



# Intramuscular vaccination with Strangvac is safe and induces protection against equine strangles caused by *Streptococcus equi*



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## ABSTRACT

The equine disease strangles, caused by *Streptococcus equi*, remains a major cause of welfare and economic cost to the global horse industry. Here we report the safety, immunogenicity and efficacy of a novel multi-component chimeric fusion protein vaccine, called Strangvac, when administered to ponies via the intramuscular route. Across the four studies, Strangvac was safe and induced robust antibody responses towards the vaccine components in blood serum and the nasopharynx, which were boosted by revaccination up to 12 months after a primary course of 2 vaccinations 4 weeks apart. The vaccine response did not cross-react with a commercial strangles iELISA, which identifies horses that have been exposed to *S. equi*, demonstrating that it was possible to differentiate infected from vaccinated animals (DIVA). Following challenge with *S. equi* strain 4047 (Se4047), all 36 control ponies that had received an adjuvant-only placebo vaccine developed clinical signs of strangles. In contrast, intramuscular vaccination with Strangvac protected ponies significantly from challenge with Se4047 at two weeks (5 of 16 ponies protected (31%),  $P = 0.04$ ) and two months (7 of 12 ponies protected (58%),  $P = 0.0046$  (including pooled control data) after second vaccination. Optimal protection (15 of 16 ponies protected (94%),  $P < 0.0001$ ) was observed following challenge at two weeks post-third vaccination. Our data demonstrate that Strangvac is safe, has DIVA capability and provides a rapid onset of protective immunity against strangles. We conclude that Strangvac is a valuable tool with which to protect horses from strangles, particularly during high-risk periods, whilst maintaining the mobility of horse populations as required by the global equine industry.

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## 1. Introduction

Strangles, caused by the Lancefield group C pathogen *Streptococcus equi*, subsp. *equi*, (*S. equi*) remains an endemic disease of horses around the world with an estimated 600 outbreaks occurring in the UK each year [1]. The disease has important economic impact through the temporary closure of facilities, cancelled events and veterinary expenses, with some outbreaks costing over £225,000. A key factor in the transmission of *S. equi* from one population of horses to another, is its ability to establish persistent infections in the guttural pouches or sinuses of a proportion of horses that have recovered from acute disease [2,3]. These

persistently infected carriers exhibit no clinical signs of disease but can shed and transmit *S. equi* to other animals with which they come into direct or indirect contact. Persistently infected horses can be identified and treated using guttural pouch endoscopy and by commercially available qPCR tests that enable the detection of *S. equi* [4,5]. Quantification of the antibody response to two fragments of *S. equi* surface proteins, SEQ\_2190 and SeM, by iELISA has a high sensitivity and specificity for the detection horses that have been exposed to *S. equi* [6]. The test enables veterinarians to minimise the number of horses that require further testing by guttural pouch endoscopy and lavage. However, it is not usually practical to screen populations of horses with guttural pouch endoscopy or serology each time they return from an event where they might have been inadvertently exposed to *S. equi*. Therefore, there is a pressing need to develop a safe and effective vaccine, which is

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compatible with diagnostics tests such that it is possible to differentiate infected from vaccinated animals (so-called DIVA).

Natural infection with *S. equi* typically provides protection from disease for an estimated 5 years [7] and live attenuated vaccines have been developed based on this principle, namely Pinnacle (licensed for use in the USA and New Zealand) [8] and Equilis StrepE (licensed for use in the European Union) [9]. These vaccines are given intranasally and sub-mucosally, respectively, but they have been associated with adverse reactions and clinical signs of strangles and they are not DIVA compatible [10–13]. We have previously published the results on the development of a novel DIVA compatible recombinant fused protein vaccine, Strangvac, which was administered via the intranasal and subcutaneous routes and conferred significant levels of protection against experimental challenge with a virulent strain of *S. equi* [14,15]. Here we present data from 4 studies in ponies that measured the safety, immunogenicity, onset and duration of immunity following basic vaccination and the restoration of immunity following re-vaccination with Strangvac when administered by intramuscular injection.

## 2. Materials and methods

### 2.1. Bacterial strains used in the study

The recombinant *S. equi* antigens used in Strangvac were derived from *S. equi* strain 1866 (Se1866) [16]. Se4047 was used as the challenge strain in this study [17]. For challenge experiments in ponies, Se4047 was grown overnight in Todd Hewitt broth containing 10% foetal calf serum (THBS) in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C, diluted 40-fold in fresh pre-warmed THBS, further cultivated and harvested at an OD<sub>600nm</sub> of 0.3 as previously described [14,15].

