RURAL INDUSTRIES RESEARCH
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Improved vaccine strategies for management of equine herpesviruses

A report for the Rural Industries Research and Development Corporation

by J. Millar Whalley and Daria N. Love

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Researcher Contact Details

Associate Professor Millar Whalley
Department of Biological Sciences
Macquarie University
Sydney
NSW 2109

Phone: 02 9850 8200
Fax: 02 9850 8200
Email: mwhalley@rna.bio.mq.edu.au

In submitting this report, the researcher has agreed to RIRDC publishing this material in its edited form.

RIRDC Contact Details

Rural Industries Research and Development Corporation
Level 1, AMA House
42 Macquarie Street
BARTON ACT 2600
PO Box 4776
KINGSTON ACT 2604

Phone: 02 6272 4539
Fax: 02 6272 5877
Email: rirdc@rirdc.gov.au.
Website: http://www.rirdc.gov.au

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Foreword

As outlined in the background to the Equine Research and Development Program of RIRDC, the horse industry is one of Australia's biggest industries and is worth more than $15 billion a year. There are more than 1.2 million horses used for racing, equestrian sports, and recreation and there is a large breeding industry, with Australia producing the second highest number of thoroughbred foals in the world, after the United States. Minimization of infectious diseases is important for effective management throughout the industry. Among infectious agents recognized as posing a serious and ongoing threat are the equine herpesviruses (EHV), in particular EHV-1 which can cause abortion in pregnant mares and neurological disease. These outcomes can be financially disastrous for breeders, while respiratory illness caused by both EHV-1 and the related EHV-4 can adversely affect racing performance.

Therefore, consistent with the first RIRDC Five-Year Plan, the objectives of the project were to improve reproductive efficiency and respiratory health in horse populations through reduction of incidence of herpesvirus abortion and respiratory infection. Prior to 1997 in Australia, we were in the unusual position of being able to investigate the epidemiology of EHV-1 and EHV-4 in unvaccinated horse populations. Building on this background, the project aimed a) to obtain updated information on the prevalence of EHV-1 in a population of horses that had been routinely vaccinated with an imported vaccine introduced in 1997 b) to investigate the use of the vaccine in the field and c) to make a preliminary assessment of the potential of new types of vaccines for EHV 1 and EHV-4 based on recombinant DNA methodology.

The report details the results of large scale serological surveys of the prevalence of antibody to EHV-1 and compares this 2000 data with that of 1995, obtained prior to vaccination. Secondly by using similar serological analysis and sampling mares and foals intensively over time, the percentage of mares and foals that respond to vaccination in the field was determined. Thirdly, new subunit vaccines based on virus protein or DNA were assessed for ability to induce antibody responses against EHV-1, and the results compared with those of the currently used whole virus vaccine. The development of new vaccines may offer extra protection against EHV-1 or EHV-4 disease and also provide the possibility of import replacement and export potential.

This project was funded from industry revenue which is matched by funds provided by the Federal Government.

This report, a new addition to RIRDC's diverse range of over 800 research publications, forms part of our Equine R&D program, which aims to assist in developing the Australian horse industry and enhancing its export potential.

Most of our publications are available for viewing, downloading or purchasing online through our website:

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- purchases at www.rirdc.gov.au/eshop

Simon Hearn
Managing Director
Rural Industries Research and Development Corporation

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Executive Summary

Improved Vaccine Strategies for Management of Equine Herpesvirus Diseases

Objectives

The objectives of the project were consistent with the first RIRDC Five-Year Plan which had as part of its overall strategy the improvement of reproductive efficiency and respiratory health in horse populations. This aim could be at least partly addressed by reducing the incidence of herpesvirus abortion and respiratory infection. In this context the project had the specific aims of a) obtaining updated epidemiological information on incidence of equine herpesviruses 1 (EHV-1) and equine herpesvirus 4 (EHV-4) in populations of horses that had been vaccinated, and b) assessing the effectiveness and use of different types of vaccines for EHV 1 and EHV-4.

Background

EHV-1 is a cause world-wide of epidemic abortion, perinatal mortality, respiratory disease and occasionally neurological signs in horses. Abortion is the most dramatic and frightening outcome of EHV-1 infection, and an epidemic outbreak can be financially disastrous for breeders, with loss of clients and large insurance payments. Respiratory illness caused by EHV-1, or the closely related EHV-4, can adversely affect racing performance. Although there is a short lived period following infection when horses are protected against EHV-1 there is generally not a sufficiently high level of long term immunity to consistently protect against EHV-1 disease. Horses can therefore be re-infected several times during their lifetime, and vaccination strategies are complicated by the ability of herpesviruses to establish a lifelong latent infection in the host animal. Prior to the introduction of a whole virus vaccine in 1997 in Australia, we were in the unusual position of being able to investigate the epidemiology of EHV-1 and EHV-4 in unvaccinated horse populations. This provided baseline data on the prevalence of EHV-1 and EHV-4 in the absence of vaccination, and supported the concept of the silent cycle of infection in which the viruses can persist in a population in the absence of abortion events.

Within this project three main bodies of research were carried out:

1. **Epidemiological studies:** These were cross-sectional studies that investigated the effects of vaccination on the circulation of EHV-1 in horses on a stud farm examined previously prior to vaccination
2. **Testing responses to whole virus vaccine in the field**: Antibody tests were used to determine the ability of the current vaccine and regimen to induce immune responses in horses in the field situation.
3. **Assessing immunogenicity of a subunit vaccine and comparing with existing vaccine:** This (a) measured the ability of a single EHV-1 virus protein (EHV-1 gD) produced in cell culture or delivered as naked DNA to induce antibody responses in horses, and (b) compared these responses with the commercially available whole virus vaccine.

Results

1. Epidemiological studies

These cross-sectional studies provided a snapshot of the EHV-1 antibody status of horses at specific times, and involved the sampling and testing of 236 mares and their foals in 2000, and comparison with 229 mares and foals in 1995, on the same large stud farm. Specific antibody tests that could discriminate between EHV-1 and EHV-4 were used, and detailed statistical analysis was applied to the results from the populations.

The prevalence of EHV-1 antibody-positive mares at February 2000 was not significantly different from the same time in 1995, even though these mares had been vaccinated in the 5^{th}, 7^{th} and 9^{th} months of gestation in the previous two years. However, there was a significant difference in the prevalence of EHV-1 antibody-positive foals. Previous analysis of the 1995 data had shown that the EHV-1 antibody-positive foals were likely to be positive due to recent infection. While the results from the 2000 study suggested a similar spread of EHV-1 infection from mares to their foals and then from these foals to other susceptible foals, the small number of EHV-1 antibody-positive foals made confirmation of this spread difficult. However, when the results from the two studies were combined, the

observations from the 1995 study were confirmed, indicating that mares had experienced a recent EHV-1 infection or recent reactivation of a previously latent EHV-1 infection and were thus a potential source of virus for their foals. In the 1995 study there was no difference in the EHV-1 ELISA absorbance or the average age of EHV-1 antibody-positive foals, whether or not their dam was antibody-positive or negative. This suggested that these antibody-positive foals had also been recently infected and were not antibody-positive due to maternally derived antibody.

The reduction in the prevalence of EHV-1 antibody-positive foals on the farm in 2000 suggested that fewer foals had become infected with EHV-1 in the period prior to the survey in February 2000. It is likely that this reduction reflected a difference in the number of mares acting as a source of EHV-1 to infect the foals. The factors that lead to reactivation of latent EHV-1 infection are not completely understood, although it is known that episodes of stress are associated with herpesvirus reactivation. Thus, differences in the mare population on the farm between 1995 and 2000, differences in various management procedures and in seasonal variables such as extremes of weather may all contribute to differences in the number of mares that reactivate a latent EHV-1 infection early in their lactation. This in turn would affect the number of foals that become infected from their dams or other mares in the group and so affect the spread of EHV-1 throughout the susceptible foal population. It is possible also that vaccination has reduced the amount of virus shedding. However the data suggests that it is likely that seasonal, nutritional, or management differences played a more significant role in the number of mares that reactivated previously latent EHV-1 infections in 1995 relative to 2000. It is important to note from related research on presence of virus DNA, that foals were still being infected on this farm prior to weaning.

A follow-up cross-sectional study in 2001 supported the results for 2000. The data obtained in 2001 suggested that changes in the prevalence of EHV-1 antibody-positive foals was not due to reduced shedding of virus by vaccinated mares, but was rather more likely to be due to environmental conditions/management practices that placed the mares under stress which resulted in reactivation of latent infections. On the farm involved in these studies the pregnant mares were kept strictly away from the mares and foals and these animals were kept separate from weanlings and yearlings. Thus, there was no opportunity for foals on this farm to come into contact with older horses, except for mares with foals at foot. Consequently, the virus source for these foals was mares/foals in their pre-weaning groups. Taken together, these cross-sectional analyses of EHV-1 antibody support the continuation of the cycle of silent EHV-1 infection in the mare and foal population in the absence of outbreaks of EHV-1 abortion.

Due to the ubiquitous presence of EHV-4 antibody throughout the population, it was not possible to dissect the circulation of EHV-4 in the same way as is possible with EHV-1. However DNA testing of nasal secretions in young foals has confirmed that EHV-4 is still infecting animals at a young age, similarly to EHV-1. Therefore, although the current vaccine can elicit immune responses in a percentage of horses (see below) and is reported as reducing clinical disease following experimental challenge, the cycle of EHV-1 (and EHV-4) infection continues in the vaccinated population in a similar manner to unvaccinated populations. It is clear that there are yearly fluctuations in prevalence of antibody-positive foals at weaning and that management practices other than the current vaccination strategy alone must be addressed in order to reduce the number of EHV-1-infected mares that consequently infect foals pre-weaning in the silent cycle of infection.

2. Testing responses to whole virus vaccine in the field

The lack of a significant change in prevalence of EHV-1antibody-positive mares and foals following the introduction of routine vaccination prompted an investigation of the antibody responses of mares and foals to vaccination. In order to obtain some knowledge of this crucial aspect of current vaccination strategies we investigated the serological responses of mares and weanlings following vaccination with the current whole virus vaccine used as per the manufacturer's recommendations.

