Vaccination of foals with a modified live, equid herpesvirus-1 gM deletion mutant (RacHΔgM) confers partial protection against infection

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Highlights

- Vaccination of foals with an EHV-1 gM$^-$ MLV stimulated CF and VN antibodies
- Vaccination resulted in reduced clinical scores on challenge infection
- EHV-1 specific mucosal antibody and CTL activity were not induced by vaccination
- This EHV-1 gM$^-$ MLV vaccine partially protected foals against challenge infection

Abstract

Equid herpesvirus-1 (EHV-1) causes respiratory and neurological disease and late gestation abortion in pregnant mares. Current vaccines contain either inactivated or live EHV-1, but fail to provide complete clinical or virological protection, namely prevention of nasopharyngeal shedding and cell-associated viraemia. Thus, the development of novel products, such as modified live virus (MLV) vaccines which stimulate virus-specific, humoral and cell mediated immune responses more effectively remains a priority. Two groups of weaned foals (n=6 each group) were used in a longitudinal, prospective, experimental study to evaluate immune responses elicited by two vaccinations with a glycoprotein M (gM) deletion mutant of EHV-1 (RacHdeltagM). Following two concurrent intranasal and intramuscular inoculations six weeks apart, vaccinated (8.4±0.2 months old) and control foals (6.2±0.4 months) were challenge infected intranasally with EHV-1 Ab4/8 four weeks after the second vaccination and clinical signs and virological replication measured. Vaccination caused no adverse events, but did stimulate significantly higher complement fixing and virus neutralizing antibodies in serum compared with control foals at either equivalent or pre-vaccination time points. Virus-specific nasopharyngeal antibody levels and cytotoxic T lymphocyte responses were not significantly different between the groups. Following challenge infection, these immune responses were associated with a reduction in clinical signs and virological replication in the vaccinated foals, including a reduction in duration and magnitude of pyrexia, nasopharyngeal shedding and cell-associated viraemia. We conclude that the RacHΔgM MLV primed EHV-1-specific humoral immune responses in weaned foals. However, complete virological protection by vaccination against EHV-1 requires further research.

Keywords: equine, equid herpesvirus-1, vaccine, deletion mutant, immune response

1. Introduction

The ubiquitous alphaherpesvirus equid herpesvirus-1 (EHV-1) is responsible for respiratory and neurological disease in all horses and abortion in pregnant mares [1]. Abortion and neurological disease are the consequences of a cell-associated viraemia and endothelial cell infection, which leads to vascular pathology in the vulnerable pregnant uterus or spinal cord [2, 3].

Vaccination against EHV-1 aims to prevent nasopharyngeal virus shedding and cell-associated viraemia, which will eliminate within herd transmission or endothelial cell infection and thrombosis respectively [4]. Experimentally, a reduction in the duration and magnitude of these virological parameters is associated with high pre-infection titres of virus neutralizing (VN) antibody, respiratory mucosal IgG4 / IgG7 isotypes and high frequencies of precursor or memory cytotoxic T lymphocytes (CTL) in blood, sometimes combined with virus-specific mucosal antibodies in the nasopharynx [4, 5]. However, current vaccines which contain either inactivated virus with licensed adjuvants or attenuated, modified live virus [6], fail to stimulate the comprehensive range of immune responses required. The efficacy of these vaccines is variable, probably due to multiple factors including host, antigenic load and challenge infection method, but none prevent cell-associated viraemia completely. Thus, in the face of ongoing outbreaks of EHV-1 abortion in vaccinated horses [7], there is an urgent need to identify novel and safe but commercially practical vaccine formulations that stimulate long-lived and effective immune responses, particularly

CTL leading to complete clinical and virological protection against EHV-1. Modified live virus vaccines (MLV) have been proposed as suitable candidates [8, 9].

Examples of the success of MLVs under field conditions include use of a modified live gE deletion mutant to eradicate bovine herpesvirus-1 (BHV-1) from some European countries [10]. In pigs, management and a gI deletion mutant of pseudorabies virus reduced the prevalence of disease in a large field study [11]. In young horses, EHV-1 gE/gI deletion mutants provided partial protection against respiratory disease [12, 13]. In Japanese Thoroughbred racehorses, a live attenuated EHV-1 / EHV-4 vaccine (ERPL, Nisseiken, Tokyo, Japan) stimulated higher VN antibody titres than the inactivated virus vaccine used previously and its use was also associated with a reduction in the frequency of EHV-1 outbreaks [14]. Live attenuated EHV-1 vaccines Rhinomune® (Boehringer Ingelheim) or Prevaccinol® (MSD Animal Health), which both contain strain RacH have been available commercially in the USA and Europe for decades.

EHV-1 strain RacH was attenuated by 256 passages in porcine embryonic kidney cells [15] from the more virulent RacL (clade 4, originally isolated from an aborted fetus; [16]). Sequencing of a plaque isolate, RacL11 has identified entire deletions of genes ORF1 (HSV homologue UL56) and ORF2 (no HSV homologue identified to date) and in-frame deletions of ORF14, ORF63, EICP0) and two regions in ORF68 (HSV US2 [17]. In the RacH MLV strain, modification of ORF67 (IR6) is mainly responsible for the attenuation, relative to the parental virus [15-20]. In horses, by comparison with placebo-vaccinated controls, intramuscular vaccination of adults with Rhinomune® (RacH) resulted in a reduction in clinical disease and the amount

of virus shed from the nasopharynx and cell associated viraemia following challenge infection [21, 22]. Intranasal vaccination of weanlings with Rhinomune® failed to stimulate either mucosal or systemic antibody after vaccination but after challenge infection, was associated with a reduction in clinical and biological signs [23]. RacH therefore has shown some promise as an MLV, and thus may benefit from further modification. RacH is an old strain but phylogenetic analysis of 80% of the genomes of 78 EHV-1 strains, the majority isolated over 35 years in the United Kingdom indicated the presence of 13 clades [19]. Further analysis indicated that EHV-1 evolves very slowly, to the extent that strains isolated currently co-circulate and show similarity to much older viruses, including RacL and RacH [15, 18, 19].

Identification of the function of individual EHV-1 proteins, including those associated with virulence or correlated with protective immunity [24-26] , enables the informed and targeted removal of individual genes: one candidate is glycoprotein gM (gM; gp21/22a). This is a non-essential glycoprotein encoded by gene 52 (homologous to Herpes simplex virus-1 UL10; [17]) which is involved in virus entry and fusion and cell-to-cell spread in both EHV-1 and Herpes Simplex Virus-1 (HSV-1; [27, 28]). However RacHΔgM retains the ability to penetrate, spread from cell-to-cell and egress from host cells [27, 29] which is crucial to the development of cell mediated immune responses, including CTL activity. *In vivo,* mice vaccinated once with a RacHΔgM deletion mutant were completely protected against challenge infection with the virulent RacL11 strain, suggesting that the RacH gM deletion mutant

stimulated protective immune responses [30] and may thus be a candidate vaccine for horses.

The design of vaccine trials to assess the effectiveness of protection against EHV-1 is complex, with multiple host, environmental and virological factors potentially influencing the outcome [4, 31]. The host complexities include recruitment of animals naïve to EHV-1 infection or at least with similar historical infections within a known time frame; this ensures similar humoral and cell mediated immunological status, but is difficult to assess. The production of specific pathogen-free foals is costly and time-consuming [32] so a pragmatic alternative to assess vaccine-induced immunity is to identify synchronized pregnant mares, unvaccinated against EHV-1, then use their offspring at weaning, when maternally-derived colostrum titres have declined, to assess vaccine-induced immunity. Even then the risk of inter-current bacterial infection following weaning and mixing of foals from different herds remains high [33, 34]. Nevertheless, using weaned foals is informative because the current guidance states that vaccines against EHV-1 / EHV-4 can be administered to foals from 4-6 months of age [35].

The current study tested the hypotheses that vaccination of weaned foals with an EHV-1 RacHΔgM mutant would stimulate immune responses and provide clinical and virological protection against challenge infection.

2. Materials and Methods

2.1 Foals

The project design, animal care and ethics and genetically modified organisms' safety complied with national laws and institutional regulations. All mares and foals were cared for at the Animal Health Trust, Newmarket and were examined by an independent clinician prior to vaccination and / or challenge infection. Pregnant mares (Welsh Mountain ponies) were kept in two herds according to their stage of gestation. Mares were vaccinated against influenza virus and with tetanus toxoid according to manufacturers' instructions, but not vaccinated against EHV-1. For the vaccinated foals, 4/6 were male and the Major Histocompatibility Complex (MHC) class I haplotypes of both parents were partially known (Table 1). The control foals originated from a second herd of outbred mares mated to a single stallion, all were female and of unknown genotype. Foals were monitored at monthly intervals from birth to plot the decline of colostrum-derived, EHV-1 specific, serum virus neutralizing antibodies and to ensure the absence of complement fixing antibodies, indicative of recent, natural EHV-1 infection. With the decline of colostral antibody and increasing vulnerability of these foals to unplanned respiratory infection, the vaccination program was initiated in the six oldest foals 3 weeks after weaning. The reasons underlying this decision included the vulnerability of these foals to respiratory bacterial infection and inflammatory airway disease, particularly when groups of animals are mixed for the first time [33, 34]. The six remaining animals were allocated to the control group. As a result, the mean age of vaccinated foals was 2.2 months older than the control group.