For gene cloning and for expression of recombinant fusion proteins *E. coli* strain BL21 was grown at 37 °C in Bacto Tryptone with Yeast extract medium supplemented with kanamycin (final conc. 50 µg/ml).

### 2.2. Construction and purification of fusion proteins

Strangvac is based on CNE, ScIC, ScIF, ScII and EAG (fused as CCE), SEQ\_0402 and SEQ\_0256 (fused as Eq85) and IdeE generated from clones where nucleotide sequences were synthesized to obtain optimal codon choice for *E. coli* and purified as previously described [15]. These proteins, with the exception of IdeE, are predicted to be localised on the bacterial surface and antibodies against them are assumed to be opsonic and block bacterial adherence to tissue surfaces. The choice of components in Strangvac is based on previous studies [14]. Briefly, for all proteins a combination of ammonium sulphate precipitation, hydrophobic interaction chromatography and ion exchange chromatography was used for purification [15].

### 2.3. Immunization of ponies

Ponies between 6 and 12 months of age (n = 56) were vaccinated with Strangvac in four separate experiments, EXP I, EXP II, EXP III and EXP IV, where they were divided into groups as shown in Table 1. Three additional groups of ponies of the same age (n = 16 + 4 + 16, respectively), one per experiment for EXP II, EXP III and EXP IV, were vaccinated with a placebo control vaccine containing only adjuvant. Ponies were maintained at pasture for the duration of the vaccination phases. Ponies were vaccinated by intramuscular administration of 2 ml of Strangvac. Each intramuscular dose contained 106 µg of CCE, 42 µg of Eq85, 32 µg of IdeE and 326 µg of Matrix C a saponin derived adjuvant, in a 2 ml volume (Novavax

**Table 1**

Number of ponies vaccinated with Strangvac or placebo per experiment.

Experiment	Number of ponies vaccinated	
	Strangvac vaccinated	Placebo vaccinated
EXP I	12	0
EXP II	16	16
EXP III	12	4
EXP IV	16	16

AB, Uppsala, Sweden). Vaccination schedules are shown in Fig. 1. The vaccination status of all ponies in EXP II, EXP III and EXP IV was blinded to study personnel throughout the experimental phases.

### 2.4. Nasal swab and blood sampling

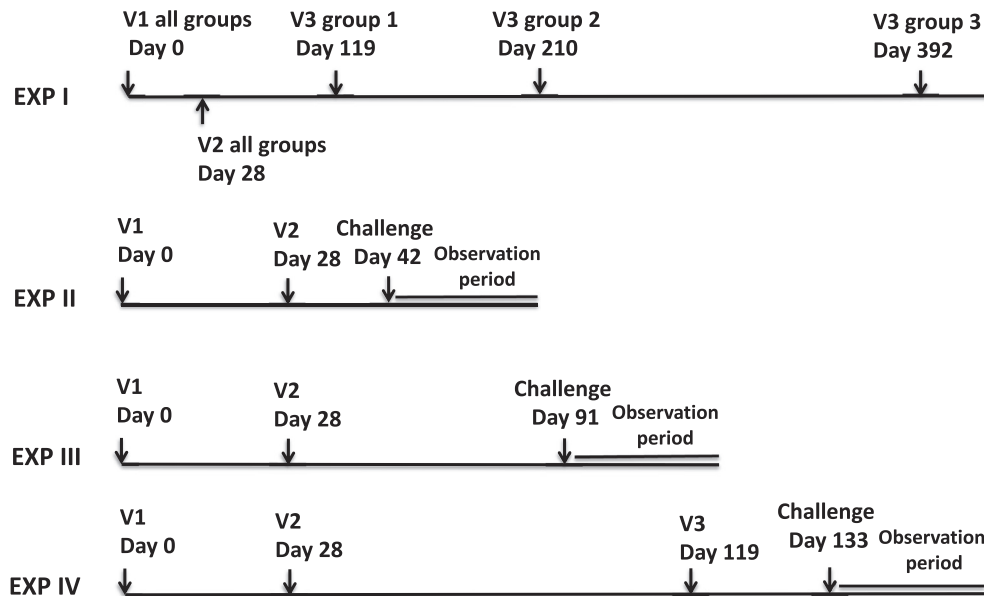
Nasal swabs and blood samples were taken pre-vaccination, pre-challenge and three times a week post-challenge. The nasal swab comprised a rayon pad on the end of a 30 cm long thin plastic tube, which was inserted into the nares of the pony to be sampled until a swallowing reflex was induced, indicating that the swab had touched the back of the nasopharynx. The plastic tube was cut and the swab pad placed into 3 ml of PBS, vortexed and then centrifuged at 1750g. The supernatant was decanted and stored at –70 °C. Blood samples, were drawn from the jugular vein and allowed to clot at room temperature for 2 h. The serum was then removed and stored at –20 °C.