Serum samples were collected from 159 mares and 101 foals resident on a stud farm in the Hunter Valley of NSW in February 2000 and again at three intervals over the next six months. Two assays were used to determine the animals' response to vaccination, based on two different components of the virus: the surface glycoprotein D (gD) that detects antibodies to both EHV-1 and EHV-4, and the gG test used above, that differentiates between antibodies to EHV-1 and EHV-4. Responses to both assays were interpreted using liberal criteria: that is, an animal needed only to show a single response to vaccination at any time during the vaccination regimen to be considered a "responder" and this response could be detected using either assay. Using gD and gG assays and the definition criteria outlined in this study, up to 29% of mares and 47% of the foal population responded to the vaccine. There was generally good agreement among categories using both assays, especially in the foal population. Seventy-nine of the 159 mares were the dams of 79 of the 101 foals. The data for this sub-population was analysed, and was shown statistically to be similar to the data from the population as a whole.

A certain percentage (~16%) of mares was found to be persistently sero-positive to EHV-1, probably reflecting the underlying experience of the general population to EHV-1 infection. These mares did not respond to vaccination. Consistent with this, vaccination responses were seen only in mares with the lower antibody levels at the time of initial vaccination. Furthermore, responder foals were more likely to have been born to responder mares than to other mares, which could be ascribed in part to the low level of antibody passed to the foal from the mare in colostrum.

One explanation for the observations in persistently sero-positive mares or any horse with high antibody levels is that the immunogenicity of the EHV-1 antigens in the vaccine was reduced by the high levels of EHV-1 antibody present at the time of vaccination preventing an active response by the mare. Non-responder foals had high pre-existing antibody levels using the gD ELISA although there was no corresponding difference between non-responders and responders using the EHV-1-specific gG ELISA. This suggested that high levels of pre-existing antibody to EHV-4 may interfere with active immunization against EHV-1.

There was no association observed between the response of mares to vaccination and the number of previous vaccinations. The manufacturer recommends that mares should be vaccinated in their 5^{th}, 7^{th} and 9^{th} month of gestation every year. This is a significant cost for the industry and identification of these non-responder mares along with persistently sero-positive mares, and reassessment of vaccination policies in these groups may represent considerable savings to the industry if management includes the multiple use of such vaccines in pregnant mares.

Age has been reported as a critical factor in determining responses to vaccination in foals. In this study low numbers of responders to vaccination were observed among young foals, with the majority of the responder foals aged four months or older. The presence of high levels of maternal antibody in younger foals to EHV-4 may inhibit a response to vaccination, suggesting that it may be difficult to effectively vaccinate foals less than five months of age against EHV-1/4 using available vaccines.

If young foals are becoming infected with EHV-1 before the time of recommended vaccination, alternative control strategies may be required in order to protect this group of horses from becoming latently infected persistently sero-positive animals. Additionally, if less than half of the foal population is responding to vaccination, the factors contributing to this need investigation.

The serological response to vaccination reported in this paper is not necessarily a measure of protection induced following vaccination. Despite a considerable body of research over many years, the precise determinants of protective immunity to EHV-1 have not been defined, nor is it known what proportion of responders is required in a population to minimise spread and provide herd immunity. However, as is known with many persistent and latent infectious diseases such as herpesviruses, population susceptibility plays a less critical role than it does in acute infections and percentage of responders and herd immunity may not be an issue in protection against EHV-1 infection. As well, parent to offspring spread is a feature of many latent infections and EHV-1 infection is no exception. Therefore the key to control of EHV-1 would appear to be prevention of infection of very young foals. Whether or not this can be achieved has yet to be demonstrated, but the emergence of a variety of new vaccine technologies that can be targeted to specific components of the immune system may eventually make this goal a reality.

3. Comparative vaccine study

A surface protein of EHV-1, glycoprotein D (gD) had been previously identified as a potential vaccine candidate, using mouse models, but had not previously been tested in horses. This 'gD subunit vaccine' was produced in cell culture and inoculated into groups of adult horses at different doses.

Following a single inoculation with any dose of the gD subunit vaccine 22 out of a total of 24 horses (> 90%) showed large increases in gD antibody. All groups showed a similar effect and there were no significant differences between the mean gD antibody levels of the different vaccine dose groups. In addition all groups had a significant rise in virus neutralising antibody, one element of a protective immune response. This experiment demonstrated the immunogenicity of the gD subunit vaccine, and the low dose was selected as suitable for further experimentation and comparison with other vaccine strategies.

To follow up the encouraging results for the gD subunit vaccine, we compared the ability of EHV-1 gD delivered either as protein (the gD subunit vaccine), as DNA alone, or as DNA followed by protein, to elicit antibody responses in horses. These EHV-1 gD formulations were also compared with antibody responses to the whole virus vaccine containing both EHV-1 and EHV-4 antigen. Horses were divided into groups of seven or eight after randomizing for levels of pre-existing antibody. Consistent with the results in the previous experiment, all seven out of seven horses in the gD subunit vaccine group responded after a single inoculation. This included strong increases in both ELISA antibody and in virus-neutralizing antibody. Similar results were obtained with the group inoculated with the whole virus vaccine, which although showing more variable ELISA antibody levels, also elicited virus neutralizing antibody in all horses. In contrast, only one horse responded to a single dose of EHV-1 gD DNA, while two horses responded after a second dose. In this DNA-only group there was no significant increase in mean virus neutralizing antibody after two inoculations, although in other experiments we had found 50% of horses to respond. However, in the prime-boost group, six out of seven gD DNA-primed horses showed increases in ELISA absorbance following boosting with gD subunit protein, and increases in virus neutralizing antibody. All vaccine formulations or strategies generated a similar IgG isotype profile, which is an indication of the balance between antibody and cell-mediated immune responses.

Hence although only a single antigen, the EHV-1 gD homologue, delivered as a recombinant protein or in a DNA-prime/protein-boost protocol, generated comparable antibody responses to those of an inactivated whole virus vaccine. It is likely that in the near future there will be a new generation of animal vaccines is likely to be based on recombinant DNA technologies. One relatively straightforward approach is that of virus subunit preparations such as the EHV-1 gD subu it vaccine tested here. These have merit for reasons of safety, flexibility in generating cocktails of vaccine components, ability to distinguish vaccine from natural infection, and production without generating large amounts of infectious virus. The ability of the EHV-1 gD to evoke similar neutralizing antibody responses to the whole virus vaccine indicates that this is an excellent candidate for inclusion in a new subunit vaccine against EHV-1. Furthermore, since antibody to EHV-1 gD also neutralizes EHV-4, it is likely that any protective responses induced by the gD subunit vaccine against EHV-1 will also act against EHV-4. Hence we believe that the results with this subunit vaccine justify further testing, in particular by challenging vaccinated horses with EHV-1 or EHV-4 and assessing whether this vaccine offers protective immunity.

Outcomes and implications:

Taken together, our data analysis support the findings of previous studies which described the cycle of silent EHV-1 infection in the mare and foal population in the absence of outbreaks of EHV-1 abortion. This is continuing even in the face of widespread vaccination of the population.

Therefore management practices other than the current vaccination strategy alone must be addressed in order to reduce the number of EHV-1-infected mares that are a source for infection of foals pre-weaning in the silent cycle of infection. This could include careful attention to eliminating practices that may facilitate transmission of viruses between animals during procedures in the crush.

Our data on responses to vaccination indicated that in a field situation, at most only 30% of mares and 50% of foals were responding to the currently used vaccine used as per manufacturer's instructions.

Identification of non-responder mares along with persistently seropositive mares using antibody testing may allow a more selective vaccination policy to be adopted, as those animals do not respond to vaccination, either initially or

subsequent to previous vaccinations. In addition the poor response to vaccination of young foals was supported in this study, with the majority of the responder foals aged four months or older. Taken together the data on non-responding mares and foals suggest that a reassessment of vaccine strategies and regimens could provide considerable savings to the industry, especially where management presently includes multiple vaccinations of pregnant mares.

The results of immune responses to the envelope glycoprotein D of EHV-1 (gD subunit vaccine) was selected as a trial antigen showed that antibody responses to gD delivered as a protein subunit were highly comparable to those elicited by the whole virus vaccine. The results support the potential of this technology to identify specific vaccine antigens that may provide equivalent or greater protection against EHV diseases than available whole virus vaccines. The study also indicated that at least at this stage the use of direct DNA as a vaccine requires further research before application to the horse. 1. Furthermore, since antibody to EHV-1 gD also neutralizes EHV-4 it is likely that any protective responses induced by the gD subunit vaccine against EHV-1 will also act against EHV-4.

The results from the project warrant further assessment of this subunit formulation and in particular it would be important to carry out challenge experiments to determine whether the immune responses elicited correlate with protection against EHV-1 and EHV-4. disease in horses. This could be addressed in the first instance by vaccinating weanling foals and then infecting via the respiratory route. A reduction in clinical signs, virus shedding and other parameters would support the development and commercialization of the gD subunit formulation as a useful vaccine against EHV-1.

Publications arising:

- Gilkerson, J. R., Whalley, J. M., Drummer, H. E., Studdert, M. J. & Love, D. N. (1999). Epidemiological studies of EHV-1 in Thoroughbred foals: a review of studies conducted in the Hunter Valley of New South Wales between 1995 and 1997. *Veterinary Microbiology* 68, 15-25.
- Ruitenberg, K. M., Love, D. N., Gilkerson, J. R., Wellington, J. E. & Whalley, J. M. (2000). EHV-1 glycoprotein D DNA inoculation in horses with pre-existing EHV-1/EHV-4 antibody. *Veterinary Microbiology* 76, 117-127.
- Gilkerson, J. R., Love, D. N. & Whalley, J. M. (2000). Incidence of equine herpesvirus 1 infection in Thoroughbred weanlings on two stud farms. *Australian Veterinary Journal* 78, 277-278.
- Foote, C. E., Love, D. N., Gilkerson, J. R., & Whalley, J. M. (2002). Serological responses of mares and weanlings following vaccination with an inactivated whole virus equine herpesvirus 1 and equine herpesvirus 4 vaccine *Veterinary Microbiology (in press)*
- Foote, C. E., Love, D. N., Gilkerson, J. R., & Whalley, J. M. Seroprevalence of equine herpesvirus 1 in mares and foals on a large Hunter Valley stud farm in years pre- and post- vaccination. *Australian Veterinary Journal* (under review).