2.2 EHV-1 glycoprotein M (gM) deletion mutant EHV-1 (RacHΔgM)

EHV-1 RacH11ΔgM, a LacZ insertion mutant that still expresses the N-terminal 130 amino acids was constructed and propagated by five passages in rabbit kidney 13 (RK13) cells [27, 29]. RacH has deletions in ORF1 (MHC class I downregulation and modulation of cytokine responses) and ORF 2 (modulation of cytokine responses) and in frame deletions in ORF14, ORF63 and ORF68. The most significant alteration which is largely responsible for the attenuation of RacH is the deletion of both copies of ORF67 (IR6; HSV US10; [15, 20]).

2.3 Vaccinations and EHV-1 challenge infection

Three weeks after weaning, vaccinates were inoculated with RacH11ΔgM (Figure 1). Vaccinated foals had a mean age of 6.1 $\pm$ 0.2 months (183.0 $\pm$ 7.1 days) at the first vaccination (Table 1) and were kept on a separate pasture from the control foals. Administration was both intramuscularly in the neck by 1” 18g needle and intranasally by aerosol spray attached to tubing and syringe and on two occasions (V1 and V2), with a 6 week interval. The vaccinated foals were monitored on days 2, 5, 7 and 9 after V1 for clinical health, including ocular and nasal discharge, pyrexia, and cell associated viraemia. Four weeks after V2 (vaccinates) and three weeks after weaning (controls), all foals were stabled in pairs within their group and immediately challenge infected concurrently. All foals were infected intranasally (day 0) with 2 x $10^{6.3}$ 50% tissue culture infectious dose ($TCID_{50}$) of EHV-1 strain Ab4/8 propagated on equine cells using an aerosol spray [36, 37]. The mean ages of vaccinated and control foals were 8.4 $\pm$ 0.2 or 6.2 $\pm$ 0.4 months respectively at challenge infection.

2.4 Serology

Serum samples were screened for complement fixing (CF) and virus neutralizing (VN) antibody at the time points shown in Figure 2. The V1+0 sample for VN antibody testing was taken immediately before first administration of the vaccine. Seroconversion was defined as a four-fold increase in titre [37].

2.5 Clinical signs, pyrexia and nasopharyngeal virus shedding

Following challenge infection, clinical signs were assessed by one operator who was blinded to the group status. The presence of mucopurulent nasal or ocular discharge at each nostril or eye were assessed and scored as 0 (absent), 1 (present mild), 2 (present, moderate) or 3 (present, severe). Swelling of the sub-mandibular lymph nodes was graded as 0 (barely palpable), 1 (slightly palpable), 2 (easily palpable) or 3 (enlarged and painful). Rectal temperatures (pyrexia $\geq$38.9^{o}C) and deep nasopharyngeal swabs extending approximately 12" were collected daily for 10 days, the latter into 2ml of virus transport medium. The Animal Health Trust is an OIE reference laboratory for EHV-1 and EHV-4, therefore swabs and blood samples were handled according to the OIR manual [36]. Swabs were squeezed using metal forceps, which were sterilized between each sample extraction by flaming in alcohol. The swab extract was not filtered before titration from neat to 10^{-10} using $\log_{10}$ dilutions across a 96 well flat-bottomed micro-titre plate, with 8 replicates per sample. Indicator cells were RK13s. Results from this endpoint dilution assay were expressed as $TCID_{50}$/ml.

2.6 Cell associated viraemia

Peripheral blood samples were collected by jugular venipuncture from vaccinates into 10ml evacuated tubes coated with sodium heparin. Blood samples were then processed according to the OIE manual [36] using RK13 indicator cells in T25 tissue culture flasks. Leukocytes were pelleted by centrifugation of supernatant plasma after spontaneous rouleaux formation; Ficoll enrichment of leukocytes was not performed. In the absence of cytopathic effect (cpe), cells were passaged a second time as described [36]. Results were expressed as positive or negative. Samples were collected on days 2, 5, 7, and 9 after V1 and on day 6 after V2. After challenge infection, all foals were sampled on alternate days until day 21.

2.7 Measurement of mucosal IgA and IgG in nasal washes

Nasal washes were performed as described [38], before V1 (vaccinates only), then in controls and vaccinates at equivalent time points after vaccination and after challenge infection. Samples were clarified by centrifugation, stored at –70^{o}C then assayed together following a method adapted from [38]. An optimised dilution of EHV-1 strain Ab4 was used as antigen and murine anti-equine IgA monoclonal antibody 3E7 (a kind gift from Dr Chris Stokes, University of Bristol), and commercial, enzyme conjugated anti-mouse or anti-horse IgG (Kirkegaard & Perry Laboratories) were used to detect the respective antibody isotypes. Optical density (OD) was read at 450nm. The mean protein concentration (triplicates) of each nasal wash was measured by comparing the OD_{295} generated using a protein assay kit (Sigma, Poole, Dorset) with a calibration curve ranging from 0.05 to 1.7 mg/ml (absorbance from 0.1 to 1 at OD_{295}). Corrected OD was calculated according to the formula: mean OD_{450} of nasal wash immunoglobulin – non-specific binding OD_{450} /

protein concentration (mg/ml). Each assay included positive and negative nasal wash controls, selected from pilot assays.

2.8 Cytotoxic T lymphocyte assay

Peripheral blood mononuclear cells (PBMC) were cryopreserved from all foals and MHC class I restricted, EHV-1 specific cytotoxic T lymphocyte activity was measured as described previously [39]. The mean of 6 replicates at each effector : target ratio (E:T; 100:1, 50:1, 25:1 and 12:1) was calculated and the percent lysis of mock infected targets subtracted from that of the autologous EHV-1 infected targets, to give a final percent specific lysis. Samples from each foal were tested in a single assay, with effector CTL from a hyper-immune pony used as a positive control. Statistical analysis was performed on CTL activity at all E:T ratios.

2.9 Statistical Analysis

For each parameter and sample time point, the mean and standard deviation for each group of foals was calculated. The data were checked for normality and homogeneity of variance before statistical analysis and were log transformed as necessary. For the endpoints (e.g. CF antibody titre) where more than one sample was collected and the data points were continuous, a randomized-block ANOVA was performed. The time points were grouped into pre-vaccination, after V1, after V2 or post-infection and analysed separately. In the analysis, time and vaccination groups were considered the factors, with the data blocked by foal identification. Where significant differences were detected, individual t-tests were performed to compare the control and vaccinated group on a particular day. For discrete endpoints, a non-parametric Mann-Whitney (Wilcoxon rank-sum) test was performed. As qualitative data, clinical scores were

assessed using a non-parametric, one-way ANOVA (Kruskal-Wallis test). CTL data were analysed by Fisher's Exact test. All statistical analysis was performed in Genstat 17.1.

3. Results

All foals completed the study, there were no modifications to the study design and there were no unexpected adverse events or side effects.

3.1 Pre- and post-vaccination monitoring

Prior to V1, CF and VN antibody titres were either undetectable or had a reciprocal titre of $\log_{10}$ 0.2 or 1.7 respectively ($\leq$1:20) in all foal samples (Figure 2a & b). Vaccinated foals showed no ocular or nasal discharge, pyrexia, clinical abnormalities or cell associated viraemia.

3.2 Serology

Mean CF antibody titres in control foals were low and stable until 2 weeks after challenge infection, when all 6 seroconverted to EHV-1 (Figure 2a). Vaccinated foals exhibited similar low titres prior to V1, then a non-significant increase in mean titre after V1 followed by a significant increase at all post V2 vaccination points compared with V1-3m. Samples taken one week after V2 demonstrated seroconversion in 5 / 6 vaccinated foals, and one foal (1B05) seroconverted 1 week later. Elevated CF antibody titres declined thereafter but all 6 vaccinates seroconverted again by 2 weeks after challenge infection. Mean CF antibody titres were significantly higher in vaccinated *versus* control foals at all sample points after V2 ($p<0.001$) and 1 week after challenge infection ($p<0.001$).

VN antibody titres were low in both groups of foals prior to the V1 sampling point (Figure 2b; Supplementary Table 1). Thereafter titres in the younger control foals declined further, reflecting the level of residual colostral antibody, but subsequently increased following challenge infection. In vaccinated foals, titres at the post V2 vaccination and post infection time points were significantly different from the titre at V1+0 ($p<0.001$). There was a significant difference between the mean VN antibody titres of vaccinated *versus* control foals at V2+2weeks ($p<0.001$), but not before V1 ($p=0.07$) or after challenge infection ($p=0.07$).

3.3 Clinical signs and virological parameters after challenge infection

Mean clinical scores amounted to 21.2$\pm$7.8 or 8.3$\pm$6.2 in the controls and vaccinated foals respectively ($p=0.03$). Pyrexia was detected on day 2 post infection in all control foals and 3/6 vaccinated foals (Figure 3a). Mean days of pyrexia were 4.5$\pm$1 in control foals and 1.2$\pm$1 in vaccinated foals ($p=0.004$), but no significance was detected for the first or last day of pyrexia. The mean rectal temperature was significantly higher in the control group compared with the vaccinated group ($p<0.001$) and at each time point on days 2, 3, 4, 5 and 8 after challenge infection ($p<0.001$).