### 2.5. Quantification of the immune response to vaccination

Serum samples and nasal wash samples were taken at regular intervals to measure IgG responses by conventional iELISA performed as previously described [14]. Samples were serially diluted two-fold and the log<sub>10</sub> value of the dilution required to obtain an absorbance value below a cut off threshold of 1.5 (for serum samples) or 1.0 (for nasal washes) was determined. The amount of IgG towards the SEQ\_2190 and SeM protein fragments used in a commercial dual iELISA for the detection of horses exposed to *S. equi* was determined as previously described [6].

### 2.6. Experimental infection of ponies and clinical assessment

Ponies were divided into groups at random and moved to one of four rooms within a purpose-built containment unit three days prior to challenge. A 2 ml culture of Se4047 containing  $5 \times 10^7$  cfu was sprayed into each nostril ( $1 \times 10^8$  cfu per pony in total). Clinical signs of infection, rectal temperature, and swelling of submandibular lymph nodes (SMLN) were followed daily for four weeks or until the time of euthanasia on reaching the humane endpoint of pyrexia and a preference for haylage and water over dried pelleted food. As a sign of inflammation, fibrinogen levels and neutrophil counts were determined, elevated fibrinogen level and neutrophil count were defined as a value exceeding the upper 2.5 percentile of the values obtained before challenge [14,18]. Post-mortem examination was performed on all ponies following euthanasia. The severity of disease and pathology was quantified according to a scoring system described previously [18] and summarized below: Retropharyngeal or submandibular lymph node abscess = 15, Retropharyngeal or submandibular lymph node microabscess = 10, empyema of guttural pouch = 5, scarring of guttural pouch = 5, enlarged lymph node = 1, Follicular hyperplasia of guttural pouch = 1. The amount of *S. equi* DNA present on swabs taken post-mortem was determined by qPCR as previously described [4].



**Fig. 1.** Immunization schedule. Ponies were vaccinated as indicated, followed by experimental infection two weeks (EXP II and EXP IV) or two months (EXP III) after the last vaccination. Challenged ponies were observed for three weeks or until the time of euthanasia. V1, V2 and V3 indicate the time of first, second and third vaccinations.

## 2.7. Statistical methods

The Mann Whitney test was used to test the significance of post-mortem scores. A two-way ANOVA was used to calculate the significance between pooled post-mortem scores of control ponies with vaccinates for EXP III. The Wilcoxon rank sum test was used to compare temperatures, fibrinogen levels, and neutrophil counts. The Last Observation Carried Forward method was used to account for ponies euthanased on welfare grounds prior to day 8 post-challenge on the assumption that their clinical signs of disease would have become more pronounced. The Fisher's exact probability test was used for the comparison of frequencies over the whole experimental time course. The Kruskal-Wallis test was used to determine statistical differences between groups of control ponies.

## 2.8. Ethical considerations

This work was conducted under the auspices of a Home Office Project License and following ethical review and approval by the Animal Health Trust's Animal Welfare and Ethical Review Body (RPP 01\_08).

## 3. Results and discussion

### 3.1. Strangvac was safe for intramuscular injection

Intramuscular vaccination was used throughout these studies since it is quick, easy and preferred by veterinarians. Table 1 shows that over the course of the four studies, 56 ponies received a total of 140 doses of Strangvac and 36 ponies received a total of 87 doses of the placebo vaccine, which contained adjuvant only. The vaccinations were well tolerated throughout the studies with a proportion (12/12, 9/32, 12/16 and 18/32, for Experiments I, II, III and IV respectively) of ponies in each study showing mild transient clinical signs that resolved completely without veterinary intervention between one and five days post-vaccination. There was no difference in the incidence of these mild injection site reaction indicators (heat, pain and swelling) between Strangvac- and placebo control-vaccinated ponies after the first vaccination (Table 2).

However, the incidence of mild injection site reaction indicators was significantly more frequent in ponies that had been vaccinated with Strangvac when compared with those vaccinated with the placebo control vaccine after the second and third vaccinations (Table 2). Therefore, the occurrence of transient heat, swelling and pain at the injection site likely reflect the development of an immune response to the specific protein components within Strangvac. No adverse events related to the administration of Strangvac or placebo control vaccines occurred; only mild side effects listed above were noted.

