Paper

Efficacy of a non-updated, Matrix-C-based equine influenza subunit-tetanus vaccine following Florida sublineage clade 2 challenge

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Assessing the ability of current equine influenza vaccines to provide cross-protection against emerging strains is important. Horses not vaccinated previously and seronegative for equine influenza based on haemagglutination inhibition (HI) assay were assigned at random to vaccinated (n=7) or non-vaccinated (control, n=5) groups. Vaccination was performed twice four weeks apart with a 1 ml influenza subunit (A/eq/Prague/1/56, A/eq/Newmarket/1/93, A/eq/Newmarket/2/93), tetanus toxoid vaccine with Matrix-C adjuvant (EquilisPrequenza Te). All the horses were challenged individually by aerosol with A/eq/Richmond/1/07 three weeks after the second vaccination. Rectal temperature, clinical signs, serology and virus excretion were monitored for 14 days after challenge. There was no pain at the injection site or increases in rectal temperature following vaccination. Increases in rectal temperature and characteristic clinical signs were recorded in the control horses. Clinical signs were minimal in vaccinated horses. Clinical (P=0.0345) and total clinical scores (P=0.0180) were significantly lower in the vaccinated than in the control horses. Vaccination had a significant effect on indicators of viraemia – the extent (P=0.0006) and duration (P=<0.0001) of virus excretion and the total amount of virus excreted (AUC, P=0.0006). Vaccination also had a significant effect (P=0.0017) on whether a horse was positive or negative for virus excretion during the study. Further research is needed to fully understand the specific properties of this vaccine that may contribute to its cross-protective capacity.

Introduction

Equine influenza is a frequently occurring, contagious respiratory infection of horses caused by equine influenza virus – an orthomyxovirus of the genus influenza virus A. Outbreaks of equine influenza occur almost all over the world in the equine population – only Iceland and New Zealand are free of this virus (Anon 2012) – and incur significant economic losses due to the cost of treatment, disruption of the equine industry and limitations on the movement of horses.

Surface glycoproteins (haemagglutinin and neuraminidase), which are used to subtype influenza A viruses, play an important role in establishing and spread of infection, as well as being the target for virus neutralising antibody. Haemagglutinin is the principal surface glycoprotein, controls the entry of virus into host cells, is a key immunogen for neutralising antibody and is used for virus typing. Neuraminidase controls the release of newly synthesised virus particles from infected cells and plays an increasingly recognised role as an immunogen, through reducing the amount of virus released from infected cells (Sylte and Suarez 2009). Random mutation leads to structural change of the surface glycoproteins, which is known as antigenic drift. Over time, if sufficient changes in amino acid composition have occurred, the invading virus may no longer be recognised by the primed immune system – the immune system of horses that have been vaccinated or infected previously – and thus, would not be neutralised by antibody. However, the situation is complex. While circulating antibody to haemagluttinin appears to correlate with protection after vaccination, antibody titres in horses following natural infection are often low even though these horses are protected against infection (Cullinane and Newton 2013).

The viruses currently circulating in horses are of the H3N8 antigenic subtype (Borchers and others 2005, Bryant and others 2009, Elton and Bryant 2011, Gildea and others 2012, Cullinane and Newton 2013). In fact, all influenza viruses isolated from horses in the last 30 years have belonged to this subtype suggesting that H7N7 viruses (ie, A/eq/Prague/56) are no longer circulating in the horse population (Elton and Bryant 2011). In the mid-1980s, H3N8 viruses split into two distinct lineages (American and Eurasian) (Endo and others 1992, Daly and others 1996). The situation has since become more complex (Daly and others 2011) with the American lineage split into three distinct sublineages – South American, Kentucky and Florida (Lai and others 2004, Lewis and others 2011). The Florida sublineage is split into two distinct clades known as Florida 1 (eg, A/eq/South Africa/4/03) and Florida 2 (eg, A/eq/Newmarket/5/03, A/eq/Richmond/1/07) (Daly and others 2011, Lewis and others 2011, Cullinane and Newton 2013). In samples from the field, 'Eurasian lineage' strains are now isolated

infrequently and 'American lineage' strains predominate (Daly and others 2011, Cullinane and Newton 2013). In Europe, the strains circulating currently are from Florida sublineage clade 2 (Barthold and others 2011, Cullinane and Newton 2013).