The mean titres of infectious virus shed from the nasopharynx were higher in the control foals (mean $10^{4.93}$/ml on day 2; Figure 3b), with all 6 animals still shedding detectable titres of virus on day 8 (mean $10^{2.67}$/ml; $p<0.01$). The amount of virus shed by the vaccinated foals peaked at a mean of $10^{2.32}$/ml on day 2, but declined rapidly thereafter with 4/6 vaccinated foals no longer shedding by day 4. One foal in this group had no detectable virus shedding (V6825). There was a significant

reduction in the total days of nasopharyngeal virus shedding in the vaccinated *versus* control groups ($p<0.01$; vaccinates mean 2.8±2.3 days, controls mean 8.3±1.6 days). There was no difference in the first day of nasopharyngeal shedding between the groups. In contrast, the last day of nasopharyngeal shedding was significantly earlier in the vaccinated group, compared with controls ($p<0.05$; Figure 3b; vaccinates day 4.0±3.6 *versus* controls day 8.8±1.0).

There was a significant difference between the vaccinated and control groups in the total days of cell associated viraemia ($p<0.002$; vaccinates mean 2.5±0.5 days, controls 6.2±1.0 days; Figure 3c). In addition, there was a significant difference in the last day of cell associated viraemia between the vaccinated and control groups ($p=0.006$; vaccinates day 8.3±2.1 *versus* controls day 15.3±3.9). Details are shown in Supplementary Table 2.

3.4 Mucosal antibody responses

The levels of nasopharyngeal IgA and IgG were similar in control and RacH11ΔgM vaccinated foals. There was a gradual but significant increase in EHV-1 specific, nasopharyngeal IgA ($p<0.001$) and IgG ($p<0.001$) over the sampling period in both groups, but no significant antibody production was induced by vaccination alone (Figure 4).

3.5 Cytotoxic T lymphocyte responses

All foals in both the control and vaccinated groups showed low (<5.6% maximum lysis at 100:1 E:T ratio) or undetectable levels of lysis at both time points prior to EHV-1 challenge infection (Figure 5). At three weeks after infection, an increase in

CTL activity above the value of the pre-vaccination sample was detectable in some control (2/6) and vaccinated (4/6) foals. By ten weeks after infection, CTL activity either increased further or remained stable in most control and vaccinated foals, but individual responses were variable. Statistical analysis showed there was a non-significant trend towards the earlier detection of CTL activity in vaccinated compared with control foals using the 100:1 E:T ratios ($p=0.06$). Analysis of data from all other effector to target ratios was also non-significant.

4. Discussion

This study demonstrated that mucosal and systemic vaccination of weaned foals with EHV-1 RacH11ΔgM, a modified live virus vaccine, caused no side effects and induced no clinical signs or detectable viral replication. Furthermore, by comparison with unvaccinated control foals, vaccination served to prime the foals' systemic humoral immune response and this was associated in vaccinates with a significant reduction in mean rectal temperature and temperatures on days 2-5 and 8 post-infection inclusive, as well as total days and last day of nasopharyngeal virus shedding and cell-associated viraemia after challenge infection. Such partial protection is found commonly with many types of commercial and experimental EHV-1 vaccines [13, 22, 40-43], but is particularly encouraging in EHV-1 naïve, weaned foals.

Undertaking EHV-1 challenge infection experiments in horses, particularly foals is difficult and expensive, especially considering the need to obtain weaned animals of approximately the same age and disease-free status. To achieve this in the current study, home-bred foals were used. In the United Kingdom, the Horserace Betting

Levy Board's Codes of Practice for Equine Infectious Disease (specifically EHV-1) recommend keeping pregnant mares of the same gestational age in small groups [44]. Thus both herds of pregnant mares were kept separate. At birth, foals were kept in the separate groups into which they had been born. This approach aimed to reduce any stress in the lactating mares and the consequent risk of reactivation of latent EHV-1, which may have transmitted infection to their foals and thus compromised the foals' EHV-1 naïve status. At weaning, the vulnerability of foals to inter-current respiratory bacterial infection and inflammatory airway disease, particularly when groups of animals are mixed for the first time, is also an ever-present risk [33, 34]. Therefore, in the current study, prior to vaccination, foals were weaned, then maintained in their original birth groups; a strength of this approach is that healthy animals which were naïve to EHV-1 were available for the vaccine trial. It did however lead to several limitations in the study design. These included a lack of randomization, leading to differences in age, sex and genetic background between the vaccinated and control groups; addressing these issues would have improved the study design, but carried the associated risk outlined previously.

Amongst the limitations was that control foals were two months younger than vaccinated foals, which may have had an impact on their ability to mount an antigen specific immune response. The generation of an immune response is reliant on many factors including the ability of antigen presenting cells to stimulate T cells, T cell numbers, a favourable cytokine microenvironment to mediate co-stimulation of T and B cells and ultimately development of immune effector functions, including antibody synthesis of an appropriate isotype and CTL activity. In terms of foal age, most literature focusses on the immune ontogeny of the adaptive immune response

to environmental antigens between birth and 6 months old. Holznagel et al [45] analysed immunoglobulin isotypes in 13 foals of mixed breeds at approximately monthly intervals from birth to 51 weeks of age, one of the few studies to monitor antibodies between 6 months of age, through puberty to adult hood. They reported stable concentrations of serum IgG1/2 (IgGa) or IgG5 (IgG(T)) and IgA at 8 weeks and 12 weeks respectively but IgG6 and IgG7 (IgGc) were not reported. Flaminio et al [46] showed that the absolute number of peripheral blood lymphocytes increased 2.5 fold during the first 3 months of life but by 4 months of age, foals had their full repertoire of CD4 and CD8 lymphocytes. In addition, foals aged 1-5 months had a higher proportion of $FoxP3^+$ cells in the circulating $CD4^+$ $CD25^+$ (T regulatory cell) population and possessed higher suppressor activity compared with their mothers [47]. Compared with adult horses, foals at 4 months of age demonstrated equivalent innate and adaptive immune functions, including lectin-mediated lymphocyte proliferation (indicative of a functional T cell receptor), lymphokine activated killer activity and oxidative burst activity [46, 48]. Similarly Major Histocompatibility Complex class II expression by T and B lymphocytes reached adult levels by 3-4 months of age [46, 49]. Ryan et al. reported a lower frequency of IFNγ and IL-4 secreting cells in 3-4 month old foals compared with adults [50]. Another study reported that IFNγ gene expression was similar in blood leukocytes from foals aged 4, 5 and 6 months [51], suggesting that this parameter had matured. IFNγ production per cell was also equivalent in 6 and 10 month old foals (8 month old foals were not tested). However the number of IFNγ^+ blood leukocytes appeared lower in foals aged 6 months compared with 10 months [51]. These authors concluded that the mean level of IFNγ protein production in foals approached adult levels by 3 months of age. Ryan et al also reported a lower frequency of IFNγ and IL-4 secreting cells in 3-4

month old foals compared with adults [50]. Demmers et. al. [52] demonstrated that total IgG concentrations were similar in 6 versus 8 month old foals. Antibody isotype analysis in foals aged 3-8 months, which encompasses the ages of the foals in the current study, showed that IgG1, IgG3 and IgG5 predominate quantitatively over IgG4/IgG7 [53]. The latter displays virus neutralizing activity [54]. Perkins & Wagner [55] re-analysed published data to show that IgG1 and IgG5 are equivalent quantitatively in foals at 6 and 8 months of age. However IgG7 (IgGc) shows a slow but gradual increase from a nadir at 3-4 months, to reach ~3g/l at 30 weeks then 4g/l by 50 weeks of age [45]. Thus the increased levels of VN antibody titre in the RacHΔgM vaccinated foals within the current study may in part be attributable to the greater capacity of older foals to synthesize this isotype compared with the younger controls. Nevertheless, this increase in IgG7 is part of immune ontogeny rather than measuring the ability of foals to respond to a viral antigen and mount primary and secondary immune responses. There is some evidence of variation in antigen-specific immune responses with age, particularly very young foals. For example, foals vaccinated with keyhole limpet haemocyanin at 3 days old, followed by booster vaccination showed a diverse immunoglobulin repertoire and the ability to switch isotype as measured by sequencing of the B cell immunoglobulin variable region [56]. Three-month-old foals vaccinated twice intra-musculary at 3 week intervals with a killed cattle respiratory viral vaccine and adjuvant, mounted lower antigen specific proliferation and humoral immune responses compared with adults [57] suggesting that the adaptive immune response to this antigen is still immature at 3-4 months of age. In contrast, the influenza and tetanus IgG1/IgG2, IgG4 and IgG5 sub-isotype responses (IgGa, IgGb and IgG(T) respectively) of 6-month-old foals to vaccination with inactivated influenza virus, tetanus toxoid and adjuvant followed the same

pattern as those shown by yearlings but titres were generally lower [58]. Further comparison of the results from control foals in the current study with data from 7 month old, EHV-1 naïve, Icelandic foals infected with strain NY03 [59], showed similar patterns of pyrexia, nasopharyngeal virus shedding and cell-associated viraemia; the key difference was the longer duration of these signs in the current study. This difference may be a reflection of the strain used for infection or other unidentified host-related factors. On balance, the sparsity of published literature means that currently, it is impossible to draw any firm or precise conclusion about the impact of a two month age difference on the ability of weaned foals to generate immune responses to either EHV-1 RacH11ΔgM and / or challenge infection. The majority of immune parameters studied appear mature at 4 months of age, with absolute IgG4 / IgG7 increasing gradually to adult hood [45]. Furthermore current guidelines states that primary vaccination of foals at 4-6 months of age is sufficient to generate an effective immune response to viral vaccines [35].