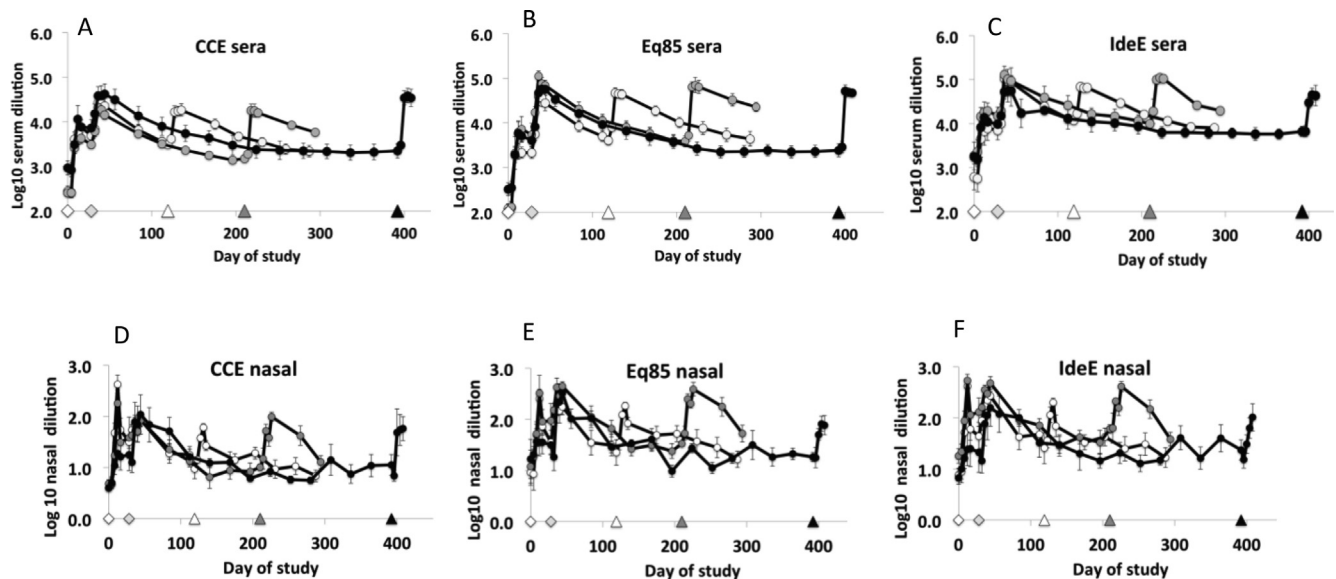
### 3.2. Strangvac was immunogenic

The vaccination schedules for the studies and numbers of ponies used are shown in Fig. 1 and Table 1, respectively. Twelve ponies were vaccinated with Strangvac on days 0 and 28 in Experiment I. Four of these ponies received a booster vaccination on day 119 (13 weeks post-second vaccination, group 1), four on day 210 (26 weeks post-second vaccination, group 2), and the remaining four ponies were boosted on day 392 (52 weeks post-second vaccination, group 3) (Fig. 2). Antibody responses to all three vaccine components in serum and nasal swab samples were significantly elevated from 8 days post-first vaccination. We have previously observed that this reflects an antibody response against each individual component of the fusion proteins (Flock et al., unpublished data). Antibody responses were boosted following the administration of the second vaccination on day 28 and third vaccination whether this was given at 13, 26 or 52 weeks post-second vaccination. A gradual decline of antibody levels over time was observed, which was quicker for nasal than for serum antibodies. However, nasal antibody levels remained elevated for at least 100 days post-second vaccination. These data provide evidence that the administration of a third vaccination at up to 52 weeks post-second vaccination induced antibody responses in sera and nasal secretions that were equivalent to those obtained following the second vaccination. Therefore, immune memory cells, which were induced following the primary course of two vaccinations persisted for at least a period of one year.

Data from the vaccination and challenge studies of Experiments II, III and IV provide further evidence in support of the

**Table 2**  
Combined injection site reaction data during vaccination phases. P values are calculated using the two sided Fisher's exact test. V1, V2 and V3 is first, second and third vaccination, respectively.