A panel of experts (expert surveillance panel (ESP)) appointed by the World Organisation for Animal Health (OIE) make recommendations for equine influenza vaccines based on the worldwide equine influenza strain surveillance programme, in place since 1993. This programme relies on the analysis of antigenic differences between strains of equine influenza based on haemagglutination inhibition (HI) assays using post-infection ferret antisera. These assays are notoriously difficult to interpret and serve as a guide (Daly and others 2011). For this reason, antigenic cartography of HI data and genetic sequencing of the haemagglutinin 1 (HA1) gene is also carried out. The rate of antigenic drift is slower for H3N8 strains than it is for human influenza A viruses. In 1995, the panel recommended that Eurasian and American lineage strains be included in equine influenza vaccines (Daly and others 2011). About a decade later, an update to the American lineage strain to include either A/eq/South Africa/4/03 or A/eq/Ohio/03 was recommended (Daly and others 2011). An update to include a clade 1 and a clade 2 virus of the Florida sublineage was recommended 5–6 years later (OIE 2010). Representatives of A/eq/Prague56 (H7N7) and of the Eurasian strain (A/eq/Newmarket/2/93) are no longer considered relevant (Cullinane and others 2010). The latest recommendations advise that representatives of clades of the Florida sub-lineage (with clade 1 represented by A/eq/South Africa/4/03-like or A/eq/Ohio/03-like viruses and clade 2 by A/eq/Richmond/1/07-like viruses) should be included (Bryant and others 2011).

Vaccination of horses against equine influenza should include an initial basic (primary) vaccination course of two vaccinations, with a first revaccination (booster, third vaccination) normally after around five months after the primary vaccination course. Some equine bodies and associations require annual revaccination, while others, such as the Fédération Equestre Internationale (FEI), require more frequent revaccination (in 6 months plus 21 days of the previous booster vaccination) for animals entering competitions (Anon 2013).

In non-vaccinated horses, disease caused by equine influenza virus infection usually has an incubation period of three to five days, and most of the affected horses show fever, depression, nasal discharge and cough with complete remission of clinical signs taking around 10–12 days. It is still possible to isolate the virus and observe clinical signs in horses in spite of them being appropriately and regularly vaccinated, because equine influenza vaccines reduce clinical signs and virus excretion after infection. Recently, equine influenza virus has been isolated from unvaccinated horses or from horses with an unknown vaccination history (Barthold and others 2011). There have been so-called vaccination breakdowns, where vaccinated horses have developed clinical signs of influenza similar in magnitude and duration to those described for non-vaccinated horses (Newton and others 2006, Martella and others 2007, Barbic and others 2009). Vaccination breakdown is a term used to describe sudden appearance of disease in an individual or herd in which immunity had previously appeared (or been assumed to be) adequate. Failure of immunisation has a number of potential causes including the vaccine (eg, inappropriate storage and/or handling), administration (eg, vaccination schedule, route of administration), host (eg, selection, stress, intercurrent disease, immunocompetence, immune status) and pathogen (antigenic drift, antigenic shift).

There is strict guidance on the requirements for demonstrating vaccine quality, safety and efficacy when an already authorised inactivated equine influenza vaccine is being updated in response to antigenic drift that has been in place for more than a decade (EMA 1998). This was set up on the basis that a regular update of equine influenza strains in vaccines may be necessary every three to five years, based on the slower rate of antigenic drift in equine influenza virus compared to human influenza virus. The studies for, and approval of, an existing inactivated influenza vaccine with an updated strain composition take a number of years, introducing a lag period between the panel of experts' recommendations and the appearance of updated strains in commercially available vaccines. Revised European Medicines Agency (EMA) regulations are currently under discussion with key stakeholders, such as

the animal health industry via the International Federation for Animal Health – Europe (IFAH-Europe) (www.ifahsec.org).

Incorporating new equine influenza strains recommended by the ESP into existing vaccines takes several years. Thus, when an updated equine influenza vaccine is granted marketing authorisation, its strain composition may no longer be completely compatible with the most recent recommendation of the ESP. It is therefore important that the efficacy of current vaccines be assessed against emerging strains under appropriate controlled conditions in horses since this is the best way to assess equine influenza vaccine efficacy (Barthold and others 2011). Therefore, the present randomised, controlled study examined whether vaccination with a non-updated vaccine containing subunits of H7N7 and European and American lineage H3N8 equine influenza plus tetanus vaccine protects equines against challenge with a Florida sublineage clade 2 strain representative of field strains found currently in Europe at onset of immunity (3 weeks after basic vaccination).

Materials and methods

The study was approved by the local animal ethics committee of MSD Animal Health in Boxmeer, The Netherlands.

Animals

Twelve Shetland ponies aged 2–17 years, not vaccinated previously and seronegative for equine influenza based on HI assay were kept separately at pasture. No other equines were housed on or in the vicinity of the farm. The horses were assigned at random to the vaccinated or non-vaccinated (control) group, based on their microchip number. Seven of the horses were vaccinated intramuscularly in the neck on two occasions four weeks apart. Two weeks after the second vaccination (week 6) these horses and five non-vaccinated horses (controls) were transferred to an isolation unit and allowed to acclimatise for one week before experimental challenge (week 7) with virulent equine influenza virus.