Conference presentations:

- Foote, CE, Love, DN, Gilkerson, JR, & Whalley, JM (2001) Improved vaccine strategies for Equine Herpesvirus 1. *The International Horse Industry Symposium*, Sydney. p231.
- Foote, CE, Love, DN, Gilkerson, JR, & Whalley, JM (2001) Serological responses of mares and weanlings following vaccination with an inactivated whole virus Equine Herpesvirus 1 and Equine Herpesvirus 4 vaccine. *23rd Bain Fallon Memorial Lectures*, Yeppoon.
- Gilkerson, JR., Foote, CE., Love, DN. and Whalley, JM. (2001) Seroepidemiological studies of EHV-1. *23rd AEVA Bain Fallon Memorial Lectures.* Yeppoon Qld.
- Gilkerson, JR., Foote, CE., Love, DN. and Whalley, JM. (2001) Epidemiological studies of equine herpesvirus 1 and a review of EHV-1 vaccines (conventional and experimental). *Australian Veterinary Association Conference.* Melbourne Vic.
- Gilkerson, JR., Foote, CE., Love, DN. and Whalley, JM. EHV-1 Epidemiology. *3rd Australian Veterinary Virology Conference.* University of Sydney. NSW.

1. Introduction

Importance of equine herpesvirus 1 to the equine industry

Equine herpesvirus-1 (EHV-1) continues to be a major cause world-wide of epidemic abortion, perinatal mortality, respiratory disease and occasionally neurological signs in horses. Abortion is the most dramatic and frightening outcome of EHV-1 infection, and an epidemic outbreak can be financially disastrous for breeders, with loss of clients and large insurance payments. Since EHV-1 was first recognised and isolated in Australia in 1977, it has become endemic in the Australian population of approximately 1.2 million horses The country has seen some of the most dramatic outbreaks of equine herpesvirus abortion, while the virus has recently manifested itself in parts of Australia in association with severe neurological disease. Respiratory illness caused by EHV-1, or the closely related EHV-4, can adversely affect racing performance. Equine herpesvirus infection appears also to render horses more susceptible to other diseases, and in young animals, strangles, and rattles are already of significance to the industry.

Therefore the Australian horse industry has viewed EHV-1-related diseases to be of major importance, and this is reflected within the RIRDC Five Year Strategic Plan in Programs 7 (Reduction of reproductive failure in mares) and 3 (Improvement of the respiratory health of horses). Strategies and targets for these programs include identification and prevention of viral (EHV-1) abortion, including epidemiological investigation and development of vaccines.

Epidemiology of EHV-1

The source of EHV-1 responsible for epidemics of viral abortion has not always been readily identifiable (Allen & Bryans, 1986). The value of serological analysis in an unvaccinated population has been elegantly demonstrated by studies of Gilkerson et al., (1997b), which were made possible by collaboration with stud farm managers and veterinarians. Using a specific antibody test for EHV-1 or EHV-4 (Crabb et al., 1995), we obtained evidence of EHV-1 infection in Thoroughbred foals as young as 30 days of age. This illustrated that EHV-1 is circulating in the mare and foal population on this farm and that this population could be a reservoir from which mares may be subsequently infected with EHV-1. Circulation of infectious EHV-1 had not been previously reported in foals of this age and this has implications for management of foals and vaccination regimens.

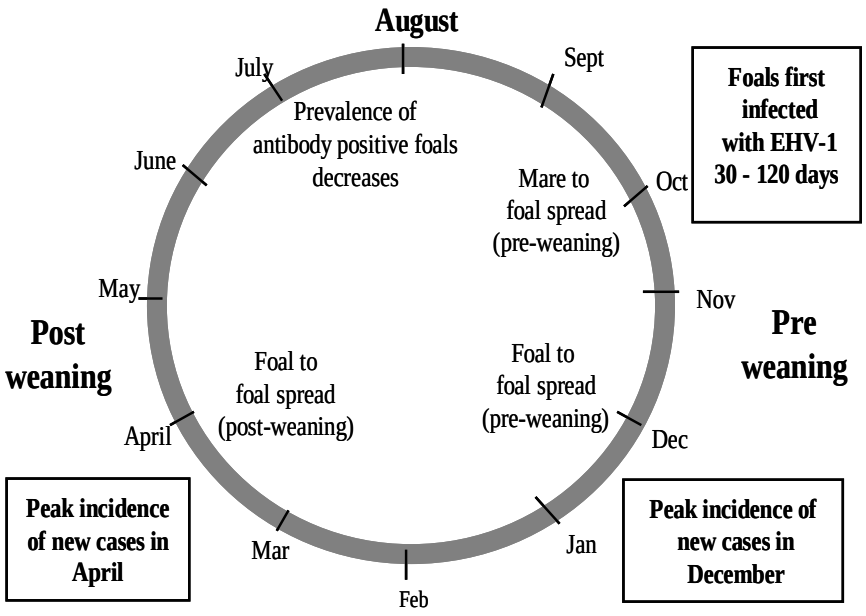

Fig. 1. Annual cycle of EHV-1 infection

Approximately 50% of older horses (broodmares and racehorses) had serological evidence of EHV-1 infection while approximately 30% of weanlings had EHV-1 antibodies. It is worth noting that the ability to sample newborn foals enabled the determination of a reliable baseline for the test and hence for the first time there were definite answers for levels of

specific EHV-1 antibody (Gilkerson et al., 1997b).

By combining data from these studies, we developed a scheme for the annual cycle of EHV-1 infection through horse populations. This schematic representation (Fig. 1) is a useful working model which will assist in designing strategies for vaccination and management.

These studies were made prior to the widespread introduction of commercial vaccination in Australia, and therefore serve as a unique set of reference data from which to assess the effect of vaccination on horse populations.

Vaccination and management

Natural immunity to EHV-1 infection following infection, while apparently reducing the severity of clinical outcomes, does not generally provide sufficiently high level of long term immunity to consistently protect against EHV-1 disease. Horses can therefore be re-infected several times during their lifetime. In common with other herpesviruses such as chicken pox and herpes simplex virus of humans, EHV-1 can establish a lifelong latent infection in its host, and may carry genes that can suppress the host's immune mechanisms. Vaccines based on whole virus have been available overseas and since 1997 in Australia, However these have historically been of limited efficacy (reviewed by Gilkerson et al., 1997a). In the case of the currently used vaccine, it has been reported recently that following experimental challenge, vaccination of foals and pregnant mares with Duvaxyn EHV1.4 reduces the risk of abortions and outbreaks of respiratory disease caused by EHV-1 and EHV-4 (Heldens et al., 2001). This suggests that vaccination should be useful in the management of EHV diseases. Nonetheless vaccinated animals were not fully protected against EHV disease. Problems associated with the use of EHV vaccines are highlighted by the report from Kentucky of a three fold increase in 1996/97 in numbers of abortions and neonatal deaths from mares vaccinated at 5, 7 and 9 months. Investigations of farms where multiple cases had occurred indicated that management practices for limiting EHV-1 abortion had not been adhered to, with introduction of mares and other horses from outside the farm and mixing of mares during the critical last three months of gestation. In a report from Belgium in 1999 (van der Meulen et al., 2000), among cases diagnosed with EHV-1 infection, there were twenty -two abortions, four neonatal foal deaths and five cases of nervous system disorders. 30% of the animals involved had been vaccinated. These examples illustrate that although available vaccines may reduce the severity or frequency of EHV-1 disease following experimental challenge (e.g. Heldens et al., 2001), the use of vaccines without appropriate management practice may be insufficient to prevent abortion.

Another unsatisfactory feature of available whole virus vaccines is that they induce a similar spectrum of antibodies to the natural infection. Therefore it is not possible to use serology to readily follow the incidence of EHV-1 infection in vaccinated horse populations. This removes a significant and valuable component for management practice. Apart from the above biological aspects, current whole vaccines require multiple doses, which in turn make them very expensive.

Hence the history of EHV-1 highlights the importance of good management strategies based on knowledge of the epidemiology of the virus and the judicious use of an appropriate vaccine. While management procedures to minimise the effects of EHV-1 disease represents a considerable annual cost to horse breeders, there has been little research into the changes in seroprevalence of this virus infection over time. In particular there have been few if any comparisons on the prevalence of EHV-1 antibody in vaccinated populations as compared with a similar population without vaccination. Having carried out extensive sampling and analysis of sera prior to the introduction of the whole virus vaccine, we were in an excellent position to investigate the effect of extensive vaccination on the presence of EHV-1 in a horse population. This component of the project was addressed by cross-sectional studies that analysed the ELISA antibody in horses on the same stud farm in 1995 and in 2000. These studies are essentially a 'snapshot' of the prevalence of EHV-1 antibody in the mare and foal population in early February in years before and following vaccination. This data was applied to reveal key features of the circulation of EHV-1 in a vaccinated population.

Potential for new vaccines

The key elements that may be required for effective vaccination against EHV-1 have been discussed by Allen *et al*. (1999). These include virus-neutralizing antibody at mucosal respiratory surfaces, systemic virus-neutralizing antibody, and EHV-1 specific cytotoxic T-lymphocytes (CTLs). In the absence of mucosal immunity, EHV-1 can replicate within 12 hours in the respiratory tract, with subsequent shedding of virus. Protection against the respiratory infection caused by both EHV-1 and EHV-4 will therefore require high levels of virus-neutralizing antibody that can act to clear virus from mucosal sites of primary or recurrent infection. Virus-neutralizing antibody will limit also the circulation of free virus particles in the blood or lymphatic systems. In addition, a cell-mediated immune response in the form of cytotoxic T cells (CTLs) is necessary to destroy infected lymphocytes in the respiratory tract and in reducing the cell-associated viraemia of EHV-1. Ideally a truly effective vaccine would elicit each of the components of this multifaceted immune response.