Any effect of gender on immune responses in foals has not been reported to date. In people, gender plays a role in susceptibility to disease and response to vaccination, but any bias is dependent on the pathogen [60]. For example Fleming et al [61] reported a higher seroprevalence against herpesvirus-2 in adult females compared with males, while the incidence of varicella zoster infection was higher in women, but age-dependent [62]. Reproductive hormone status can also have an impact on the immune response, as many white blood cells express receptors for steroid and gonadal hormones [60]. For example, in adult people, oestrogens promote the Th2 cytokine bias and thus activation of antibody production, but androgens tend to promote Th1 –dependent responses and activate CD8+ T lymphocytes, although

progesterone can have immune-suppressive effects [63-65]. In the current study, the weaned foals were pre-pubertal; puberty in this species begins at approximately 10 months and until then, is characterized by little testicular activity, but ovaries do have active steroidogenesis and thus low gonadotrophin and steroid concentrations [66-68]. Thus the influence of reproductive hormones on the immune responses of the control and vaccinated foals, particularly females remains undetermined.

As part of an unrelated project, the MHC class I haplotypes expressed by some foals were characterized. For intracellular pathogens such as EHV-1, MHC class I expression is crucial in antigen processing and ultimately in primed animals, the presentation and recognition of short viral peptides by cytotoxic T lymphocytes. Here, the two groups of foals were each sired by a single but different Welsh Mountain pony stallion and the genetics of the foals also differed, with one group expressing unknown MHC Class I haplotypes and the other showing a predominance of the MHC class I A3 and A7 haplotypes. Both these haplotypes are common in the Welsh Mountain pony breed (Prof D.F. Antczak, Cornell University, Personal Communication), thus it is likely that at least some of the control foals expressed these haplotypes and their associated alleles, making them potentially more genetically similar to the vaccinated group. In addition all vaccinates were heterozygous so both groups may have had similar genetic diversity, but this remains uncharacterized. Nevertheless a potential impact of genetic background must be considered when evaluating the outcome of this vaccine trial.

There is evidence that infection of horses with different wild type strains of EHV-1 will stimulate cytokine responses with subtle differences [69]. Thus, the parent strain of

an MLV may affect the nature and route of antigen presentation and thus the immune response generated. Here, vaccination with the prototype MLV based on RacH11 primed humoral immune responses effectively. Vaccination of a third group of foals with the parent RacH strain would have enable more detailed characterization of the precise role of gM in immune-enhancement and protection against EHV-1 challenge infection as reported in mice [29]. However experimental infection of yearling or 2-4 year old ponies with either strain Ab4ΔORF1/2 or Ab4ΔORF2 resulted in pyrexia and nasopharyngeal shedding of a lower magnitude and shorter duration compared with wild type Ab4, but no difference in cell associated viraemia [70, 71]. These data are in contrast to the current study where RacHΔgM vaccination (in which ORF 1/ 2 are deleted) did result in reduced cell-associated viraemia on challenge in infection, along with reduced clinical signs and nasopharyngeal shedding.

It had been anticipated that the MLV vaccine would prime antigen specific cell mediated immunity *via* its replication then presentation of viral antigens to CTL by MHC class I molecules. Strain RacL11, from which RacH was derived, also causes moderate MHC class I downregulation in infected host cells *in vitro* compared with strain Ab4; this is largely due to the absence of ORF1 (EHV-1 UL1; pUL56 HSV homologue) [72]. ORF2 (EHV-1 UL2; no HSV homologue identified) which modulates cytokine responses is also absent [17]. The ORF17 (gene pUL43) collaborates with ORF1 (pUL56) in the downregulation of MHC class I in strain Ab4 [73]. However, ORF17 is present in strain RacL [17] and to the author's knowledge, there are no reports to suggest it has been deleted during the attenuation of RacH. Thus the impact of RacHΔgM on MHC class I expression is likely to reflect the

parental RacL strain. Nevertheless, *in vivo* the replication of EHV-1 occurs sequentially at primary and secondary sites, so it is unlikely that downregulation of MHC class I occurs in all infected cells simultaneously. Thus antigen presentation by MHC class I molecules to CTL is predicted to continue. Despite this prediction and although there was a suggestion that RacH11ΔgM vaccination primed CTL activity, this was insignificant and individual responses were variable. One potential explanation and in common with other viruses and species [74, 75], is that the host's genetic background, particularly MHC class I haplotypes may impact the magnitude of the CTL response. Unlike in-bred mice, the recruitment of genetically identical horses is challenging and despite being the same breed, the foals in the current study were not in-bred. This potential confounding factor needs consideration when interpreting the data.

Despite deletion of EHV-1 gM, RacHΔgM retains the ability to penetrate, spread from cell-to-cell and egress from host cells [29]. This glycoprotein is also a moderate tetherin antagonist, potentially permitting release of virions trapped on host cells and allowing further dissemination [76]. It may be that the RacHΔgM vaccination resulted in a more limited release of virions from host cells, but antigen processing and presentation continued, permitting the development of robust systemic humoral immunity but a more limited priming of CTLs. Vaccination with the RacH vector alone would have permitted further insights into the role of gM in the generation of selected immune responses.

Intranasal administration of the RacHΔgM mutant in combination with an intramuscular prime was predicted to result in the stimulation of mucosal antibody,

particularly IgA and thus contribute to protection against EHV-1 challenge infection in vaccinated foals. Simultaneous intranasal and intramuscular administration of an MLV provided good protection against challenge infection with bovine herpesvirus-1 [77]. Here, no significant difference in corrected OD values for nasopharyngeal IgA and IgG was detected in the control *versus* vaccinated foals. Instead there was a general increase in mean corrected OD values for IgA and IgG throughout the sample period. A similar failure of an intranasally administered, live attenuated vaccine (Rhinomune®; RacH strain) administered exclusively within the nares to stimulate EHV-1 specific mucosal IgA or IgG or serum antibody in weaned foals was reported previously [23]. This was in contrast to the intranasal administration of strain A183, which stimulated virus specific mucosal IgA effectively, for a duration of at least 13 weeks [23]. However Dolby et al [78] reported that intramuscular administration of EHV-1 strain V592 with Freunds' complete and incomplete adjuvants successfully primed the mucosal immune response, resulting in detectable mucosal IgA on challenge infection. Thus, as well as the route of administration and antigen, adjuvants are likely to play an important role in the generation of mucosal immunity [79, 80]. Another contributing factor to the relative absence of nasopharyngeal antibody may be the nasopharyngeal muco-ciliary clearance system, particularly the mucus component, which may have trapped and removed the majority of RacHΔgM virions at this location. The main component of mucus is secreted mucins and these are composed of heavily glycosylated glycoproteins which form a complex mesh, with pore size dictating the size and charge of particles which can enter the mucus [81]. In horses, MUC5B and MUC5AC form the major and minor components in equine respiratory secretions respectively [82]. *In vitro*, the movement of pseudorabies virus, was 59 fold slower in porcine mucus compared

with water and this hindrance was related to charge, rather than size alone (Yang et al 2012). EHV-1 has a zeta potential of -33mV (J.H. Kydd, unpublished data) and is approximately 266nm in diameter which is similar to pseudorabies virus (zeta charge -31.8±1.5mV; [83]. Thus the respiratory mucus may have limited access of the RacH11ΔgM vaccine to the underlying epithelial cells and local immune system. This in turn may have resulted in EHV-1 failing to reach the lamina propria and stimulate IgA production by cognate plasma cells and transudation or FcRn mediated transport of selected IgG isotypes to the apical aspect of the mucosal epithelium and thus prohibit any post-vaccination increase in mucosal antibody [84-86]. The absence of mucosal IgA and IgG following vaccination of ponies with a ΔgM deletion mutant may also be explained by the virus failing to, or at least showing limited binding and entry to the respiratory epithelium. An equine respiratory epithelial cell model (EREC) has shown that EHV-1 uses cellular N-linked glycans as receptors for initial EHV-1 binding and entry and thus infection of epithelial cells. These receptors are located on the basolateral aspect of ERECs and are normally inaccessible to EHV-1 when the epithelium is intact [87]. This phenomenon would also prevent antigen reaching plasma cells within the lamina propria and explain the absence of mucosal antibody. Yet another contributing factor to the relative absence of nasopharyngeal antibody may be a failure of RacH11ΔgM to replicate sufficiently, perhaps due to degradation by mucosal enzymes, such as lysozyme [88]. Thus it may be that a combination of mucus and intact respiratory epithelium limited replication in the respiratory tract following intranasal vaccination with a RacHΔgM deletion mutant, thus failing to prime mucosal immunity with no consequent exponential increase in virus specific IgA and IgG responses. The form (MLV and / or inactivated) and route (intranasal and / or intramuscular) of concurrent inoculation can influence protection against

challenge infection, as highlighted for BHV-1 [77]. Moreover, in horses, separate intramuscular prime, followed by an intranasal boost by vaccination or infection did stimulate equine influenza or EHV-1 specific mucosal antibody responses [38, 78]. However, although intranasal vaccination of foals with the RacH11ΔgM failed to stimulate mucosal antibody, it may have generated effector CTL activity in respiratory lymphoid tissue, as has been reported for strain A183 [89], contributing to partial protection.