Vaccination

The 1 ml vaccine (Equilis Prequenza Te, MSD Animal Health, Intervet International bv, Boxmeer, The Netherlands) contains purified antigens of the A/eq/Prague/56, A/eq/Newmarket/1/93, and A/eq/Newmarket/2/93 strains, tetanus toxoid and Matrix-C adjuvant.

Challenge

Horses were challenged as described previously (Ragni-Alunni and van de Zande 2006, Ragni-Alunni and van de Zande 2008, van de Zande and Ragni-Alunni 2008, Heldens and others 2009, Heldens and others 2010) using the Florida sublineage clade 2 A/eq/Richmond/1/07 strain kindly provided by the Animal Health Trust, Newmarket (UK). Each horse was challenged individually by exposure to an aerosol via a nebuliser that delivered a 50 per cent egg infective dose (EID_{50}) of equine influenza virus of $10^{8.5}$ per horse.

Clinical signs

The horses underwent a physical examination on the day before challenge, the day of challenge, and daily for 14 days after challenge. Assessments were made by trained personnel using a standardised scoring system developed more than a decade ago (Heldens and others 2010) with the scorer blinded to the vaccination status of the animals. Rectal temperature was measured and a score for severity awarded (0–4, Table 1). Clinical signs were assessed and a score for severity awarded (Table 1), with a maximal possible score of 30.

Serology

Blood samples were taken from the jugular vein. The blood was allowed to clot and serum was separated and heat-inactivated for 30 minutes at 56°C before storing at −20°C until analysis. HI antibody titres against the vaccine strains and virus neutralisation (VN) antibody levels against the A/eq/Richmond/1/07-challenge strain were determined.
After kaolin and tween ether treatment of the sera, HI titres against haemagglutinin of influenza strains A/eq/Prague/56, A/eq/Newmarket/1/93 and A/eq/Newmarket/2/93 were determined using a validated micro-HI assay against chicken erythrocytes. The assay

TABLE 1: Scoring key for rectal temperature and the severity of clinical signs of equine influenza in horses					
	0	1	2	3	4
Rectal - °C temperature	<38.5	38.5 to 39.0	39.1 to 39.5	39.6 to 40.0	>40.0
General health	Appears normal	Malaise, depression, normal appetite	Malaise, depression, reduced appetite, dehydration	–	Anorexia
Respiratory character	Normal	–	Hyperpnoea	–	Dyspnoea
Cough	None	2–5 times in 10 minutes	6–20 times in 10 minutes	>20 times in 10 minutes	–
Ocular discharge	None	Lacrimation	Mild, mucopurulent	–	Marked, mucopurulent
Conjunctivitis	None	–	Mild	–	Severe
Nasal discharge	None	Serous	Mild, mucopurulent	–	Marked, mucopurulent
Sneezing	None	2–5 times in 10 minutes	6–20 times in 10 minutes	>20 times in 10 minutes	–

was performed by a single laboratory, by the same personnel using the same reagents. HI titres were expressed as log2 values of the reciprocal of the highest serum dilution that still had the capability to completely neutralise the virus.

VN antibody titres against the challenge strain were determined by the constant virus-varying antibody level method. Serial dilutions of sera were mixed with an equal volume of equine influenza virus with a concentration of 10^3 EID_{50}/ml. After incubation for two hours at 37°C, 0.2 ml of each virus/serum mixture was inoculated into specific pathogen-free 9 to 11-day-old embryonated hens' eggs (two eggs per dilution). After incubation for three days at 37°C, the haemagglutinating activity of the allantoic fluid of each egg was assessed, with a positive reaction indicating virus replication and a negative reaction indicating VN (Anon 2012). VN titres were expressed as log2 values of the reciprocal of the highest serum dilution that still had the capability to completely neutralise the virus.

Virus excretion

Nasopharyngeal swabs were taken from each horse daily after challenge to detect excreted virus, an indicator of viraemia. The infectious virus content of the swab was titrated in embryonated hens' eggs (Anon 2012).

Statistical analysis

Statistical analysis was performed using SAS V.9.1 (SAS Institute, Cary, North Carolina, USA).