Against the background of what may be termed 'traditional' vaccines described above, a variety of contemporary approaches are being used to analyse immune responses to individual virus components. This offers the prospect of eliciting more defined and targeted immune responses as required for protection (Allen et al., 1999). In particular the vaccine potential of envelope glycoproteins of EHV-1 has been investigated using a murine model of EHV-1 respiratory disease (Awan et al., 1991, Osterrieder et al., 1995; Packiarajah et al., 1998; Stokes et al., 1997; Tewari et al., 1994). Of the glycoproteins analysed, EHV-1 glycoprotein D (EHV-1 gD) appears to provide among highest levels of protection in the models used. EHV-1 gD delivered as protein elicits high levels of ELISA and virus neutralising antibody, as well as delayed type hypersensitivity (DTH) and proliferative lymphocyte responses to EHV-1 antigen (Stokes et al., 1997; Tewari et al., 1994; Zhang et al., 1998). When all these results are interpreted, of the individual antigens studied the most effective appears to gD at least in EHV-1 mouse models. EHV-1 gD delivered as DNA led to similar protective responses (Ruitenberg et al., 1999). A prime-boost strategy in which inoculation of gD DNA was followed by recombinant gD as protein led to significantly higher neutralizing antibody titres and faster clearance of challenge virus than either recombinant protein or gD DNA alone (Ruitenberg et al., 2000b). As yet there are few reports of any testing of these recombinant proteins in the horse. Audonnet et al. (1999) reported the use of a canarypoxvirus to express gB, gC and gD of EHV-1 (Kentucky D strain), with some reduction in virus shedding after experimental challenge. Previous studies in our laboratory showed that when adult horses which had pre-existing antibodies to EHV-1 and EHV-4 were inoculated intramuscularly (i.m.) with EHV-1 gD DNA, a rise in ELISA and EHV-1 neutralising antibodies occurred in over fifty percent of horses (Ruitenberg et al., 2000a).

The use of recombinant protein subunits allows a great deal of flexibility in formulation with the possibility of using mixtures of antigens from one or several different viruses or even other types of pathogens. Complex cocktails can be designed to optimize responses to different arms of the immune system. Furthermore, such proteins can be used in the prime-boost strategy described below.

Direct inoculation of DNA encoding a vaccine antigen can elicit a wide spectrum of antibody and cell-mediated immune responses (Robinson & Pertmer, 2000), and this approach is being pursued in livestock and other domesticated animals (Littel-van den Hurk et al., 2000). We have shown that EHV-1 gD delivered intramuscularly as DNA can induce specific antibody responses in adult horses including neutralizing antibody (Ruitenberg et al., 2000a). The antibody responses in horses to DNA were induced in the face of pre-existing antibodies, supporting the premise that DNA vaccination may be able to stimulate the immune system early in life. This may be extremely advantageous, given that foals are being infected at a very young age when they have high levels of maternally-derived antibody.

Other data from the mouse model indicate that a prime-boost strategy (using DNA followed by a protein boost using subunit vaccine products) enhances antibody responses yet maintains the desired Th1 response (Ruitenberg et al., 2000b). Prior to this project, there are no reports of this strategy applied to the horse.

2. Objectives

In this project we have built on the above experimental and conceptual background to carry out three studies with the following aims:

1. to investigate the effects of vaccination on the circulation of EHV-1 in horses on the stud farm examined previously prior to vaccination **(Cross-sectional studies in 1995 and 2000)**
2. to determine the ability of the current vaccine and regimen to induce immune responses in horses in the field situation **(Responses to whole virus vaccine in the field)**
3. to measure the ability of EHV-1 gD antigen to induce antibody responses in horses, and to compare these responses with the commercially available whole virus vaccine. This encompasses a number of the essential elements to improve vaccine strategies for EHV-1 **(Comparative vaccine study)**

3. Methodology

Experimental designs

(1) Cross-sectional studies in 1995 and 2000

The studies were performed on a large Hunter Valley stud farm where all mares and their foals are mustered annually for routine management procedures in early February. In February 1995, samples had been collected from 229 mares and their foals (Gilkerson et al., 1999b), and none of these animals had been vaccinated. In February 2000 in this project samples were collected from 236 mares and their foals. All mares sampled in 2000 had been vaccinated in 1999 with the EHV-1 and EHV-4 vaccine (Duvaxyn ™, Fort Dodge) according to the manufacturer's recommendations in the 5th, 7th and 9th months of gestation. No foal had received any vaccination prior to sample collection.

(2) Responses to whole virus vaccine in the field

During a six month period in the year 2000 mares received three doses of the combined EHV-1 and EHV-4 inactivated whole virus vaccine (Duvaxyn™, Fort Dodge), while foals received two doses, at approximately one month intervals as per stud farm protocol and in accordance with the manufacturer's recommendations. Blood samples were collected from 159 mares and 101 foals resident on a stud farm in the Hunter Valley of NSW in February 2000 and again at three intervals over the next six months.

Vaccination and Sampling Routine. Foaling reports, procedure listings and horse listings were obtained from the stud farm and information was collected on age of mares, date of birth of foals, and vaccination schedules of mares from the time vaccination was introduced into the farm protocol in 1997. In February 2000 (week 0) mares (aged four to 17 years) and foals (aged three to six months) were vaccinated. Foals were re-vaccinated in March (week 4). As per farm protocol, mares received vaccinations during their 5th, 7th and 9th months of gestation. The vaccination which occurred in February (week 0) was counted by the farm management as the 5th month gestation vaccination. The 7th month vaccination occurred during weeks 8-12 (April/ May), and the 9th month vaccination at week 20 (July). A blood sample was taken from each mare in February 2000 (week 0) and again at weeks 4, 16 and 24. A blood sample was also collected from each foal in February, 2000 (week 0) and again at weeks 4, 8 and 16.

(3) Comparative vaccine study

Thirty horses were allocated to four groups, according to their EHV-1 antibody status (2.2.1). Group 1 received two inoculations of gD subunit vaccine; group 2 received two inoculations of gD DNA; group 3 received one inoculation of gD DNA then one of gD subunit vaccine (the prime-boost strategy), and group 4 received two inoculations of whole virus vaccine. The two inoculations for all groups were administered four weeks apart.

gD subunit vaccine. Sf9 insect cells were infected with a recombinant baculovirus expressing full-length EHV-1 gD (Whalley et al., 1991) as described previously (Love et al., 1993) suspended in phosphate-buffered saline (PBS) and treated to inactivate baculovirus. High, medium and low doses of gD subunit vaccine were prepared in 1 ml PBS from 5 x 10^7, 1 x 10^7, or 2 x 10^6 insect cells respectively and contained the adjuvant Iscomatrix (CSL Limited, Australia).

***EHV-1 gD DNA*. Plasmid pRc/CMV-gD (Wellington et al., 1996), which expresses EHV-1 gD under the control of the human cytomegalovirus immediate early promoter, was grown in *E. coli* DH5α and DNA was extracted and purified using a Plasmid Mega Kit (Qiagen). DNA in PBS was heated at 70°C for 10 min before subsequent inoculation i.m. as single 500 µl doses containing 500 µg.**

Whole virus EHV-1/4 vaccine. Inactivated whole virus vaccine (Duvaxyn ™, Fort Dodge) was administered according to the manufacturer's specifications.

Sample collection

Blood samples from horses were collected into sterile, non-additive Vacutainer tubes (Becton Dickinson) and allowed to clot at room temperature. Serum was removed from the tubes and transferred to 5mL sterile screw-capped tubes and stored at -20°C until required.

Antibody tests

(1) gG. Sera were tested using a type-specific ELISA for EHV-1 and EHV-4 based on glycoprotein G (gG), essentially as described by Crabb et al. (1995). Positive control serum from a known EHV-1 highly positive horse and negative control serum from pre-suckle newborn foals with no IgG were incorporated onto each plate as an internal comparison to control for variation between plates or between ELISA tests performed on different days and to validate the identification of EHV-1 and EHV-4 antibody-positive samples. A serum sample was classified as EHV-1 antibody-positive if the mean absorbance of three replicate wells was greater than 0.129, while the positive cut-off absorbance for an EHV-4 antibody-positive sample was 0.246 (Gilkerson et al., 1997b).

(2) gD. An ELISA based on the envelope glycoprotein D (gD) (Ruitenberg et al., 2000b) was used to measure anti-EHV gD antibodies. gD is an essential component of the virus and is therefore a useful antigen for monitoring the responses to whole virus either vaccine or by infection. This test was used so supplement data from the gG ELISA described above. Due to extensive homology between the gD homologues of EHV-1 and EHV-4, cross-reacting antibodies to EHV-4 gD are measured in this system. EHV-1 antibodies were determined specifically using the gG ELISA of Crabb et al. (1995) which differentiates between antibodies to EHV-1 and EHV-4.

Immunoglobulin isotyping Monoclonal antibodies (Mabs) for equine IgGa, IgGb, IgGc, IgG(T) (Sheoran et al., 1998; kindly provided by Dr Sheoran) and IgA (kindly provided by Dr C. Stokes) were used in the gD-specific ELISA as described by (Ruitenberg et al., 2000a) to determine gD-specific Ig isotypes

Virus neutralizing antibody The ability of serum to neutralize virus infectivity was measured by an end-point assay on the continuous cell line RK13, as the highest dilution of the serum that prevented virus-specific plaques and cytopathic effects. A standard sera of known virus-neutralizing titre was included as a standard in each assay.

Data and statistical analysis.

All data were analysed using the Epi Info 6 statistics program (Dean et al., 1994). For categorical data, contingency tables were constructed to compare observed variables and an odds ratio (OR) calculated using the method of Mehta et al.(1985) to measure the extent of association between the variables. An odds ratio that did not include 1.0 in the 95% exact confidence limits (CL) indicated a significant association. For comparisons of proportions in contingency tables, the Yates corrected Chi Square (χ^2) test statistic was used to measure the association between two variables. The overall χ^2 test statistic was used to measure the association between more than two variables in a contingency table. For prevalence values the exact binomial 95% confidence interval (95% CI) was calculated. For comparison of the mean absorbances of two groups, either a Student's T-test (Minitab®) was used, or alternately the non-parametric Wilcoxon two sample test statistic, as the EHV-1 ELISA absorbances of the test groups was not always normally distributed.

4.Results

(1) Cross-sectional studies in 1995 and 2000

In order to obtain information on the effect of widespread routine vaccination with the currently available vaccine on the prevalence of EHV-1 in a stud farm population, these cross-sectional studies compared the prevalence of EHV-1 antibody in 1995 (pre-vaccination) with that in 2000 (all horses vaccinated). This study employed the EHV-1/EHV-4 specific gG ELISA (Crabb et al., 1995).

1995 - As reported previously (Gilkerson et al., 1999b), of the 229 samples collected from mares in February 1995, 60 samples were EHV-1 antibody-positive (26.2%, exact binomial 95% confidence interval: 20.6 – 32.4), while 26 samples collected from the 229 foals were EHV-1 antibody-positive (11.4%). Mares were significantly more likely to be EHV-1 antibody-positive at the time of sampling than their foals ($\chi^2 = 15.59$, $P < 0.001$). Serum samples from mares were approximately 2.8 times more likely to be EHV-1 antibody-positive than samples from their unweaned foals.