The protective efficacy of other MLV or gene deletion mutant vaccines against respiratory viruses has been reported in horses. For example, intramuscular vaccination of adult horses with MLV Prevaccinol® or Rhinomune® reduced nasopharyngeal shedding and viral DNA or infectious virus in blood [21, 22, 90, 91]. van de Walle and colleagues generated an MLV (NY03ΔIR6/Δ1) that mimicked mutations in EHV-1 strain RacH [42]. Intramuscular and subcutaneous vaccination of susceptible horses aged 2 to 18 years generated high titres of virus neutralizing antibody. On challenge infection, the amount and duration of nasopharyngeal shedding was significantly shorter compared with controls and no pyrexia or cell associated viraemia was detected. Experimentally, some EHV-1 gene deletion mutants (e.g. gene 38 (thymidine kinase) and gE / gI deletions) have shown virulence after vaccination [92-94], while others gave no or partial protection on challenge infection [12, 95]. In Japanese Thoroughbred racehorses, a live attenuated EHV-1 / EHV-4 vaccine (ERPL, Nisseiken, Tokyo, Japan) which is licensed for prevention of respiratory disease and abortion has been introduced successfully at one training centre [14]. This vaccine stimulated higher VN antibody titres than the inactivated virus vaccine used previously and was also associated with a reduction in

the frequency of EHV-1 outbreaks [14]. Of note, several studies which used Ficoll gradient centrifugation to isolate PBMC report a high viral load as assessed by quantitative PCR, but no infectious virus was detected during co-cultivation on indicator cells [21, 23], which appears illogical. Overall, commercial EHV-1 vaccines containing either MLV or inactivated virus can stimulate components of humoral and cell mediated immune responses effectively, when administered individually [22] and some are associated with partial virological protection against respiratory disease . Consequently, to exploit these known benefits and improve protection, it may be timely to re-evaluate conventional vaccination strategies and consider for example, the combined use of MLV and inactivated virus vaccines, as well as optimization of adjuvants and routes, timing and frequency of administration [59, 88].

In conclusion, intramuscular and intranasal vaccination of weaned foals with an EHV-1 RacH11ΔgM mutant primed systemic humoral immune responses, but no significant mucosal antibody or CTL activity could be detected. By comparison with control foals, these responses were associated with improved but incomplete protection of vaccinated foals against challenge infection. Complete prevention of nasopharyngeal virus shedding and cell associated viraemia by vaccination remains a challenging goal for future EHV-1 research.

Conflict of interest statement

The funding source had no involvement in study design, the collection, analysis and interpretation of data, the writing of the report and in the decision to submit the article for publication.

Disclosure statement

JHK, DH and RR conducted the experiments and acquired, analysed and interpreted the data. DH and NO conceived and designed the study and NO provided the gM$^-$ deletion mutant and permissive cells for its production. NB revised the article critically for important intellectual content. JHK drafted the article and all co-authors approved the final version of the manuscript.

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Table 1. Details of weaned foals used in the EHV-1 RacH11ΔgM vaccine trial. Major histocompatibility Complex (MHC) class I haplotype had been determined previously by microcytotoxic lymphocyte assay. V1 = first vaccination. CI = challenge infection. n/a = not applicable. M = month. * two stallions were used and each sired the foals in one group only.

Foal no. (Group)	Gender	Date of birth	Age at V1 (days)	Age at CI (days)	Dam MHC	Sire MHC*
Controls						
144D	Female	24th June	n/a	210	unknown	unknown
6633	Female	16th July	n/a	188	unknown	unknown
4033	Female	17th July	n/a	187	unknown	unknown
4933	Female	17th July	n/a	187	unknown	unknown
4150	Female	24th July	n/a	180	unknown	unknown
4B05	Female	30th July	n/a	174	unknown	unknown
Mean ± standard deviation				187.7±12.2 6.2±0.4 m		
Vaccinates						
4568	Male	11th April	184	254	A3/x	A7/x
6825	Male	30th April	195	265	A3/x	A7/x
1B05	Male	10th May	185	255	A3/x	A7/x
6A77	Female	14th May	181	251	A7/x	A7/x
1105	Female	16th May	179	249	A3/x	A7/x
7728	Male	21st May	174	244	A3/x	A7/x
Mean ± standard deviation			183±7.1 6.1±0.2 m	253±7.1 8.4±0.2m		

Figure legends.

Figure 1. Timeline of EHV-1 RacHΔgM vaccine trial in weaned foals. V1 and V2 = first and second vaccinations respectively.

Figure 2. EHV-1 specific antibody titres in serum samples collected from control and vaccinated foals at different stages of the EHV-1 RacHΔgM vaccine trial. Seroconversion of either complement fixing (CF) or virus neutralizing (VN) antibody was defined as an increase of $\log_{10}$ 0.6 in titre. * indicates a significant difference ($p<0.001$) between the antibody titres in the control group compared with the vaccinated group. m= months, w = weeks.

a) complement fixing antibody. A reciprocal CF titre of $\log_{10}$ 1.9 (> 1:80) is indicative of exposure. Comparison of V1-3m with other time points in vaccinated foals: “a” not significant, “b”, “c”, “d” indicate $p<0.05$, with significance levels of a<b<c<d, with d the highest level of significance at $p<0.001$.

b) virus neutralizing antibody. Comparison of V1+0 (pre-vaccination) with other time points in vaccinated foals: “a” not significant; “b” $p<0.001$.

Figure 3. Clinical and virological parameters in control and RacHΔgM vaccinated foals following intranasal challenge infection with EHV-1 strain Ab4/8. a) rectal temperature; b) nasopharyngeal virus shedding; c) percent of foals with detectable cell associated viraemia. Samples for viraemia testing used buffy coat cells, which were not purified on sucrose density gradients. No samples were collected on Day 8 after infection.

Figure 4. EHV-1 specific IgA and IgG immunoglobulins in nasal washes from control and vaccinated foals at different stages of the EHV-1 RacHΔgM vaccine trial. Data are expressed as corrected optical densities (OD) namely relevant immunoglobulin (Ig) OD – non specific binding / protein concentration in neat nasal wash (mg/ml). Mean corrected positive and negative control OD values for each group assayed were: IgA unvaccinated foals 0.51±0.20 and 0.20±0.04; IgA vaccinated foals 0.57±0.14 and 0.09±0.03; IgG unvaccinated foals 0.37± 0.17 and 0.07±0.03; IgG vaccinated foals 0.30±0.19 and 0.06±0.01 respectively. a) IgA control foals; b) IgA vaccinated foals; c) IgG control foals; d) IgG vaccinated foals.

Figure 5. EHV-1 specific cytotoxic T lymphocyte (CTL) responses in peripheral blood lymphocytes collected from control and vaccinated foals at different stages of the EHV-1 RacHΔgM vaccine trial. Final mean percent specific lysis by effector cells after subtraction of percent lysis against mock infected target cells is shown. Each sample was assayed in triplicate. Effector to target ratios are shown. a) control foals; b) vaccinated foals.

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Figure 1.

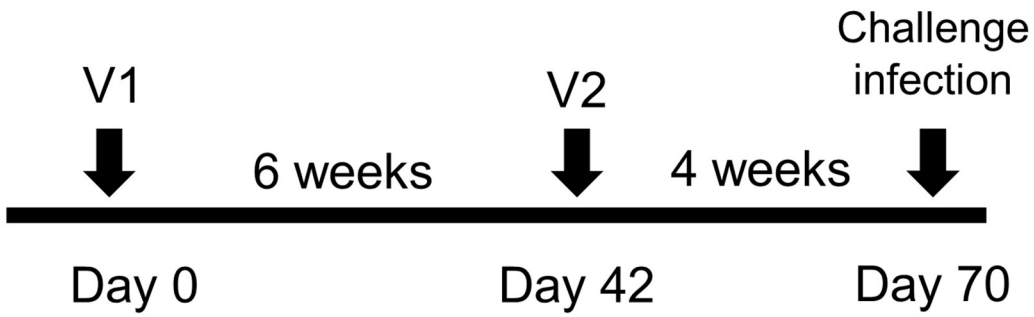
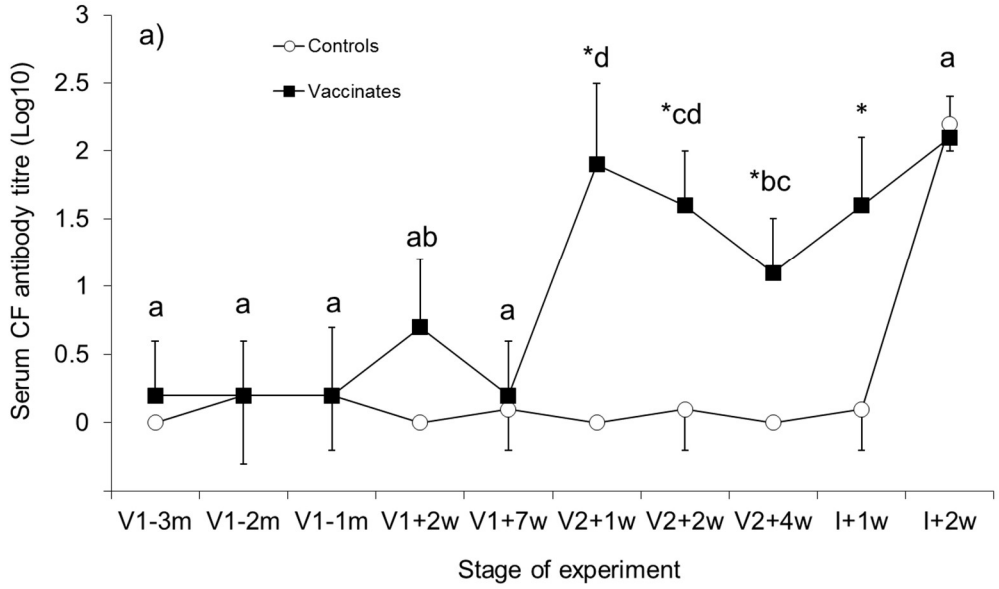