Linear mixed models were used for rectal temperature and amount (titre) of virus excretion over time. Analysis of variance (ANOVA) was used for the peak increase in rectal temperature from baseline (mean of prechallenge rectal temperature), the AUC of this change, the duration of virus excretion in days and the AUC of virus excreted (titre) versus time (calculated using the linear trapezoidal rule). Generalised Estimating Equations (GEE, multinomial distribution and cumulative logit as link function) were used for the clinical and total clinical scores

(rectal temperature score plus clinical score) over time were analysed by means of (Agresti 2002) and the OR and associated 95% CI were calculated. Where appropriate (linear mixed models, GEE), repeated measures in a subject were taken into account. GEE was also used for virus isolation (by sample). Logistic regression using exact methods was used for virus isolation (by horse, positive or negative).

The level of significance α was set at 0.05, and tests were two-sided.

Results

Vaccination

None of the vaccinated horses showed pain upon palpation of the injection site after vaccination. There were no significant changes in rectal temperature following vaccination in any of the horses.

Rectal temperature and clinical signs

After challenge, all the control horses had an increase of rectal temperature for six days (median, range 1–11 days), peaking at 40.3°C (median, range 40.3–40.8°C) on day 2 postchallenge (Fig 1). Rectal temperature exceeded 40.0°C on two days in two control animals. Rectal temperature scores were 0 on all but 3/98 occasions in the vaccinated horses; on day 3 postchallenge, three vaccinated horses had a rectal temperature score of 1 (38.6°C, 38.8°C, 39.0°C). The difference in rectal temperature between the two groups was statistically significant whether rectal temperature over time (P=0.0076), peak rectal temperature (1.8°C difference, P=<0.0001) or AUC (P=0.0093) were compared.

Typical signs of equine influenza (including marked mucopurulent nasal discharge and a hard, dry cough) were seen in the controls from day 3 to day 14 postchallenge (Table 2). Cough was recorded for one vaccinated horse only (Table 2). The clinical scores were significantly lower in the vaccinated horses than in the controls. Total clinical score peaked on day 9 (mean 2.2) in the vaccinated group, and on day 7 (mean 7.4) in the control group (Table 2, Fig 2). Vaccination
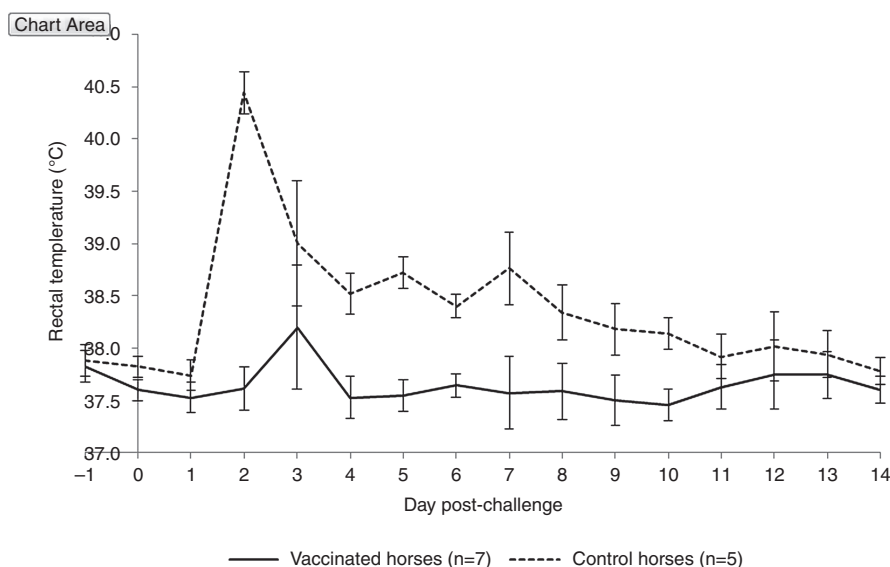

FIG 1: Rectal temperature (mean, SD) in vaccinated and control horses postchallenge

TABLE 2: Daily scores for rectal temperature and the severity of clinical signs of equine influenza in vaccinated and control horses