2000 - The prevalence of EHV-1 antibody-positive mares was significantly higher than the prevalence of EHV-1 antibody-positive foals ($\chi^2 = 34.33$, $P<0.01$). Of the 236 serum samples collected from mares in February 2000, 53 were EHV-1 antibody-positive (22.5%). In contrast, 9 of the 236 samples collected from their foals were identified as EHV-1 antibody-positive (3.8%). Samples collected from mares were 7.3 times more likely to be EHV-1 antibody-positive than those collected from their foals.

Table 1. Prevalence of EHV-1 antibody-positive mares sampled in 1995 and 2000

EHV-1 ELISA	Year of sampling 2000	1995	Total
Positive	53	60	113
Negative	183	169	352
Total	236	229	465

Odds Ratio = 0.82 (Exact 95% confidence interval: 0.52-1.28) $\chi^2 = 0.69$ P = 0.405

EHV-1 antibody prevalence of mares: 1995 versus 2000 -When the prevalence of EHV-1 antibody-positive mares in 2000 was compared with that in 1995 a small difference was observed (26.4% in 1995 compared to 22.5% in 2000). However, this difference was not statistically significant (Table 1).

EHV-1 antibody prevalence of foals: 1995 versus 2000 - Foals sampled in February 2000 were significantly less likely to be EHV-1 antibody-positive than foals sampled in February 1995 (Table 2). The prevalence of EHV-1 antibody-positive foals sampled in 1995 (11.4%) was significantly greater than that observed in foals sampled in 2000 (3.8%). Foals born in 1999 and sampled in February 2000 were approximately 3.2 times less likely to be EHV-1 antibody-positive than foals born in 1994 and sampled in February 1995.

Table 2. Prevalence of EHV-1 antibody-positive foals sampled in 1995 and 2000

EHV-1 ELISA	Year of sampling 2000	1995	Total
Positive	9	26	35
Negative	227	203	430
Total	236	229	465

Odds Ratio = 0.31 (Exact 95% confidence interval: 0.12-0.70)
$\chi^2 = 8.44$ P = 0.004*
* Statistically significant at the 99% confidence limit

Average age of foals at the time of sampling: 1995 versus 2000 There was no significant difference in the average age of the foals sampled in 1995, compared with those sampled in 2000 (P=0.78). The average age of the 236 foals sampled in the 2000 study was 122.8 days; the youngest foal was 58 days of age and oldest foal was 183 days of age at sampling. In 1995 the average age of the 229 foals sampled was 123.9 days; the youngest foal was 61 days old, and the oldest foal was 183 days at the time of sampling.

Prevalence of EHV-1 antibody in mares sampled during both studies (1995 and 2000)

Fifty mares were sampled in both 1995 and 2000. When the data from the 50 mares were analysed, the prevalence of EHV-1 antibody-positive samples in 1995 was 24% (12 positive samples, exact binomial 95% confidence interval: 13.1 – 38.2), while the prevalence of EHV-1 antibody-positive samples collected in 2000 was 16% (8 positive samples, exact binomial 95% confidence interval: 7.2 – 29.1). Of the 38 mares that were EHV-1 antibody negative in 1995, 4 mares had seroconverted to EHV-1 in the intervening 5 years, while only 4 of the 12 mares that were EHV-1 antibody-positive in
1995 were still antibody-positive when sampled again in 2000 (Figure 2). Eight of the 12 EHV-1 antibody-positive mares in 1995 were antibody negative when sampled again in 2000, while 34 of the 50 mares sampled in both studies were EHV-1 antibody negative in both 1995 and 2000.

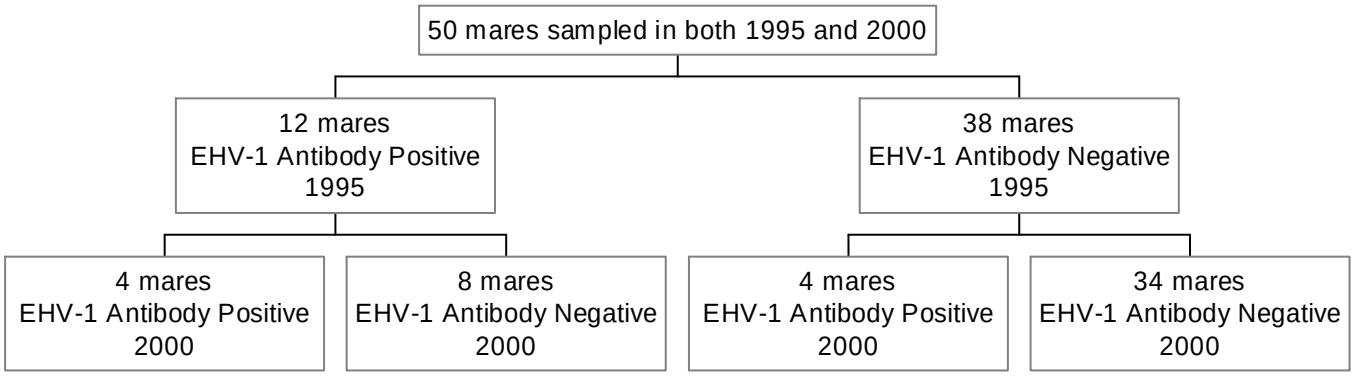

Fig. 2. EHV-1 antibody results of 50 mares sampled in both cross-sectional studies (1995 and 2000)

Relationship between mare and foal EHV-1 status. To further investigate the epidemiology of EHV-1 in this population, the mares and foals were categorised into 4 groups (A, B, C and D) based on the EHV-1 antibody status of each mare and her foal, as per Gilkerson et al.(1999b).

GROUP A: EHV-1 antibody-positive foals from EHV-1 antibody-positive dams
GROUP B: EHV-1 antibody-positive foals from EHV-1 antibody negative dams
GROUP C: EHV-1 antibody negative foals from EHV-1 antibody-positive dams
GROUP D: EHV-1 antibody negative foals from EHV-1 antibody negative dams

1995 - This analysis from data collected in 1995 was reported previously (Gilkerson et al., 1999b) and these data are presented in Tables 3 and 4. Briefly, the EHV-1 ELISA absorbance of EHV-1 antibody-positive foals (Groups A and B) was not significantly different irrespective of the EHV-1 ELISA status of the mare (from Table 3, 0.189 ± 0.057 versus 0.193 ± 0.063, P=0.864). Similarly, there was no significant difference in antibody levels for EHV-1 ELISA negative foals (Groups C and D) regardless of the antibody status of the mare (from Table 3, 0.040 ± 0.042 versus 0.028 ± 0.038, P= 0.065).

In contrast to the foals, the average antibody levels of the two groups of EHV-1 ELISA positive mares (Groups A and C) was significantly different depending on the EHV-1 antibody status of the foals (from Table 3, 0.477 ± 0.156 versus 0.281 ± 0.145, P = 0.009).

2000 - In the 2000 cross-sectional study, the small number of EHV-1 antibody-positive foals (Groups A and B) made meaningful statistical analysis difficult. Only nine EHV-1 antibody-positive samples were collected from the 236 foals sampled. There was no significant difference in the average EHV-1 absorbance between the two groups of EHV-1 antibody-positive foals (0.160 ± 0.034 versus 0.260 ± 0.089) regardless of the antibody status of the mare (from Table 3). There was a statistically significant difference between the EHV-1 ELISA absorbances of the two groups of EHV-1antibody negative foals sampled in 2000 (Table 3).

Negative foals from positive mares (Group C) foals had a higher average EHV-1 ELISA absorbance than EHV-1 antibody negative (Group D) foals from negative mares (from Table 3, 0.089 ± 0.032 versus 0.016 ± 0.024). The small numbers of EHV-1 antibody-positive foals relative to the number of positive mares (53/236 samples collected from mares in 2000) made these data difficult to analyse also. Although there

was an observable difference between the two groups of EHV-1 antibody-positive mares (from Table 3, Group A 0.452 ± 0.280 versus Group C 0.286 ±0.170), this difference was not statistically significant.

Table 3. Average EHV-1 ELISA absorbance classified according to the EHV-1 antibody status of the mare and foal.

Study	Group	EHV-1 Antibody Status	Number	Average EHV-1 Absorbance (foal)	Average EHV-1 Absorbance (mare)
	A	Positive foal & Positive Mare	14	0.189 ± 0.057 range: (0.133 to 0.318)	0.447 ± 0.156 range: (0.212 to 0.656)
	B	Positive foal & Negative mare	12	0.193 ± 0.063 range: (0.131 to 0.334)	0.041 ± 0.038 range: (-0.009 to 0.122)
1995 Data	**C**	Negative foal & Positive mare	46	0.040 ± 0.042 range: (-0.037 to 0.127)	0.281 ± 0.145 range: (0.131 to 0.661)
	D	Negative foal & Negative mare	157	0.028 ± 0.038 range: (- 0.113 to 0.128)	0.34 ± 0.050 range: (-0.174 to 0.125)
	A	Positive foal & Positive Mare	3	0.160 ± 0.034 range: (0.137 to 0.200)	0.452 ± 0.280 range: (0.138 to 0.678)
	B	Positive foal & Negative mare	6	0.260 ± 0.089 range: (0.131 to 0.362)	0.051 ± 0.043 range: (0.009 to 0.127)
2000 Data	**C**	Negative foal & Positive mare	50	0.089 ± 0.032 range: (-0.009 to 0.119)	0.286 ± 0.170 range: (0.131 to 0.877)
	D	Negative foal & Negative mare	177	0.016 ± 0.024 range: (-0.051 to 0.097)	0.024 ± 0.048 range: (-0.259 to 0.121)
	A	Positive foal & Positive Mare	17	0.183 ± 0.054 range: (0.132 to 0.318)	0.448 ± 0.172 range: (0.138 to 0.678)
	B	Positive foal & Negative mare	18	0.197 ± 0.071 range: (0.131 to 0.362)	0.044 ± 0.039 range: (-0.009 to 0.127)
All Data Com-bined	**C**	Negative foal & Positive mare	96	0.041 ± 0.037 range: (-0.037 to 0.127)	0.283 ± 0.157 range: (0.131 to 0.877)
	D	Negative foal & Negative mare	334	0.022 ± 0.032 range: (-0.113-0.129)	0.029 ± 0.049 range: (-0.259 to 0.125)