		Vaccination point					
Injection site measure	Pony vaccination	V1	P value	V2	P value	V3	P value
Heat > 1	Placebo-vaccinated	4/36	1	1/36	<0.001	1/15*	<0.001
	Strangvac-vaccinated	7/56		18/56		17/28	
Pain	Placebo-vaccinated	6/36	1	3/36	<0.001	1/15*	0.0012
	Strangvac-vaccinated	9/56		24/56		16/28	
Swelling	Placebo-vaccinated	8/36	0.8	3/36	<0.001	2/15*	<0.001
	Strangvac-vaccinated	14/56		27/56		20/28	



**Fig. 2.** Antibody levels in serum and nasal wash samples collected from ponies in EXP I for groups A and B. Log<sub>10</sub> dilution of antibody levels in serum (A. CCE, B. Eq85 and C. IdeE) and nasal wash samples (D. CCE, E. Eq85 and F. IdeE) for vaccination groups 1, 2 and 3 collected during vaccination in study EXP I. Error bars show standard deviation. Open circles: group 1. Grey circles: group 2. Filled circles: group 3. Vaccination time points are indicated by open diamond: V1 groups 1, 2 and 3. Grey diamond: V2 groups 1, 2 and 3. Open triangle: V3 group 1. Grey triangle: V3 group 2. Filled triangle: V3 group 3. The third vaccination was given 13, 26 and 52 weeks after V2 in groups 1, 2 and 3, respectively.

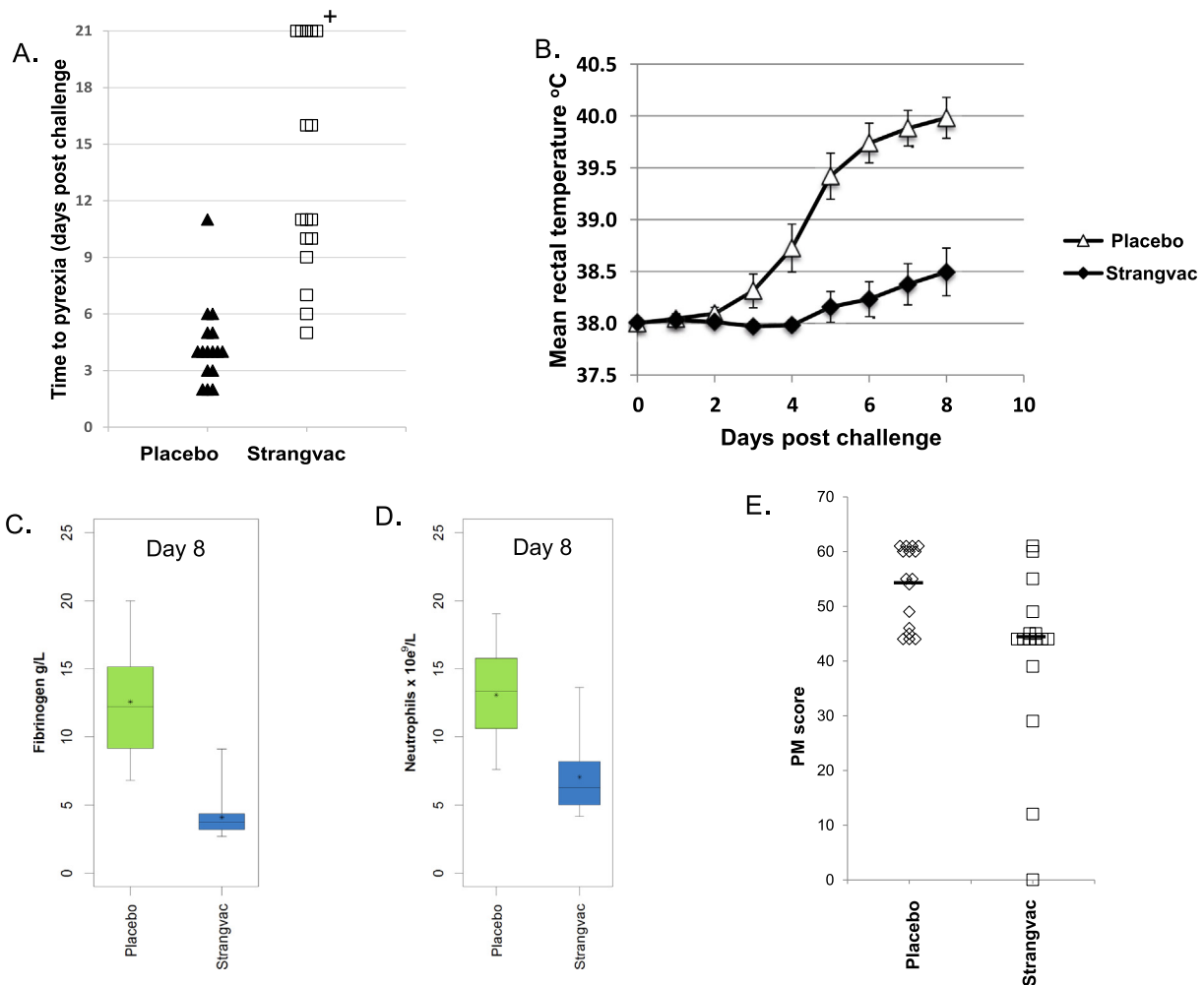
immunogenicity of Strangvac (Fig. S1). High levels of antibodies against the vaccine components were detected in all of the ponies that received Strangvac, with the earliest responses detected 7 days post-first vaccination. The administration of the second vaccination on day 28 consistently boosted antibody responses to the vaccine components of Strangvac-, but not placebo-vaccinated ponies. A similar increase in antibody levels was observed post-third vaccination on day 119 (13 weeks post-second vaccination) in Experiment IV (Fig. S1). We conclude that the intramuscular administration of Strangvac consistently induced the production of antibodies against the vaccine components in all animals. A large proportion of antibodies in mucosa may be due to transudation of plasma IgG through the mucosa. However, when comparing antibody response in sera against mucosal response, no correlation was found (data not shown) implying independent responses in sera and mucosa. The induction of mucosal antibody responses and protection against challenge following the intramuscular vaccination of horses with vaccines against equine herpes virus 1 or equine influenza virus has been demonstrated previously [19,20].