Day	Horse #	Vaccinates							Controls					Day	Vaccinates							Controls				
		2	3	6	10	14	16	17	5	7	13	18	19		2	3	6	10	14	16	17	5	7	13	18	19
−1	GH	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0
	RES	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	2	1	0	2	2
	OC	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	2	1	0	0	0
	NAS	0	0	0	0	1	1	0	0	1	0	0	1		1	4	0	1	1	0	1	4	4	4	4	4
	TEMP	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	2	1	0	3	1
	TOTAL	0	0	0	0	1	1	0	0	1	0	0	1		1	4	0	1	1	0	1	10	7	4	9	7
0	GH	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0
	RES	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	3	2	0	0	1
	OC	0	0	2	0	2	0	0	2	0	0	0	0		0	0	2	0	2	0	0	1	0	0	0	0
	NAS	0	1	0	0	1	0	0	0	0	0	1	1		1	4	1	1	2	1	1	4	4	4	0	2
	TEMP	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	3	1	0	0	0
	TOTAL	0	1	2	0	3	0	0	2	0	0	1	1		1	4	3	1	4	1	1	11	7	4	0	3
1	GH	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	0	0
	RES	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	3	1	0	0	0
	OC	0	0	2	0	1	0	0	2	0	0	0	0		0	0	2	0	2	2	2	2	0	0	0	0
	NAS	1	0	0	2	1	0	0	0	1	0	1	0		1	2	1	0	1	1	2	4	4	1	2	1
	TEMP	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	3	0	0	0	0
	TOTAL	1	0	2	2	2	0	0	2	1	0	1	0		1	2	3	0	3	3	4	12	5	1	2	1
2	GH	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0
	RES	0	0	0	0	0	0	0	0	0	0	0	0		0	0	0	0	0	0	0	3	0	0	0	2
	OC	0	0	0	0	0	0	0	2	0	0	0	0		0	0	0	0	2	0	1	2	0	0	0	0
	NAS	1	0	0	0	1	0	0	1	2	2	0	1		0	1	1	0	1	2	4	4	2	2	2	2
	TEMP	0	0	0	0	0	0	0	4	4	4	4	4		0	0	0	0	0	0	0	2	0	0	0	1
	TOTAL	1	0	0	0	1	0	0	7	6	6	4	5		0	1	1	0	3	2	5	11	2	2	2	5
3	GH	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	1	0	0	0	0
	RES	0	0	0	0	0	0	0	1	0	0	0	0		0	0	0	0	0	0	0	0	1	0	0	0
	OC	0	0	0	0	0	0	0	2	0	0	0	0		0	0	0	0	2	0	0	4	0	0	0	0
	NAS	1	0	0	0	0	0	0	2	4	0	0	1		0	1	0	0	1	1	2	4	2	1	2	2
	TEMP	0	1	0	0	0	1	1	1	1	0	4	4		0	0	0	0	0	0	0	2	0	0	0	0
	TOTAL	1	1	0	0	0	1	1	6	5	0	4	4		0	1	0	0	3	1	2	11	3	1	2	2
4	GH	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	1	0	0	0	0
	RES	0	0	0	0	0	0	0	1	1	0	0	0		0	0	0	0	0	0	0	3	0	0	0	0
	OC	0	4	0	0	0	0	0	4	2	0	0	0		0	0	0	0	0	0	2	2	0	0	0	0
	NAS	1	2	0	0	1	0	2	2	4	2	4	0		1	0	1	1	2	1	2	4	1	0	1	0
	TEMP	0	0	0	0	0	0	0	0	2	0	2	1		0	0	0	0	0	0	0	3	0	0	0	0
	TOTAL	1	6	0	0	1	0	2	7	9	2	6	1		1	0	1	1	2	1	4	13	1	0	1	0
5	GH	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0
	RES	0	0	0	0	1	0	0	3	1	0	1	1		0	0	0	0	0	0	0	3	0	0	0	0
	OC	0	0	0	0	0	2	0	2	0	0	0	0		0	0	2	0	0	2	0	2	0	0	0	0
	NAS	1	2	0	1	1	1	1	2	4	2	2	2		1	1	1	1	1	1	2	4	0	0	1	1
	TEMP	0	0	0	0	0	0	0	3	0	0	3	1		0	0	0	0	0	0	0	2	0	0	0	0
	TOTAL	1	2	0	1	2	3	1	10	5	2	6	4		1	1	3	1	1	3	2	11	0	0	1	1
6	GH	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0
	RES	0	0	0	0	0	0	0	1	1	0	0	1		0	0	0	0	0	0	0	2	0	0	0	0
	OC	0	0	0	0	2	0	2	2	0	0	2	0		0	0	2	0	1	0	0	4	0	0	2	0
	NAS	1	0	0	1	1	1	1	4	4	4	4	4		0	0	0	1	1	0	1	4	0	0	1	0
	TEMP	0	0	0	0	0	0	0	1	0	0	1	1		0	0	0	0	0	0	0	0	0	0	0	0
	TOTAL	1	0	0	1	3	1	3	8	5	4	7	6		0	0	2	1	2	0	1	10	0	0	3	0