Table 4. Comparison of the average EHV-1 ELISA absorbances of groups of mares and foals

Study	Test Groups	Foals H value+	Foals P value	Mares H value+	Mares P value
1995	A vs B	0.011	0.918	18.673	<0.001**
	A vs C	31.682	<0.001**	10.343	0.001*
	A vs D	38.345	<0.001**	38.343	<0.001**
	B vs C	28.076	<0.001**	28.071	<0.001**
	B vs D	33.254	<0.001**	0.173	0.677
	C vs D	2.090	0.148	106.216	<0.001**
2000	A vs B	0.600	0.439	5.400	0.020*
	A vs C	8.341	0.004*	0.784	0.376
	A vs D	8.804	0.003*	8.802	0.003*
	B vs C	15.802	<0.001**	15.791	<0.001**
	B vs D	17.321	<0.001**	1.632	0.201
	C vs D	28.609	<0.001**	116.456	<0.001**
Data from 1995 and 2000 combined	A vs B	0.184	0.668	25.504	<0.001**
	A vs C	42.958	<0.001**	12.038	0.001*
	A vs D	48.403	<0.001**	48.396	<0.001**
	B vs C	45.089	<0.001**	45.052	<0.001**
	B vs D	51.104	<0.001**	1.571	0.210
	C vs D	22.210	<0.001**	223.194	<0.001**

\+ Wilcoxon two sample test statistic
* Significant difference at the 95% level of significance
** Significant difference at the 99% level of significance

1995 and 2000 data combined- When the data from both studies were combined, the analysis confirmed the pattern observed in the 1995 study alone (Table 3). There was no statistical difference between the average absorbances of the EHV-1 ELISA positive foals from mares of different antibody status (Table 3, 0.183 ± 0.054 versus 0.197 ± 0.071). When the data for the mares from the 1995 and 2000 cross-sectional studies were combined, the associations observed from the 1995 study were strengthened (Table 3). EHV-1 ELISA positive mares with positive foals (Group A) had significantly higher EHV-1 ELISA absorbances than the EHV-1 antibody-positive mares with EHV-1 antibody negative foals (Group C) which indicates a more recent EHV-1 infection and/or reactivation. Both of the groups of EHV-1 antibody-positive mares had significantly greater average EHV-1 ELISA absorbances than either of the groups of negative mares (Groups B and D). There was no statistically significant difference in the average EHV-1 ELISA absorbance between the two groups of negative mares.

Relationship between month of foaling and EHV-1 antibody in the mares and foals.

Table 5. Association between month of foaling and EHV-1 antibody positivity of the mare in February 2000

Month of Foaling	EHV-1 Antibody Positive	EHV-1 Antibody Negative	Total
ugust	10	33	43
September	14	51	65
October	21	66	87
November[a]	8	33	41
Total	**53**	**183**	**236**

Overall $\chi^2 = 0.39$, 3 df, P= 0.942
a - one mare foaled on the 5th of December but was included in the November data

There was no statistical association between the month of foaling and the likelihood that a mare would be EHV-1 antibody-positive at the time of sampling in February 2000 (Table 5). There was also no relationship ($\chi^2 = 5.12$, p = 0.162) between the month in which a foal was born and the likelihood that the foal would be EHV-1 antibody-positive when sampled in February 2000 (Table 6). Although six of the nine EHV-1 antibody-positive samples were collected from foals born in October 1999, this association was not statistically significant

(p = 0.079) due to the small number of EHV-1 antibody-positive foals. When data from both the 1995 and 2000 studies were combined there was still no association between the month of foaling and the likelihood of the mare or the foal being EHV-1 antibody-positive in February 2000 (data not shown).

Table 6. Association between month of foaling and EHV-1 antibody positivity of the foal in February 2000

Month of Foaling	EHV-1 Antibody		Total
	Positive	Negative	
August	2	41	43
September	0	65	65
October	6	81	87
November[a]	1	40	41
Total	**9**	**227**	**236**

Overall $\chi^2 = 5.12$, 3 df, P = 0.162

a One foal was born on the 5th of December 1999 and was included in the November data

Relationship between the age of foals and EHV-1 antibody There was no significant statistical association between the age of the foal (in months) and the likelihood that the foal would be EHV-1 antibody-positive when sampled in February 2000 (Table 7).

Table 7. Association between the age of foals sampled in February 2000 (in months) and EHV-1 antibody positivity

Age of foal (months)	EHV-1 Antibody		Total
	Positive	Negative	
2	0	9	9
3	3	54	57
4	4	83	82
5	1	60	61
6	1	21	22
Total	**9**	**227**	**236**

Overall $\chi^2 = 1.65$, 4 df, P = 0.800

(2) Responses to whole virus vaccine in the field

The lack of a significant change in prevalence of ELISA antibody-positive mares and foals following the introduction of routine vaccination as described above prompted an investigation of the antibody responses of mares and foals to vaccination. In order to obtain some knowledge of this crucial aspect of current vaccination strategies we investigated the serological responses of mares and weanlings following vaccination with Duvaxyn used as per the manufacturer's recommendations. The study measured antibody responses to vaccination of 159 mares (aged four to seventeen years) and 101 foals (aged three to six months) on a large stud farm, using both the EHV gD -specific ELISA and the type-specific gG ELISA.

Definitions of responders and non-responders. The criteria used to classify responders and non-responders were a result of close examination of the data of individual horses in this study and previous studies of a smaller number of horses in a closed herd situation inoculated with the inactivated vaccine.
gD ELISA: Using this test a responder was classified as any horse whose ELISA absorbance value increased by at least 50% following any vaccination.
gG ELISA: Using this test a responder was classified as any horse whose ELISA absorbance value increased at least eight-fold following any vaccination.
These definitions resulted in a similar number of horses being classed as responders using both assays.
A non-responder was any mare or foal that displayed no response to vaccination (as defined above).
A mare was classified as persistently seropositive if she had high ELISA absorbances in both the gD (≥ 0.200) and the gG (≥ 0.129; (Gilkerson et al., 1997b) assays for the duration of the study.

Note: The ELISA assays used did not differentiate vaccination responses from responses to infection, and for the purposes of this study, it was assumed that all increases in absorbance values during the vaccination period resulted from vaccination. It was not feasible to have a separate non-vaccinated group as a control, since the study by its nature was conducted on a commercial farm where all horses were vaccinated.

Seventy-nine of the 159 mares were the dams of 79 of the 101 foals. This sub-population was analysed to determine if there was any relationship between mares and their foals in response to vaccination. To ensure validity of this comparison and thus to enable the sub-population to represent the whole, characteristics of this sub-population were compared and statistical comparisons drawn with the whole population.
The categorisation of mares and foals is shown in Table 8.

Table 8. Response status of vaccinated mares and foals

	gD ELISA assay				gG ELISA assay			
MARES	**Whole herd**		**Sub-population**		**Whole herd**		**Sub-population**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Responders	22	13.8	12	15.2	46	28.9	25	31.6
Non-responders	111	69.8	56	70.9	85	53.5	41	51.9
persistently seropositive	26	16.4	11	13.9	28	17.7	13	16.5
Total	159	100	79	100	159	100	79	100
FOALS	**Whole herd**		**Sub-population**		**Whole herd**		**Sub-population**	
	No.	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Responders	47	46.5	36	45.5	43	42.6	35	44.3
Non-responders	54	53.5	43	54.5	58	57.4	44	55.7
Total	101	100	79	100	101	100	79	100

For clarity the data is initially listed for each test and for mares and foals.
Mares – gD ELISA Using the gD ELISA, 13.8% of mares were classified as responders to vaccination while 69.8% mares were classified as non-responders.

Also using this test, 16.4% of mares were classified as persistently seropositive, none of whom showed a response to vaccination as defined above Persistently seropositive mares had significantly higher mean gD ELISA absorbances at week 0 than responder mares ($P < 0.01$) and non-responder mares ($P < 0.01$).

Analysis of stud records of the total number of vaccinations each mare had received from the commencement of the vaccination program in 1997 until the end of the study in 2000, showed no significant association (data not shown) between response to vaccination in 2000 and the number of vaccinations the mare had received in previous years. However mares aged 7 years or less were 4.2 times more likely (OR = 4.2; 95% CL 1.44 – 13.92) to respond to vaccination than mares aged 8 years or more (Table 10).

Table 9. Mean EHV gD and EHV-1 gG ELISA absorbances before and after vaccination of mares

Classification	**No.**	**Week 0**		**Week 24**		
gD ELISA		**Mean**	**SD**	**Mean**	**SD**	**P Value**
Responders	22	1.194[ab]	0.296	1.436	0.474	0.163
Non-Responders	111	1.691[ac]	0.347	1.703	0.423	0.817
Persistently seropositive	26	2.464[bc]	0.179	2.446	0.259	0.772
gG ELISA						
Responders	46	0.010[de]	0.053	0.034	0.137	0.275
Non-Responders	85	0.055[df]	0.067	0.069	0.143	0.405
Persistently seropositive	28	0.357[ef]	0.201	0.391	0.219	0.549

Numbers with same superscripts were significantly different ($P<0.01$).
SD – Standard deviation

Table 10. Association between age of mare and response to vaccination with EHV-1/4 using the gD EHV ELISA assay.

Age	**Number of Mares**			
(years)	**Responders**	**Non-responders**	**Carriers**	**Total**
≤ 7	16	44	9	69
≥ 8	6	67	17	90
Total	**22**	**111**	**26**	**159**

Overall $\chi^2 = 6.84$; $P < 0.01$

Mares – gG ELISA Using this test, 28.9% of mares responded to vaccination, while 53.5% were classified as non-responders (Table 8). Responder mares had significantly lower mean week 0 gG ELISA absorbances than non-responder mares (Table 9). Also using this test, 17.6% of mares were classified as persistently seropositive (Table 8); again none of these animals showed a response to vaccination by the criteria used. As can be seen in Table 9, persistently seropositive mares had significantly higher gG ELISA absorbances at week 0 than responder mares and than non-responder mares.

There was no significant difference between the total number of previous vaccinations received and response to vaccination in individual mares (data not shown).