### 3.3. Strangvac induced protection within two-weeks post-second vaccination

In Experiment II, 32 ponies were given two intramuscular injections consisting of either Strangvac or the placebo control vaccine (Fig. 1). Ponies were challenged 14 days post-second vaccination

by the administration of  $1 \times 10^8$  colony forming units (cfu) of Se4047 and monitored closely for the onset of clinical signs of disease.

Five of 16 Strangvac vaccinated ponies reached the end of the study at day 21 post-challenge, the primary end-point, without developing pyrexia (defined as a rectal temperature of 39.0 °C or above that occurs on two out of three consecutive days post-challenge), whilst all 16 of the placebo vaccinated ponies became pyretic  $P = 0.04$ . The mean time to the onset of pyrexia for Strangvac-vaccinated ponies was 13.6 days, compared to a mean of 4.3 days for control ponies ( $P = 0.0001$ ) (Fig. 3A and B). Strangvac-vaccinated ponies also benefitted from a significant reduction in the levels of fibrinogen (Fig. 3C) measured on day 8 post-challenge ( $5.7 \text{ g L}^{-1}$  compared to  $11.8 \text{ g L}^{-1}$  ( $P < 0.0001$ ) and neutrophils counts (Fig. 3D) on day 8,  $7.6 \times 10^9 \text{ cells L}^{-1}$  compared to  $13.6 \times 10^9 \text{ cells L}^{-1}$  ( $P < 0.0001$ ). At day 8 post-challenge, three of 16 Strangvac-vaccinated ponies had developed elevated levels of fibrinogen compared with 14 of 16 controls ( $P = 0.0002$ ) and 2 Strangvac-vaccinated ponies had developed elevated neutrophil levels compared with 12 controls ( $P = 0.001$ ). Although 14 Strangvac-vaccinated ponies developed at least one lymph node abscess, the overall level of pathology observed at post-mortem examination was significantly reduced in those ponies that had received Strangvac ( $P = 0.006$ ) (Fig. 3E). The occurrence of elevated lymph node score ( $P = 0.001$ ), swallow score ( $P = 0.04$ ), feeding score ( $P = 0.0434$ ) and demeanour score ( $P = 0.04$ ) were also



**Fig. 3.** Pyrexia, average rectal temperature, fibrinogen, neutrophil and post-mortem data in Experiment II. (A) Time to onset of pyrexia. (B) Average rectal temperatures up to day 8, error bars indicate 95% confidence intervals. (C) Fibrinogen data for placebo and vaccinated ponies on day 8, boxes represent 10% and 90% percentiles, means are represented by the horizontal bar, medians are represented by an \*, and the error bars represent the standard error. D. Neutrophil data for placebo and vaccinated ponies on days 8, boxes represent 10% and 90% percentiles, means are represented by the horizontal bar, medians are represented by an \*, and the error bars represent the standard error. E. Accumulated post mortem score of placebo and vaccinated ponies, medians are represented by a horizontal bar.

significantly reduced in Strangvac-vaccinated ponies (Supplementary table 1). We conclude that two doses of Strangvac significantly delayed the onset and reduced the severity of disease induced by challenge two-weeks post-second vaccination.

#### 3.4. Strangvac protected ponies for at least two months post-second vaccination

Experiment III was performed to determine the level of protection conferred by two doses of Strangvac two months after the administration of the second vaccination (Fig. 1). Ponies were again challenged by the administration of  $1 \times 10^8$  cfu of Se4047 and monitored closely for the onset of clinical signs of disease.