GH, general health; NAS, nasal discharge/sneezing; OC, ocular discharge/conjunctivitis; RES, respiratory character/cough; TEMP, rectal temperature.
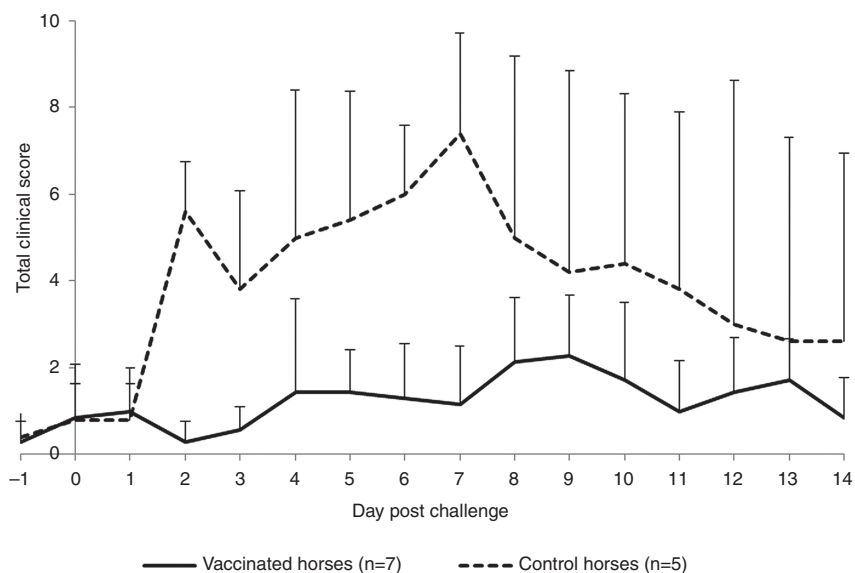

FIG 2: Total clinical score (mean, SD; maximum possible score 28)
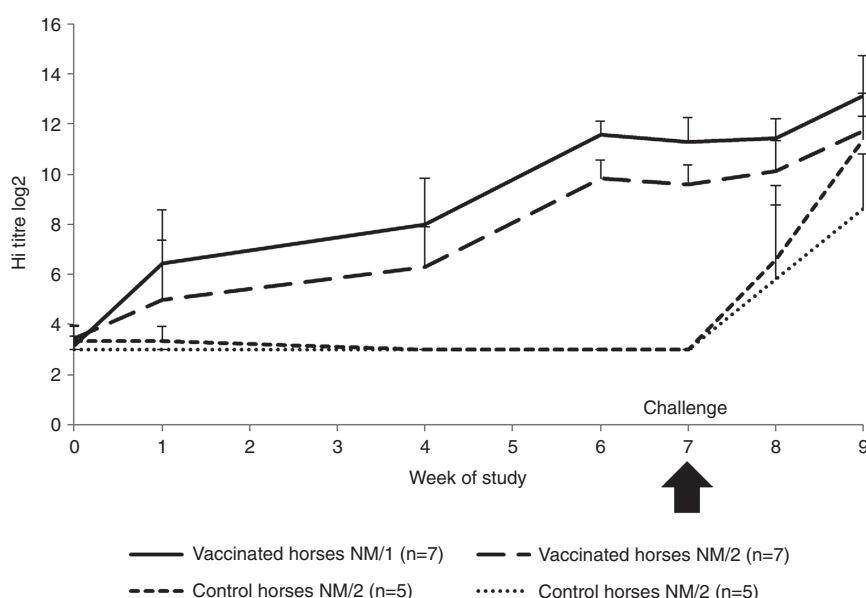
FIG 3: Haemagglutination inhibition antibody titre for Newmarket (NM)/1 and /2 in vaccinated and control horses

significantly reduced clinical score (P=0.0345) and total clinical score (rectal temperature plus clinical score, P=0.0180).

Serology

One of the vaccinated horses showed an anamnestic serological response one week after first vaccination, and was excluded from the statistical analysis. The antibody response peaked at two weeks after the second vaccination for all three strains. All but two of the vaccinated horses seroconverted (increase of at least 2 log) postchallenge meaning that the high antibody titre following vaccination did not prevent infection. In week six, the mean HI titre against A/eq/Newmarket/1/93 in the vaccinated horses was 11.6 (log2) and the mean VN titre against A/eq/Richmond/1/07 was 11.8. On the day of challenge (week 7), the mean HI titre against A/eq/Newmarket/1/93 in the vaccinated horses was 11.3 while all the non-vaccinated horses were seronegative (Fig 3). Two weeks postchallenge, all the non-vaccinated horses were seropositive for A/eq/Newmarket/1/93 and A/eq/Newmarket/2/93 confirming that the challenge virus had infected all the control horses (Fig 3).