Foals – gD ELISA Using this test, 46.6% of foals responded to vaccination (Table 8). Responder foals had significantly lower mean week 0 gD ELISA absorbances ($P = 0.04$) than non-responder foals (Table 11).

Table 11. ELISA absorbances for responder and non-responder foals before and after vaccination

Classification		Week 0		Week 8		Week 16		
gD ELISA	No.	Mean	SD	Mean	SD	Mean	SD	P Value
Responders	47	0.584[a]	0.335	1.329	0.248	1.137	0.583	<0.01
Non-responders	54	0.782[a]	0.382	0.636	0.359	0.626	0.492	0.13
gG ELISA								
Responders	43	0.024	0.031	0.209	0.228	0.139	0.120	<0.01
Non-responders	58	0.031	0.033	0.048	0.122	0.090	0.080	0.31

Numbers with same superscripts are significantly different (P<0.01). P values compare week 0 and week 8.

There was a significant association between the age of the foal and its response to vaccination ($P < 0.01$; Table 12). Foals aged four months or more were 4.2 times more likely to respond to the vaccination than foals aged three months or less.

Table 12. Association between age of foal and response to vaccination using gD ELISA assay.

Age (months)	**Number of Foals**	
	Responders	**Non-responders**
≤ 3	8	25
4	28	24
≥ 5	11	5
Total	**47**	**54**

$\chi^2 = 10.88$; $P < 0.01$

Foals – gG ELISA. Using this test, 42.6% of foals responded to vaccination (Table 8). Responder foals had significantly higher mean gG ELISA absorbances ($P < 0.01$) at week 8 than pre-vaccination at week 0 (Table 11). There was no significant difference between the mean gG ELISA absorbances of responder foals and non-responder foals at week 0. There was no significant association between age of the foal and its response to vaccination ($P = 0.69$; data not shown).

Relationship between mares and their foals and response to vaccination:

Response status of the sub-population compared to the response status of the whole population. The frequency of responder, non-responder and persistently seropositive mares is shown in Table 8 for all mares and foals sampled as well as for the sub-population of mares and their foals sampled. The proportion of responders to non-responders in the total study population was not statistically different from the proportion of responders to non-responders in the sub-population (data not shown) and thus, the sub-population was representative of the total herd studied.

Likelihood of responder mares having foals categorised as responders

gD ELISA - There was no statistically significant association between the response status of the mare and the response status of her foal using this test (data not shown). However, responder mares were three times more likely to have responder foals than other mares (OR = 2.79; 95% CL = 0.66 – 13.74), and persistently seropositive mares were 4.5 times more likely to have non-responder foals than other mares (OR = 4.50; 95% CL = 0.83 – 45.06), which suggests some biological, even if not statistical difference between these variables.

gG ELISA - Responder mares were 10 times more likely to have responder foals than were other mares (Table 13), and non-responder mares were six times more likely to have non-responder foals than other mares. These associations were statistically highly significant. Persistently seropositive mares, however, were equally likely to have responder or non-responder foals compared with all other mares (Table 13).

Table 13. Response status of mares and their foals using the EHV-1 gG ELISA assay

	Foal		**Total**	**OR**	**CL**	χ^2	**P value**
Mare	Responder	Non-responder					
Responder	20	5	25				
Other	15	39	54				
Total	**35**	**44**	**79**	**10.40**	**2.98 – 40.72**	**16.83**	**<0.01**
Non-responder	10	31	41				
Other	25	13	38				
Total	**35**	**44**	**79**	**5.96**	**2.03 – 17.90**	**12.07**	**<0.01**
Persistently seropositive	5	8	13				
Other	30	36	66				
Total	**35**	**44**	**79**	**0.75**	**0.17 – 2.94**	**0.03**	**0.87**

(3) Comparative vaccine study

This study compared the ability of the envelope glycoprotein D of equine herpesvirus 1 (EHV-1 gD), delivered either as protein (gD subunit vaccine), as DNA, or as DNA followed by protein (prime-boost), to elicit antibody responses in horses. The EHV-1 gD formulations were also compared with antibody responses to the whole-virus vaccine Duvaxyn that contained both EHV-1 and EHV-4 antigens.

Immunogenicity of gD subunit vaccine

Thirty two horses were recruited, and the EHV-1 antibody status of each horse was determined using the type-specific EHV-1/4 gG ELISA (Crabb et al., 1995) and horses distributed equally between four treatment groups to randomize antibody levels for valid comparisons between groups. Groups 1, 2 and 3 received two inoculations four weeks apart of high, medium or low doses of the gD subunit vaccine. Group 4 horses were untreated controls.

Following a single inoculation with any dose of the gD subunit vaccine 22 out of the total of 24 horses showed large increases in gD ELISA antibody. All groups showed a similar effect and there were no significant differences between the mean gD ELISA absorbances of the different vaccine dose groups. The data for individual horses in the low dose group (Group 1) are shown (Fig 3). This exemplifies the responses across all three vaccinated groups. Following the second inoculation four weeks later, there was no further subsequent increase in average absorbance at week eight, and no increases were evident in the few horses (in Groups 2 and 3) that had not shown a response to the first inoculation. In the control Group 4 there was no significant increase in anti-gD antibody levels between week zero and week four or eight.

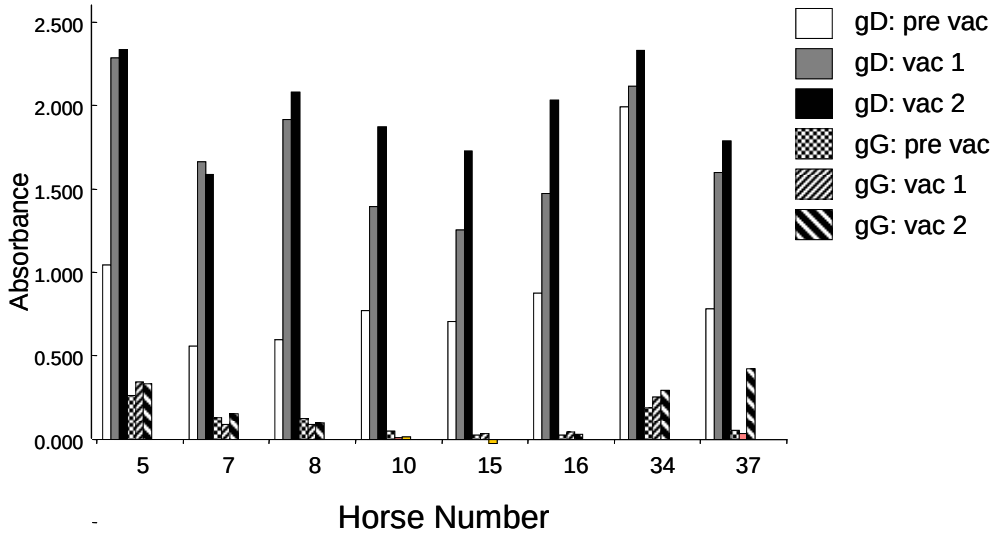

Fig. 3. *Antibody responses to gD subunit vaccine – individual horses in low dose group*

Twenty two of the twenty four horses (92%) across all groups that received the gD subunit vaccine displayed increases in virus neutralising antibody titres. Fig. 4 illustrates the mean absorbance as well as mean virus neutralizing antibody titres for all three dose groups.

In all groups there was a significant increase in mean neutralising titre between week zero and week four with no significant subsequent increase in the group mean at week eight. Consistent with the ELISA results, there were no significant differences in mean neutralizing antibody titres between the three inoculated groups at weeks zero, four or eight. Although one horse in control Group 4 showed an increased virus neutralising titre at week eight, there was no significant change in mean virus neutralising titre between week zero and weeks four or eight in this uninoculated group.

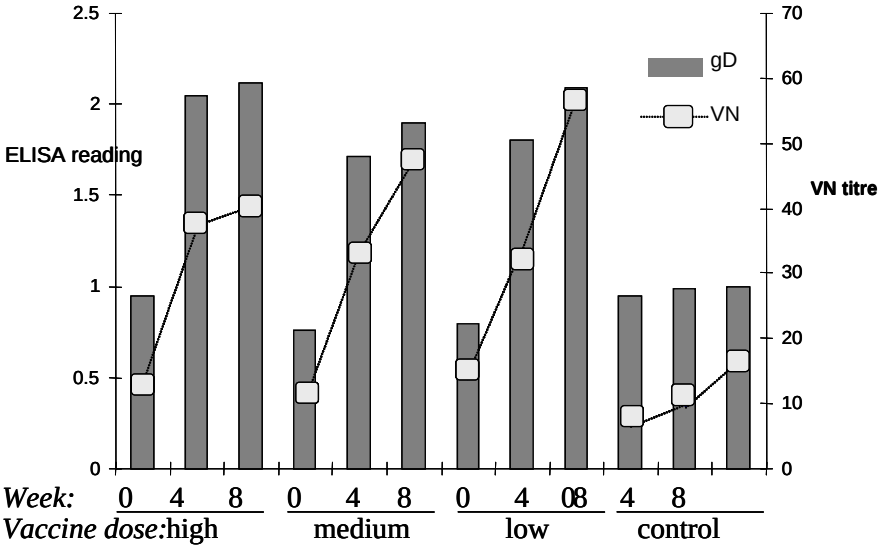

Fig 4. *gD ELISA and virus neutralizing (VN) antibody responses: Averages from each group*

Using the EHV-1/EHV-4 gG ELISA one horse in each of groups 1, 2 and 4 and two horses in group 3 had EHV-1 absorbances defined as positive prior to vaccination using the criteria of Gilkerson et al. (Gilkerson et al., 1997b), and maintained their positive absorbance values throughout the study period. Two horses in each of groups 2, 3 and 4 and one horse in group 1 were identified as sero-converting to EHV-1 positive by week eight. These data suggested that these horses had experienced an infection or re-infection with EHV-

1, despite the population being a closed herd. However since these animals were distributed evenly across the treatment groups, this did not affect the overall interpretation that the gD subunit vaccine was able to elicit strong antibody responses in a high percentage of horses. Individual animals that had the smallest increases in anti-gD antibody following inoculation with the gD subunit vaccine either had very high initial gD and/or EHV-1 gG ELISA absorbances.

Since analysis of the gD ELISA absorbances and virus-neutralizing titres confirmed that there was no significant difference in response to the different doses of the gD subunit vaccine the low dose was used in the subsequent comparative vaccine study.