Seven of twelve ponies vaccinated with Strangvac (58%) did not become pyretic post-challenge compared with all four ponies vaccinated with the placebo control vaccine ( $P = 0.09$ ). Analysis on day 8 showed that Strangvac-vaccinated ponies had lower mean rectal temperatures ( $38.6^\circ\text{C}$  compared to  $40.2^\circ\text{C}$ ), mean ocular score (0.8 compared to 2.5,  $P = 0.1$ ), mean nasal score (0.3 compared to 2.0,  $P = 0.13$ ) and mean coughing score (0.2 compared to 1.0,  $P = 0.004$ ). Strangvac-vaccinated ponies also had lower mean swallow score (0.3 compared to 1.0,  $P = 0.03$ ), mean feeding score (0.3 compared to 1.3,  $P = 0.07$ ) and mean demeanour score (0.2

compared to 1.5,  $P = 0.01$ ) when compared to placebo-vaccinated controls. The mean time to onset of pyrexia for Strangvac-vaccinated ponies was 14.3 days, compared to a mean of 3.5 days for controls. Fibrinogen and neutrophil levels on day 8 post-challenge were reduced in Strangvac vaccinated ponies:  $7.6 \text{ g L}^{-1}$  compared to  $12.1 \text{ g L}^{-1}$  ( $P = 0.002$ ) and  $9.4 \times 10^9 \text{ cells L}^{-1}$  compared to  $13.3 \times 10^9 \text{ cells L}^{-1}$  ( $P = 0.02$ ), respectively (measured on day 8). All 4 control ponies developed at least one lymph node abscess compared to 6 of the 12 Strangvac-vaccinated ponies. The post-mortem score was also reduced in Strangvac-vaccinated ponies with a mean PM score of 17.2 in vaccinates and 49 in controls ( $P = 0.006$ ). We conclude that the vaccination of ponies with Strangvac reduced the amount of clinical disease induced by challenge with Se4047 two-months post-second vaccination.

#### 3.5. Strangvac conferred excellent levels of protection following re-vaccination of ponies

In Experiment IV, 32 ponies were vaccinated with Strangvac or a placebo control vaccine, followed by a second dose 4 weeks post-first vaccination and a third dose 13 weeks post-second vaccination (Fig. 1). Ponies were challenged by the administration of  $1 \times 10^8$  cfu of Se4047 two weeks post-third vaccination and



### 3.6. The strangles challenge system is reproducible and robust

The data obtained from placebo control vaccinated ponies that were challenged in Experiment II (16 ponies), III (4 ponies) and IV (15 ponies) were analysed to determine if there was a statistically significant difference between these groups. Comparison of the time to pyrexia for the control groups of ponies in each of the studies identified that there was no significant difference between the studies ( $P = 0.15$ ) (supplementary Fig. 2a). Comparison of the pool of placebo control data from Experiments II and IV to the four control ponies in Experiment III did not identify a statistical difference ( $P = 0.94$ ). Similarly, there was no significant difference in the pathology score obtained from the three groups of control ponies at post-mortem examination ( $P = 0.88$ ) (supplementary Fig. 2b) or from the pool of placebo controls compared to the controls in EXP III ( $P = 0.66$ ). This supports the view that the infection model used in these studies is robust and reproducible.

### 3.7. The use of pooled control data can reduce the number of ponies required

Three groups (groups 1–3) of eight placebo vaccinated control ponies were constructed by random selection from studies Experiment II (16 ponies) and IV (15 ponies). Data from these three groups were then each added to the Experiment III analysis and used to recalculate the level of statistical significance in an otherwise underpowered study. Plotting the average rectal temperature data for Strangvac vaccinated and control ponies (Fig. 5) showed that the addition of pooled control data from groups 1–3 (Fig. 5, panels B, C and D) did not alter the onset or severity of pyrexia compared with the original EXP III control data (Fig. 5, panel A). All of the control ponies in the pools from Experiment IV and II became pyretic post-challenge. Therefore, the addition of any of