Virus isolation

Virus was isolated from four of the seven vaccinated horses on 1 (n=2, days 2 and 5), 2 (n=1, days 1 and 2) or three days (n=1, days 2, 5 and 7), with an average duration of virus excretion of 1.0 days and a mean peak value of $10^{1.3}$ EID_{50}/mL on day 2 postchallenge (Fig 4). Virus was isolated from all the control horses for 5 (n=1), 6 (n=1) or 7 days (n=3) between day 1 and day 7 postchallenge, with an average
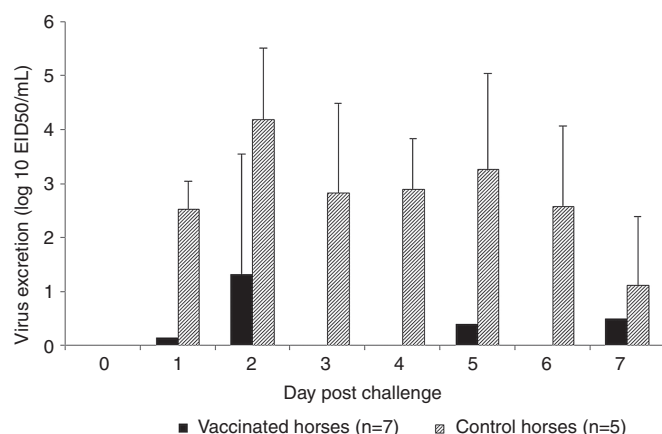
of 6.4 days of virus excretion (Fig 4). Peak values were found on days 2 (mean $10^{4.2}$ EID_{50}/ml) and 5 ($10^{3.3}$ EID_{50}/ml) postchallenge (Fig 4). Vaccination had a significant effect (OR 0.0040 95% CI (0.0002 to 0.0732), P=0.0017) on whether a horse was positive or negative for virus excretion. Similarly, the effect of the vaccination on the extent of virus excretion was significant (P=0.0006) as was the effect on the duration of virus excretion (P=<0.0001) and the total amount of virus excreted (AUC, P=0.0006).

Discussion

Previous studies have assessed the efficacy of this vaccine (non-updated (H7N7, H3N8 Eurasian and American lineages) Equilis Prequenza Te) against Florida sublineage clade 1 strains A/eq/South Africa/4/03 and A/eq/Ohio/03 and a Florida sublineage clade 2 strain A/eq/Newmarket/5/03 three weeks after a basic vaccination course (two vaccinations) and against the Kentucky strain A/eq/Kentucky/9/95 four weeks and five months after a basic vaccination course, and one year after a third vaccination (Ragni-Alunni and van de Zande 2006, Ragni-Alunni and van de Zande 2008, van de Zande and Ragni-Alunni 2008, Heldens and others 2009, Heldens and others 2010). The present challenge study assessed the efficacy of this vaccine against A/eq/Richmond/1/07 a Florida sublineage clade 2 strain that is representative of the most recent epidemiological evolution of the equine influenza virus.

Vaccination did not induce detectable local or systemic adverse reactions in the vaccinated horses. All the vaccinated horses developed high antibody titres against all four equine influenza antigens, the three vaccine strains (HI titres) and the challenge strain (VN titre). Only the titres corresponding to the American-type clade 2 (A/eq/Richmond/1/07) strain will be discussed further here.

Serum antibody concentrations are usually quantified as a measure of specific immunity to infectious agents and often correlate well, depending on the virus, with protection from the occurrence of disease or decreased severity of clinical signs. In experimental studies, HI and single radial haemolysis (SRH) are considered suitable immunochemical methods for determining antibody titre, and have equivalent sensitivity for measurement of antibody to influenza A viruses (Wood and others 1994). HI has been used extensively in experimental studies because it is technically simple. It has been used extensively to evaluate antibody responses to vaccination with this vaccine (Ragni-Alunni and van de Zande 2006, Ragni-Alunni and van de Zande 2008, van de Zande and Ragni-Alunni 2008, Heldens and others 2009, Heldens and others 2010) and has good reproducibility within our laboratory. SRH is more technically challenging, but is preferred in the field based on less variability between laboratories (Wood and others 1994) and correlation with clinical and virological protection in

FIG 4: Virus excretion (mean, SD) following challenge of vaccinated and control horses

the field (Newton and others 2000). Neutralising antibodies, depending on the virus, prevent infection or the release of virus from infected cells, and are thought to provide optimum protection to challenged animals. For influenza A viruses, the majority of neutralising antibodies are produced against haemagglutinin, and some against the neuraminidase. However, VN is a rather laborious test and was only performed on a single occasion in the present study, and was of a similar magnitude to HI. It has been shown previously that equine influenza virus antibody titres measured using SRH and HI assays were highly correlated (Wood and others 1994) as well as with VN titres (Morley and others 1995).