Figure 2.


a)
Controls
Vaccinates
Serum CF antibody titre (Log10)
3
2.5
2
1.5
1
0.5
0
a
a
a
ab
a
*d
*cd
*bc
*
a
V1-3m
V1-2m
V1-1m
V1+2w
V1+7w
V2+1w
V2+2w
V2+4w
I+1w
I+2w
Stage of experiment

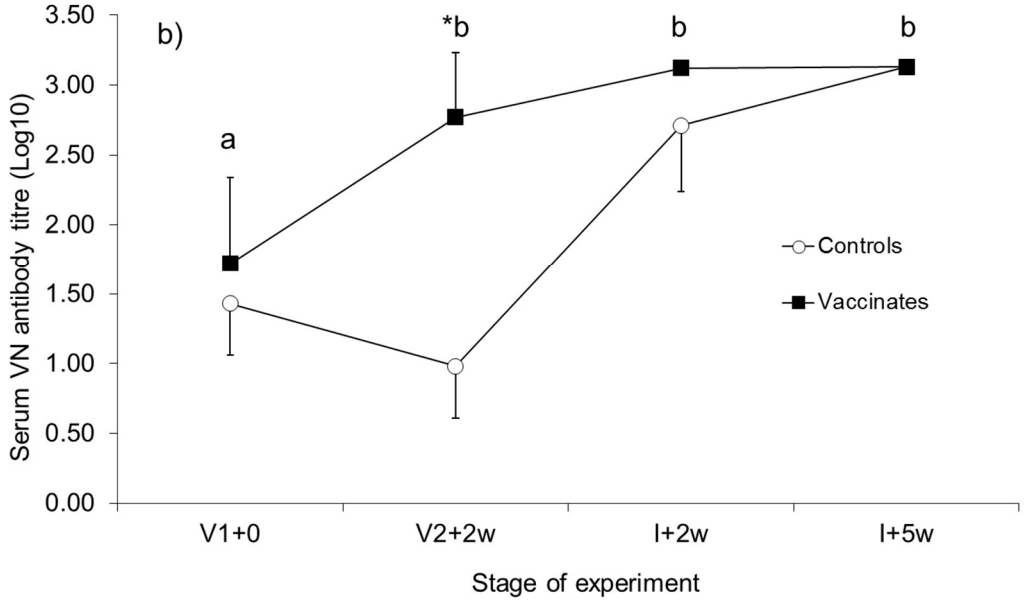
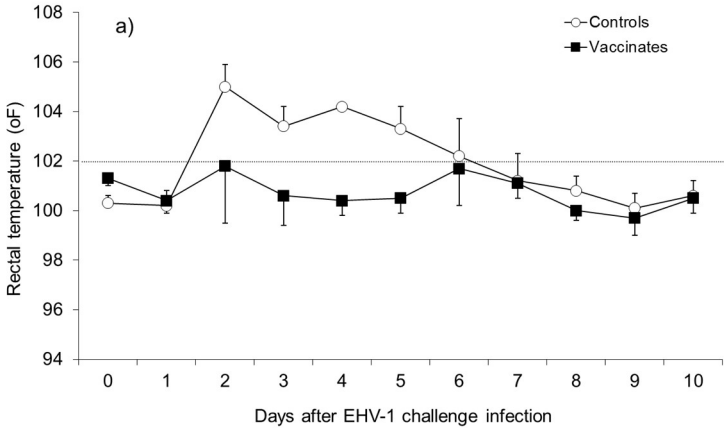
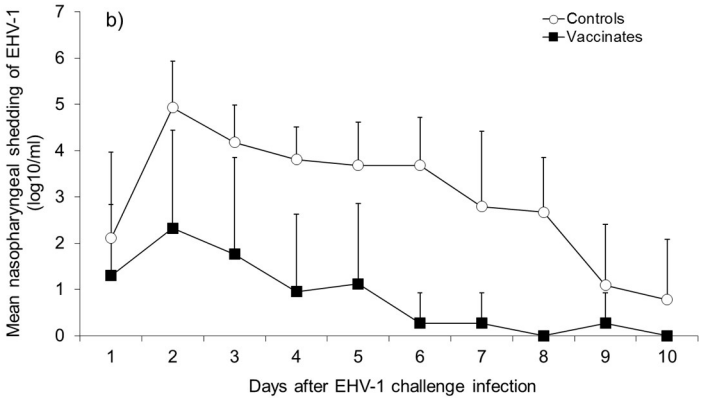

b)
Serum VN antibody titre (Log10)
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
*b
b
b
Controls
Vaccinates
V1+0
V2+2w
I+2w
I+5w
Stage of experiment

Figure 3


a)
Controls
Vaccinates
Rectal temperature (oF)
108
106
104
102
100
98
96
94
0 1 2 3 4 5 6 7 8 9 10
Days after EHV-1 challenge infection


b)
Controls
Vaccinates
Mean nasopharyngeal shedding of EHV-1 (log10/ml)
7
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
Days after EHV-1 challenge infection

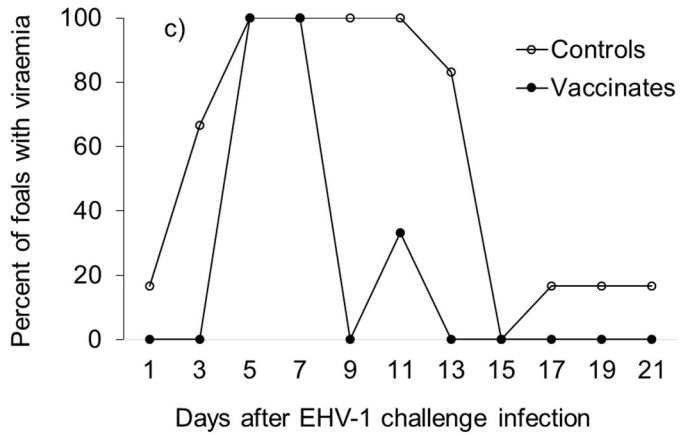
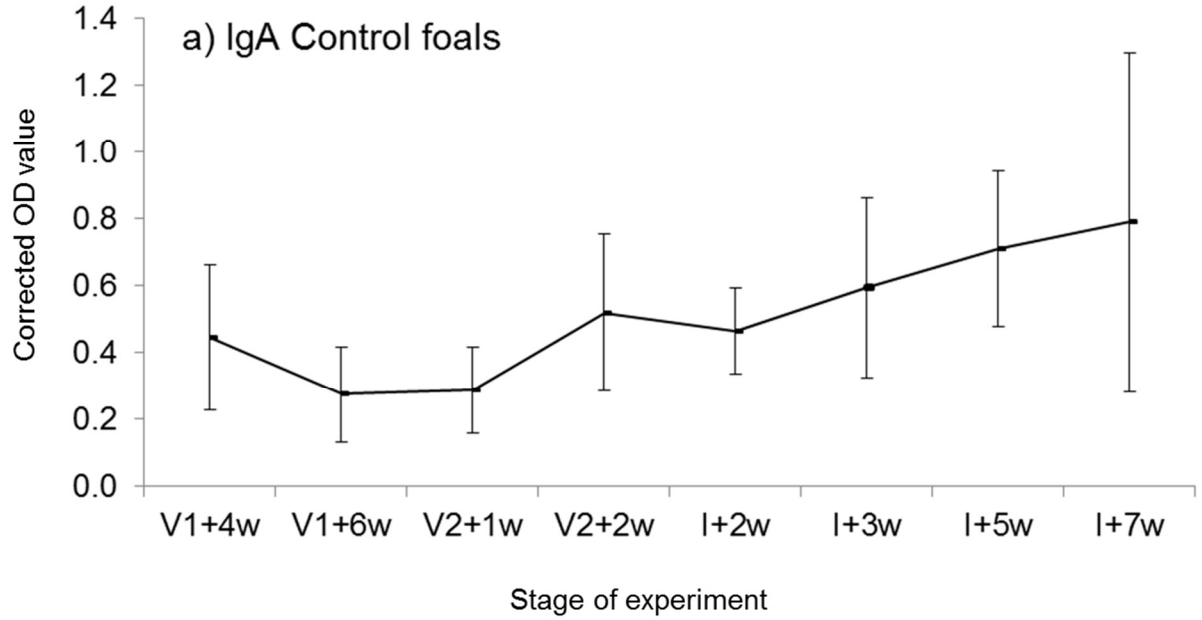

c)
Controls
Vaccinates
Percent of foals with viraemia
100
80
60
40
20
0
1 3 5 7 9 11 13 15 17 19 21
Days after EHV-1 challenge infection

