered from ponies with antibody $> 116$ $mm^2$, except from one pony which showed no seroconversion postchallenge (Fig. 4*a*). There was also an inverse correlation ($r = -0{\cdot}62$) between prechallenge antibody and duration of pyrexia with significant temperature responses occurring only in ponies with prechallenge antibody of 84 $mm^2$ or less (Fig. 4*b*). Similarly, there was an inverse correlation ($r = -0{\cdot}72$) between prechallenge antibody and duration of coughing, with coughing occurring only in ponies with SRH antibody of 64 $mm^2$ or less (Fig. 4*c*).

When ponies were ranked according to prechallenge antibody it was found that all those with antibody of 154 $mm^2$ or greater were protected against infection and that all those with antibody of 85 $mm^2$ or greater were protected against clinical signs of disease.

These data illustrate the close relationship between circulating antibody to haemagluttinin (as measured by SRH) and the duration of clinical responses and virus excretion in individuals following challenge.

## DISCUSSION

This study was carried out to compare inactivated equine influenza vaccines containing Carbomer adjuvant with an inactivated vaccine containing aluminium phosphate as adjuvant and to assess the acceptability of Carbomer as an adjuvant for horses. Differences in the composition of the four vaccines containing Carbomer adjuvant provided the opportunity to examine the effect of the tetanus component on the responses to influenza antigens, the effect of mixing the tetanus adjuvanted with aluminium phosphate with the influenza and Carbomer fraction, and to examine the effect of omitting the original H3N8 prototype strain (Miami/63) from the vaccine.

The antigenicity of Vaccines B, C and D which contained the Carbomer adjuvant was consistently superior to that of Vaccine A which contained aluminium phosphate, both in terms of levels and duration of antibody response stimulated to all three viruses (A/Prague/56, A/Miami/63 and A/Kentucky/81) although the differences were more marked with H3N8 strains than with the H7N7 strain. There were no significant differences between the antibody responses stimulated by Vaccines B, C and D, thus the concomitant administration of tetanus toxoid adsorbed with aluminium phosphate at a separate site or the absence of 15 $\mu$gHA of A/Miami/63 virus in Vaccine D did not markedly influence the antigenicity of these vaccines. However, when ponies were injected with influenza vaccine containing Carbomer adjuvant pre-mixed with tetanus toxoid adsorbed with aluminium phosphate (Vaccine E), weaker antibody responses were stimulated by two doses of vaccine as compared with two doses of Vaccines B and C containing equivalent influenza antigen. This was later shown to be associated with a formulation problem with the two adjuvants forming a highly viscous

suspension, and in further studies with vaccines containing equine influenza with Carbomer combined with tetanus adsorbed to aluminium phosphate, antibody responses similar to Vaccine C were demonstrated (unpublished data). The Carbomer adjuvant was well tolerated by the experimental ponies and has subsequently been shown to be acceptable in thoroughbred horses (unpublished).

In groups B, C and D, the decline in antibody was slower after the third dose, whereas antibody stimulated in group A declined at a similar rate after the second and third doses. Thus, the difference in antibody levels between group A, as compared with B, C and D at the time of challenge 19 weeks after the third dose of vaccine, was greater than the differences seen between peak responses two weeks after the third dose of vaccine. The plateau in the antibody response after the third dose has not been observed with conventional vaccines and represents a real improvement in equine influenza vaccines.

It was of interest that the Carbomer vaccines (B, C and D) also stimulated consistently high antibody levels within groups. This is an important characteristic as poor responders are likely to be significant in the spread of infection and development of herd immunity.

In previous studies, protection against challenge with both homologous and heterologous strains of H3N8 virus has been shown to correlate with circulating antibody to HA as measured by the single radial haemolysis test [3, 5]. In this study, in which four of the vaccines contained both the A/Miami/63 and A/Kentucky/81 virus, it was not possible to determine how each strain contributed to the antibody response seen since the SRH test measures both cross-reacting and strain-specific antibody [9]. Both types of antibody are thought to contribute to immunity, although it has been shown that strain-specific antibody to human influenza is more effective in passive protection studies in mice than cross-reacting antibody [10, 11].

In previous challenge studies with H3N8 virus, ponies were infected by intranasal instillation of virus and it was found that animals with prechallenge antibody $> 74\ mm^2$ were completely protected against infection [3, 5]. In the present study, where ponies were challenged by exposure to nebulized aerosol at a dose equivalent to $10^{5\cdot6}$ minimum infectious doses [8], only those with antibody $> 154\ mm^2$ were completely protected against infection as judged by the absence of raised antibody levels following challenge. However, clinical protection (i.e. absence of febrile responses or coughing) was demonstrated in ponies with antibody $> 85\ mm^2$ (Fig. 5). Thus it would appear that exposure to aerosol provides a more severe challenge and demands higher levels of antibody to provide protection.

When protection against challenge was examined in relation to vaccine groups it was clear that those ponies receiving three doses of vaccine containing Carbomer were clinically immune 20 weeks after the third dose of vaccine, in spite of exposure to a very high dose of virus, whereas only partial immunity was provided by the vaccine containing aluminium phosphate. By extrapolation of protective levels of antibody it can be calculated that, using this challenge system, complete protection persisted for 5–7 weeks following two doses of Carbomer Vaccines B, C and D and for 7–11 weeks following a third dose. The duration of protection provided by the aluminium phosphate adjuvanted vaccine was much less, lasting

for only 3–4 weeks following the third dose. Similarly, clinical protection could be expected to persist for 16–18 weeks following two doses of Carbomer vaccine and for approximately 9 weeks following vaccination with the aluminium phosphate adjuvanted vaccine.

Based on these observations, it appears that, if horses are vaccinated with conventional adjuvant vaccines according to a schedule of two primary doses 4–6 weeks apart followed by a booster dose at 6 months, there would be a period between the second and third dose when animals would be susceptible both to infection and clinical disease, and more frequent vaccination would be required to maintain immunity in young animals. However, it is uncertain how the challenge dose used in this experiment relates to the challenge dose received under field conditions. It is probable that the number of infectious particles inhaled by ponies in the experimental situation greatly exceeds those inhaled in a field situation and on this basis the challenge could be regarded as severe. However, it is not known whether culture in embryonated eggs may have attenuated the virus.

The current study confirms previous observations that circulating SRH antibody levels to haemagglutinin stimulated by inactivated influenza vaccines correlates closely with protection against infection with H3N8 virus and that this correlation is sustained 20 weeks after vaccination. The contributions of local antibody and cell-mediated immunity to the protection stimulated by these inactivated vaccines have not yet been examined although the ability of inactivated vaccines to stimulate mucosal antibody has been confirmed [12]. The presence of cytotoxic T cells active against influenza has been reported following influenza infections in horses [13] and these studies are now being extended to include T cell immunity stimulated by vaccines.

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