Equine influenza vaccines reduce clinical signs and virus excretion, an indicator of viraemia, after infection. In the present study, the magnitude and duration of rectal temperature increases, and clinical signs after experimental challenge were significantly reduced in vaccinated compared to control horses, as were the total amount and duration of virus excretion. The total clinical score in the control group peaked at an average of 7.4 and, while this was only a fraction of the total possible score of 30, was higher than has been seen in previous studies with the same vaccine (see eg, Heldens and others 2010) and significantly different than the vaccinated group. Clearly, there is a difference in the magnitude and duration of clinical signs, depending on the challenge virus (Ragni-Alunni and van de Zande 2006, Ragni-Alunni and van de Zande 2008, van de Zande and Ragni-Alunni 2008, Heldens and others 2009, Heldens and others 2010), reflecting differences between challenge strains, since all these previous studies and the present study use that same target challenge dose of around $10^{8.0}$ EID$_{50}$. Experimental challenge by aerosol delivered individually which produces tiny droplets containing relatively large amounts of virulent live virus directly into the respiratory tract, reflects a more standardised challenge of each individual than would occur during natural infection under field conditions, and allows comparison with non-vaccinated control animals. There were significant differences between vaccinated and control horses even under these stringent experimental challenge conditions. The data show that the combination of the influenza strains and adjuvant in this vaccine was able to reduce clinical signs and virus excretion after infection with a recent, heterologous equine influenza virus strain. This was demonstrated when antibody titres were high, three weeks after second vaccination. The vaccine produces high antibody titres, and this can help to improve protection against heterologous viruses (Yates and Mumford 2000). However, the duration of immunity against heterologous challenge with recent strains remains to be proven. Virus isolation was using the gold standard (sensitive and specific) methodology using hens' eggs. Reverse transcriptase PCR (rtPCR) is more rapid and has been shown to be more sensitive than the gold standard methodology (Quinlivan and others 2005). This means that it can be used to demonstrate infection (by detection of viral nucleic acid in respiratory secretions) particularly where rapid results are required. It may be possible to demonstrate viral nucleic acid on additional days, but this is only demonstrated as viable virus using hens' eggs. As such, rtPCR does not offer an advantage over traditional methodology in controlled studies of this type.

All equine influenza vaccines marketed currently in Europe rely on adjuvants, which are added to a vaccine to stimulate the host's immune response to the target antigen(s). Different types of adjuvant have slightly different modes of action. Matrix-C is composed of a specific subfraction of saponins which, together with cholesterol and phospholipids, form spherical open cage-like structures (typically 40 nm in diameter). Matrix-C is unique, and its specific composition is hydrophilic and lipophilic allowing for rapid uptake by cells of the immune system (Pearse and Drane 2005). Within a few hours after vaccination, the adjuvant has moved from the vaccination site to lymph nodes draining it, and from there on to the spleen and bone marrow where most of the immune cells reside (Pearse and Drane 2004, Pearse and Drane 2005). This type of adjuvant has been shown to stimulate antibody and a cellular immune response in mammals, including the horse (Pearse and Drane 2004, Paillot and Prowse 2012).

Unlike in seasonal human influenza vaccines, the provision of protection against equine influenza is due to a combination of antigen content, strain and adjuvant (Korsman 2006). Vaccine strategies

against influenza have traditionally focussed on generating robust antibody responses against the surface glycoproteins, particularly haemagglutinin (Cullinane and Newton 2013). Recent outbreaks have shown that other factors including strain pathogenicity are also involved. Moreover, the development in horses of clinical signs of influenza appears not be a direct result of virus replication but related to the duration and level of cytokine (interferon, interleukin-6) responses (Wattrang and others 2003), as in other species (Daly and others 2011). While a low antibody titre to haemagglutinin can indicate whether a vaccination breakdown may occur, it gives no indication of the potential of an influenza strain to cause severe disease or to become widespread (Daly and others 2011, Couch and others 2013). That requires knowledge of the interaction between the host and the infecting virus as well as of the whole immune response to the target antigens in vaccine.

Protecting horses from equine influenza is an integral and economically important part of the equine industry worldwide (Timoney 1996, Paillot and others 2006). Achieving and maintaining so-called herd (or population) immunity is challenging. Moreover, equine influenza viruses continue to evolve and, thus, evade even the primed immune system. Thus, outbreaks of equine influenza continue to occur, even in vaccinated horses due to multiple factors impacting disease prevention, and the complexity of obtaining and maintaining so-called herd (or population) immunity (Daly and others 2011). The data presented here show that the combination of the influenza antigen content, strains and adjuvant in this vaccine were able to reduce clinical signs and virus excretion after infection with a recent equine influenza virus strain, at least in the short term, and when antibody titres were high. Further research is needed to fully understand the specific properties of Equilis Prequenza Te that contribute to this vaccine's cross-protective capacity.

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