Figure 4.


a) IgA Control foals
Corrected OD value
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
V1+4w
V1+6w
V2+1w
V2+2w
I+2w
I+3w
I+5w
I+7w
Stage of experiment

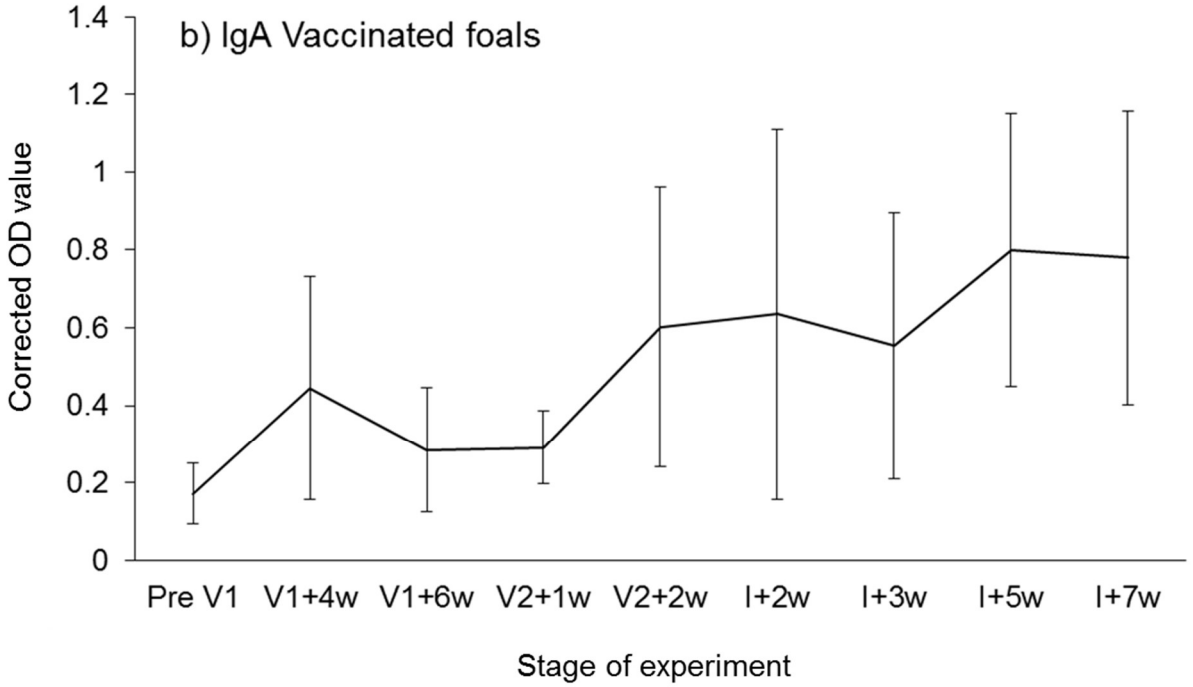
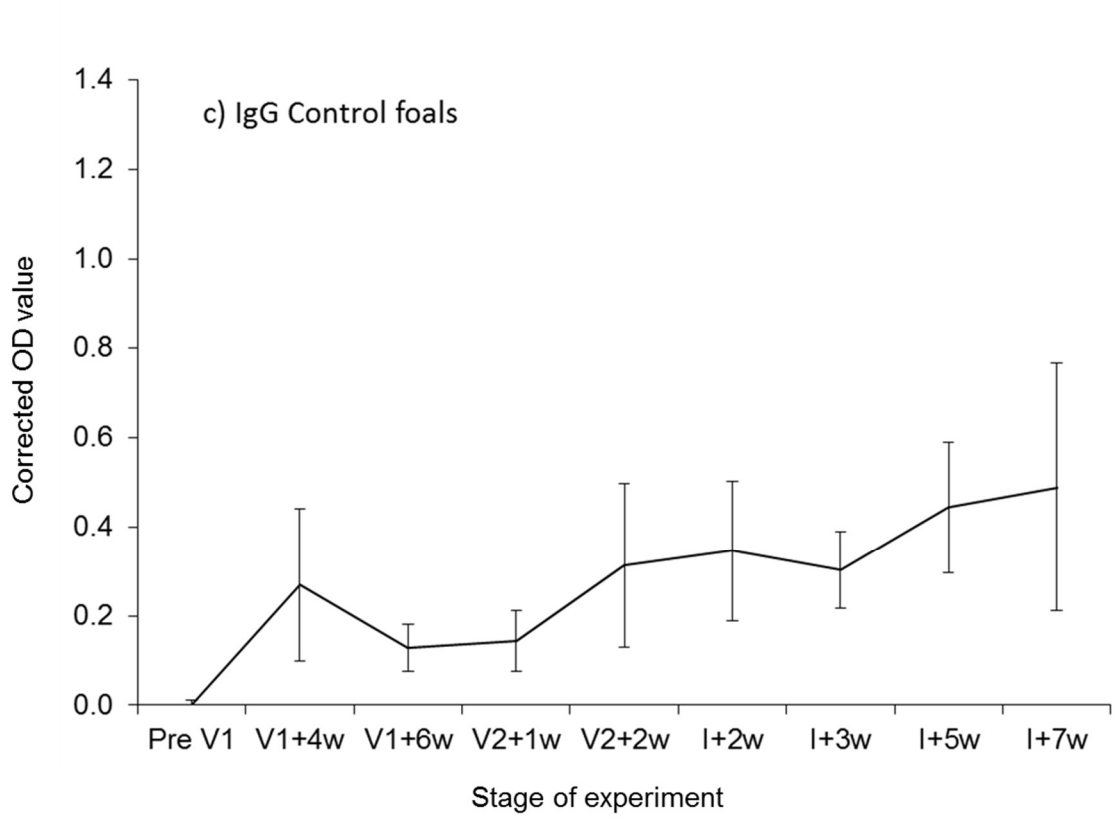

b) IgA Vaccinated foals
Corrected OD value
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pre V1
V1+4w
V1+6w
V2+1w
V2+2w
I+2w
I+3w
I+5w
I+7w
Stage of experiment

Figure 4 continued.



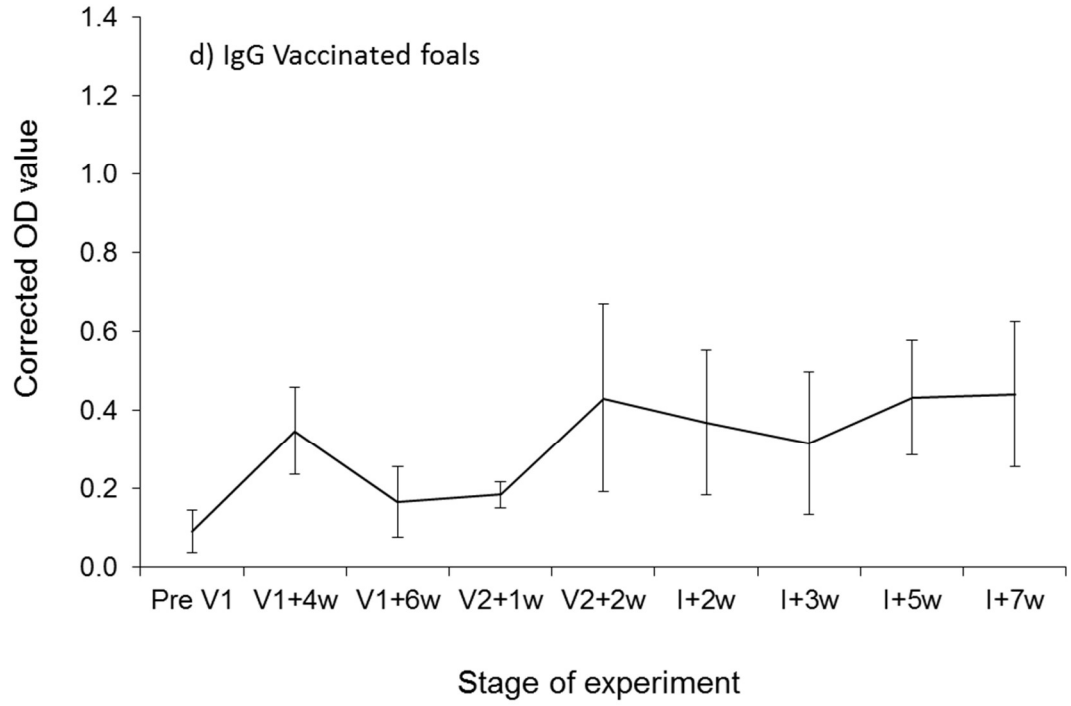


Figure 5a.