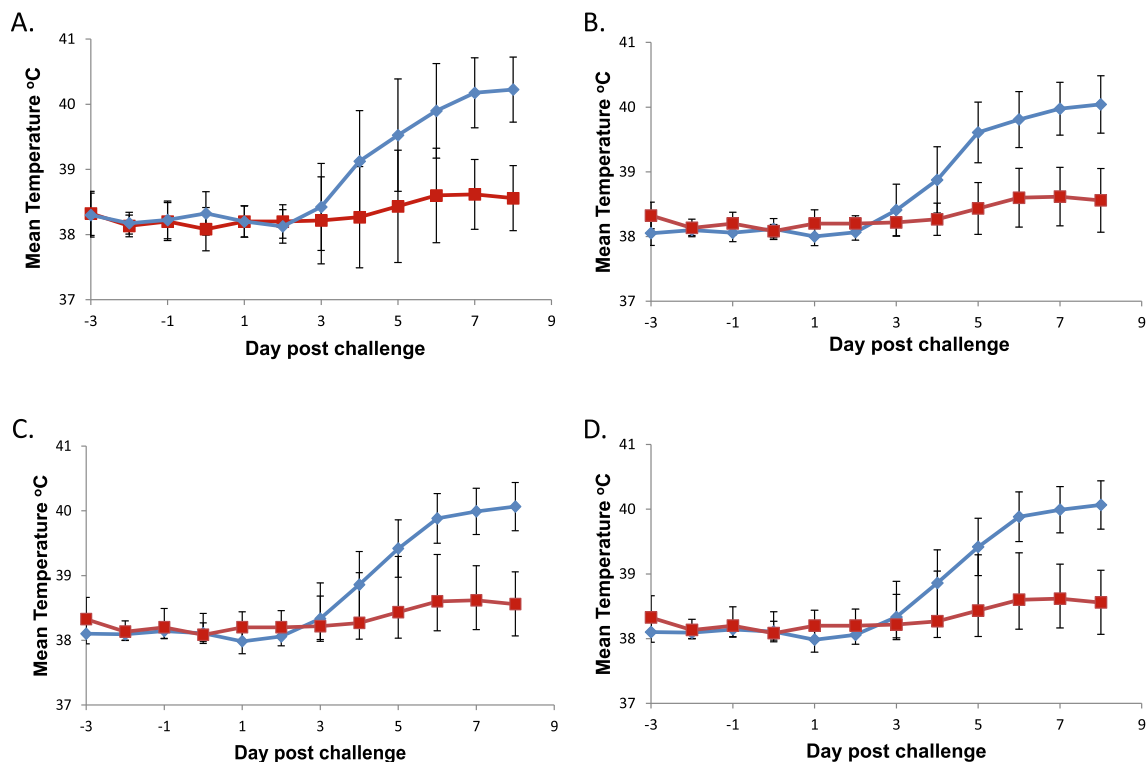
the three pooled control samples showed a statistically significant difference in the incidence of pyrexia between Strangvac-vaccinated in Experiment III and the placebo control-vaccinated groups (all  $P = 0.005$ ), regardless of which control ponies were selected as controls in the pools. The addition of pooled control data confirmed the significance of post-mortem pathology score data (all  $P < 0.0001$ ) (Fig. 6). We conclude that the challenge of Welsh mountain ponies with a dose of  $1 \times 10^8$  cfu of Se4047 consistently induces clinical signs that reproduce the natural disease. Large numbers of control ponies are often required for each study to 'stand-alone' and reach statistical significance. Therefore, the use of pooled data from placebo vaccinated control ponies is an effective approach to reduce the number of ponies required in accordance with the principles of the 3Rs.

### 3.8. Correlation between different clinical observation parameters

The discrimination between infected and non-infected animals was based on the definition of pyrexia as an elevated rectal temperature ( $>38.9$  °C) for two days during any three-day period. This definition as a predictor of disease is justified by its significant correlation with post-mortem score ( $P < 0.001$ ), combined clinical score ( $P < 0.001$ ), fibrinogen level ( $P < 0.001$ ) and neutrophil count ( $P < 0.001$ ) as assessed on day 8 post-challenge.

### 3.9. Strangvac has DIVA capability

The analysis using a commercial dual antigen iELISA [6] of serum samples that were collected after vaccination with Strangvac, but pre-challenge, demonstrated that the vaccination of ponies with Strangvac did not interfere with this widely-used diagnostic assay. Furthermore, none of the Strangvac-vaccinated ponies returned a positive qPCR test result pre-challenge using a triplex



**Fig. 5.** Rectal temperatures using pooled control ponies. Average rectal temperature post-challenge for the 12 Strangvac vaccinated ponies compared to: (A) the original 4 control ponies in EXP III. (B) the original 4 control ponies in EXP III plus historical data from 8 pool 1 control ponies. (C) the original 4 control ponies in EXP III plus historical data from 8 pool 2 control ponies. (D) the original 4 control ponies in EXP III plus historical data from 8 pool 3 control ponies. Error bars indicate 95% confidence intervals.