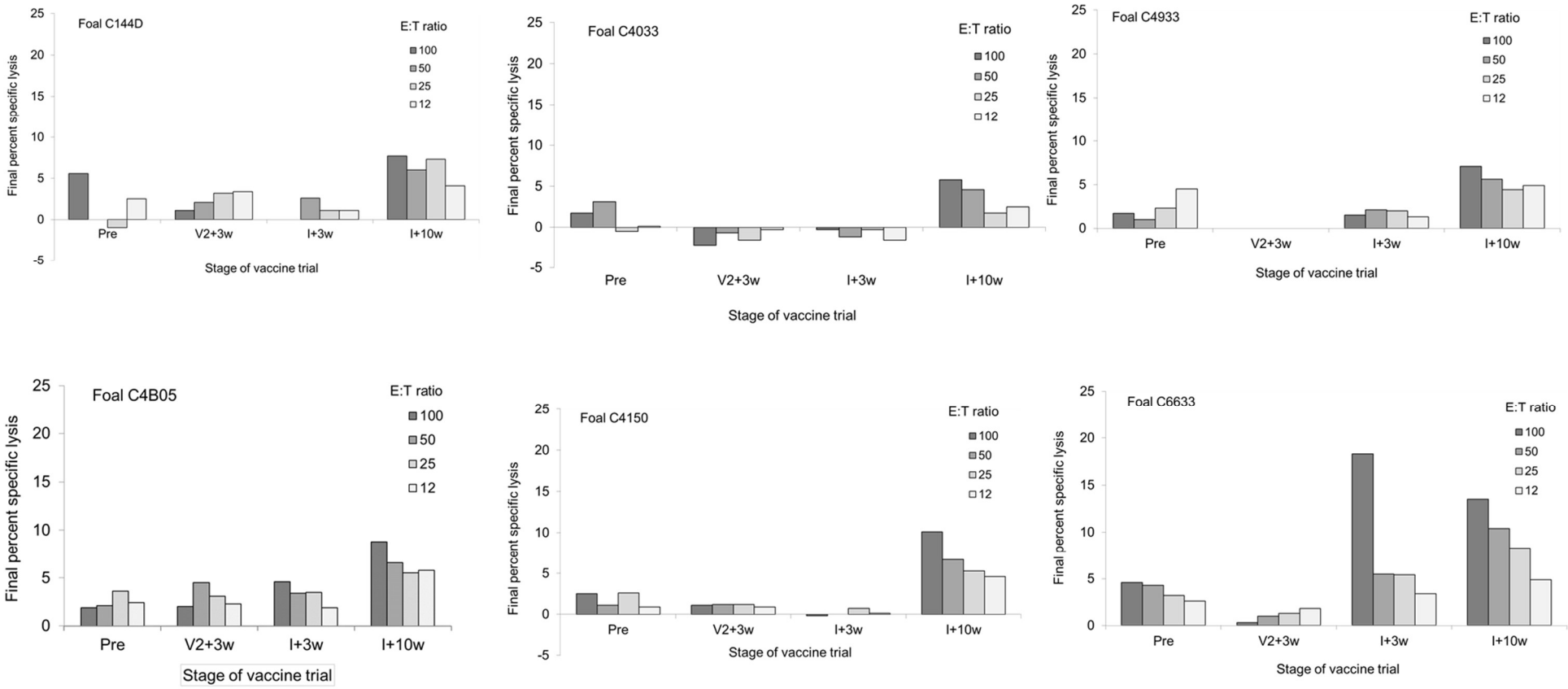
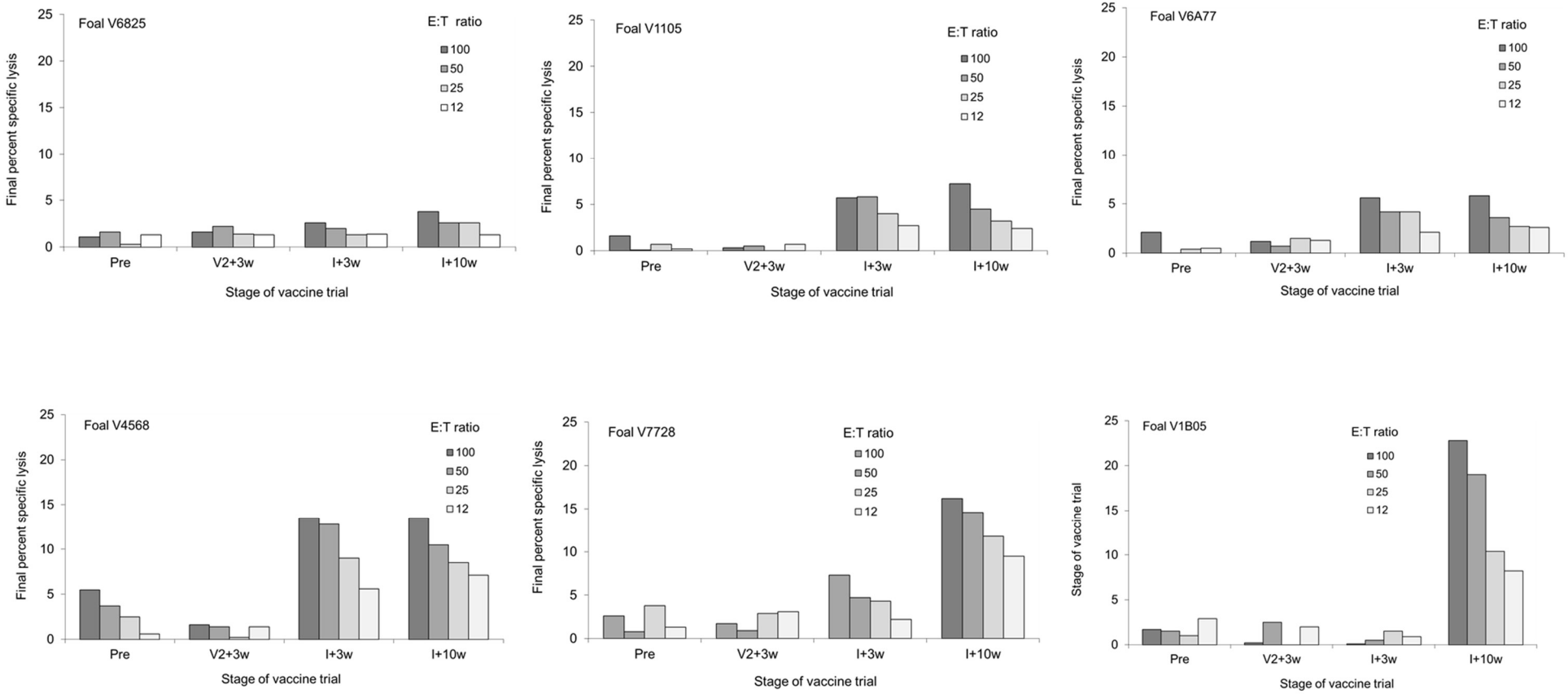

Foal C144D
Foal C4033
Foal C4933
Foal C4B05
Foal C4150
Foal C6633
E:T ratio
100
50
25
12
Final percent specific lysis
Pre
V2+3w
I+3w
I+10w
Stage of vaccine trial

Figure 5b


Foal V6825
E:T ratio
100
50
25
12
Final percent specific lysis
Pre
V2+3w
I+3w
I+10w
Stage of vaccine trial
Foal V1105
Foal V6A77
Foal V4568
Foal V7728
Foal V1B05
Stage of vaccine trial

Supplementary Table 1. Log_{10} VN antibody titres/ml before and after vaccination and following EHV-1 challenge infection

Foal Number	Sample Point			
	Pre V1	V2+2w	I+2w	I+5w
Controls:				
4B05	2.15	1.55	1.95	>3.15
4033	1.44	1.14	>3.15	>3.15
4933	1.35	0.75	2.45	3.05
6633	1.35	0.55	2.64	>3.15
144D	1.14	1.14	>3.15	>3.15
4150	1.14	0.75	2.94	>3.15
Mean	1.48±0.37	0.98±0.37	2.71±0.47	3.13±0.04
Vaccinates:				
7728	0.75	2.55	3.05	>3.15
1B05	2.25	>3.15	>3.15	>3.15
6825	2.15	>3.15	>3.15	>3.15
4568	2.15	2.94	>3.15	>3.15
1105	1.85	1.95	3.05	3.05
6A77	1.14	2.85	>3.15	>3.15
Mean	1.71±0.62	2.76±0.46	3.12±0.05	3.13±0.04

Supplementary Table 2. Mean clinical scores, pyrexia, nasopharyngeal (NP) shedding and cell associated viraemia in control or gM-vaccinated foals following intranasal challenge infection with EHV-1, strain Ab4/8. The presence of mucopurulent nasal or ocular discharge at each nostril or eye and submandibular lymph node enlargement were assessed and scored as 0 (absent), 1 (present, mild), 2 (present, moderate) or 3 (present, severe). Total = total number of days, then first and last day of detection.

Foal status	Clinical Score	Pyrexia (Days)			NP virus shedding (Days)			Cell associated viraemia (Days)		
		Total	First	Last	Total	First	Last	Total	First	Last
Controls:										
Mean	21.2	4.5	2.0	5.5	8.3	1.3	8.8	6.2	3.0	15.3
St Dev	7.8	1.0	0	1.0	1.6	0.5	1.0	1.0	1.3	3.9
Vaccinates:										
Mean	8.3	1.2	3.0	3.7	2.8	1.2	4.0	2.5	4.7	8.3
St Dev	6.2	1.0	2.4	2.7	2.3	0.7	3.6	0.5	0.8	2.1

1