Comparison of different vaccines

Fig 5. summarizes the ELISA antibody results obtained for groups of horses inoculated with gD subunit vaccine, gD DNA, DNA followed by subunit vaccine (prime-boost) or the commercially available whole virus EHV-1/EHV-4 vaccine (Duvaxyn).

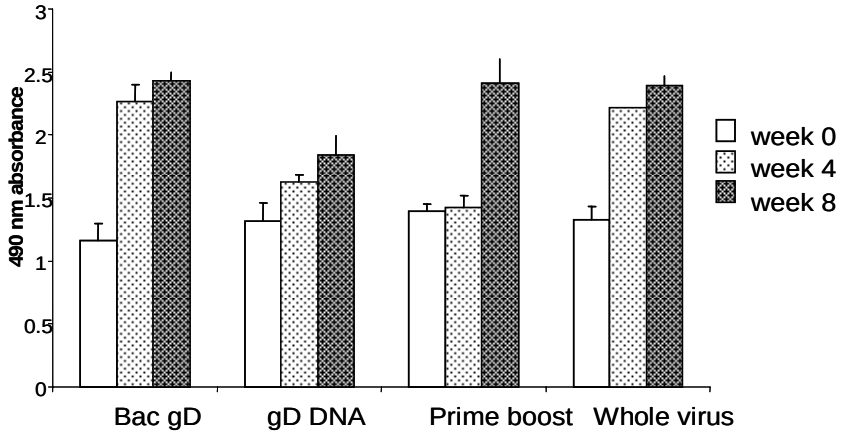

Fig 5. *Mean gD ELISA absorbances for vaccinated groups*

Group 1: gD subunit vaccine. Consistent with the results above, seven out of seven horses (100%) in Group 1 responded to inoculation with the gD subunit vaccine by week four, with a highly significant increase in mean gD ELISA absorbance, and no further significant increase at week eight. No significant increases in EHV-1 or EHV-4 gG absorbances were observed in this group, indicating that the increases in gD antibody were due to the subunit vaccine and not to infection.

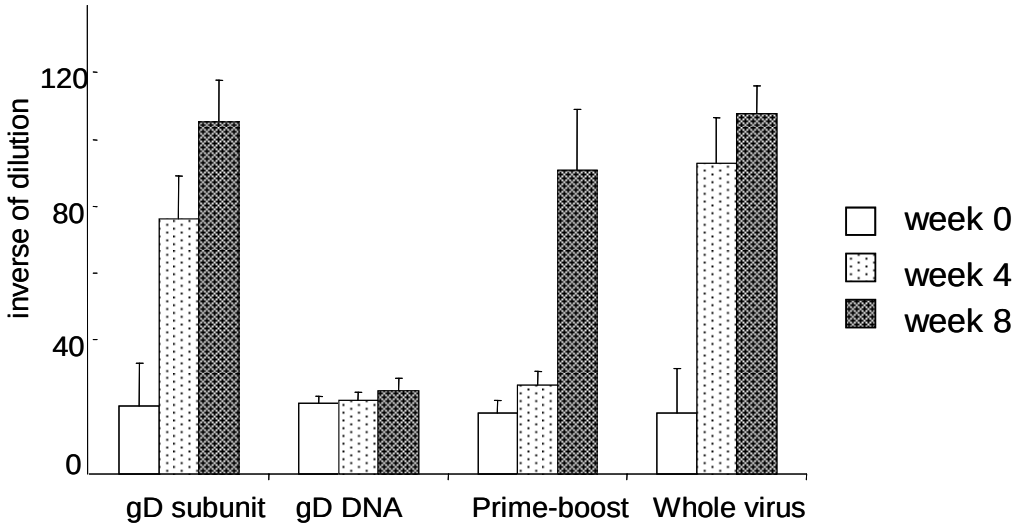

Fig. 6. *Mean virus neutralizing antibody titres for each vaccine group*

All seven animals displayed increases in virus neutralising titres at week four (Fig 6), and the increase in mean titre was highly significant. The second inoculation of the gD subunit vaccine did not lead to any

significant increase in neutralising titres at week eight. Isotype analysis showed that all horses had relatively strong increases in IgGa and IgGb to gD at week four, relative to IgGc, IgG(T) and IgGA.

Group 2: gD DNA. Only one horse in group 2 responded by week four using the gD ELISA absorbance criteria, while two horses responded by week eight. There was no significant increase in mean gD ELISA absorbance for the group between week zero and week four, and between week four and week eight. However, overall the two doses of gD DNA resulted in a significant increase in mean gD ELISA absorbance between week zero and week eight (Fig 5).

Four horses showed a small increase in virus neutralising titre following vaccination with DNA at four weeks, and this increase was sustained at week eight. However this was not sufficient to ascribe a significant increase in mean virus neutralising titres to the group, even after two doses of DNA.

Group 3: Prime-boost vaccination. No horse in group three responded to DNA ('priming') vaccination by week four. However, six out of seven horses responded by week eight, following 'boosting' with gD subunit vaccine. This resulted in a significant increase in mean gD ELISA absorbance between week four and week eight and an overall significant increase in gD ELISA absorbance between week zero and week eight (Fig 5). After the gD subunit boost, five of the six animals showing ELISA responses had correspondingly large increases in virus neutralising titres by week eight resulting in the mean titres shown in Fig 6. No significant increase in EHV-1 or EHV-4 gG absorbances were observed in this group although two horses had positive EHV-1 gG ELISA absorbances prior to and throughout the sampling period.

Group 4: whole virus vaccine. Four horses had increases in gD ELISA absorbances by week four and the mean absorbance increase was significant (Fig 5). The second inoculation did not significantly increase the mean absorbance value. Three horses had positive EHV-1 ELISA absorbances prior to and throughout the sampling period. Despite only 50% of horses showing response in the gD ELISA, the whole virus vaccine resulted in an increase in virus neutralising titres in all animals, with a significant increase in mean virus neutralising titres by week four (Fig 6). The second inoculation did not significantly enhance neutralising titres measured at week eight. All horses in this group had relatively strong increases in IgGa and IgGb antibody isotypes by week four.

5. Discussion of results

(1) Cross-sectional studies in 1995 and 2000

The seroepidemiology of EHV-1 in Australia has been complicated by the recent commercial release in 1997 of the EHV-1/EHV-4 whole virus vaccine. Despite its use, however, the prevalence of EHV-1 ELISA antibody-positive mares at February 2000 was not significantly different from the same time in 1995, even though these mares had been vaccinated in the 5th, 7th and 9th months of gestation in the previous two years. However there was a significant difference in the prevalence of EHV-1 antibody-positive foals. Previous analysis of the 1995 data (Gilkerson et al., 1999b) showed that the EHV-1 antibody-positive foals were likely to be positive due to recent infection. While the results from the 2000 study suggested a similar spread of EHV-1 infection from mares to their foals and then from these foals to other susceptible foals, the small number of EHV-1 antibody-positive foals made confirmation of this spread difficult. However, when the results from the two studies were combined, the observations from the 1995 study were confirmed, ie, that the EHV-1 antibody-positive mares with positive foals (Group A) had significantly higher EHV-1 ELISA absorbances on average than the other group of EHV-1 antibody-positive mares. This indicated that the Group A mares had experienced a recent EHV-1 infection or recent reactivation of a previously latent EHV-1 infection and were thus a potential source of virus for their foals. In the 1995 study there was no difference in the EHV-1 ELISA absorbance or the average age of EHV-1 antibody-positive foals, whether or not their dam was antibody-positive or negative. This suggested that these antibody-positive foals had also been recently infected and were not antibody-positive due to maternally derived antibody.

The reduction in the prevalence of EHV-1 antibody-positive foals on the farm in 2000 suggested that fewer foals had become infected with EHV-1 in the period prior to the survey in February 2000. It is important to note, however, that foals were still being infected on this farm prior to weaning which supports the results of previous studies (Gilkerson et al., 1999a).

It is likely that the difference in the prevalence of EHV-1 antibody-positive foals between 1995 and 2000 reflects a difference in the number of mares acting as a source of EHV-1 to infect the foals. The factors that lead to reactivation of latent EHV-1 infection are not completely understood, although it is known that episodes of stress are associated with herpesvirus reactivation. Thus, differences in the mare population on the farm between 1995 and 2000, differences in various management procedures and differences in seasonal variables such as different extremes of weather may all contribute to differences in the number of mares that reactivate a latent EHV-1 infection early in their lactation. This in turn would affect the number of foals that become infected from their dams or other mares in the group and so affect the spread of EHV-1 throughout the susceptible foal population. As the most likely source of EHV-1 for these foals is the mare (Gilkerson et al., 1999b), these results suggest that there were fewer mares reactivating a previously latent EHV-1 infection prior to the survey in 2000 than was the case in 1995. It is possible also that vaccination has reduced the amount of virus shedding. However the data suggests that it is likely that seasonal, nutritional, or management differences played a more significant role in the number of mares that reactivated previously latent EHV-1 infections in 1995 relative to 2000.

It is possible that either that the vaccine was not stimulating an immune response of sufficiently long term duration to be considered positive by the gG ELISA, or alternately, that there was insufficient glycoprotein G antigen in the vaccine preparation to elicit a positive level of gG antibody. This latter possibility is perhaps supported by a recent report where horses vaccinated with a formalin-inactivated unadjuvanted whole virus EHV-1 vaccine did not show measurable increases in gG ELISA antibodies, but did so after horses had been infected naturally with EHV-1 (Yasunaga et al., 2000). The small decrease in the prevalence of EHV1- antibody-positive mares between 1995 and 2000 supports this conclusion, as 100% of the mares had received 3 vaccinations in the 12 months prior to sampling in February 2000, while only 22.5% of the mares were EHV-1 antibody-positive using the gG ELISA. Also, of the 50 mares sampled in both 1995 and 2000 there was no statistically significant difference in the seroprevalence of EHV-1 antibody. This is not to say that horses were not being infected with EHV-1 during the period between studies. Four of the 50 mares that were EHV-1 antibody negative in 1995 were positive by gG ELISA in 2000, indicating that EHV-1 infection was actively circulating in this population during this period. This supports the findings of previous studies which described the cycle of silent EHV-1 infection

in the mare and foal population in the absence of outbreaks of EHV-1 abortion (Gilkerson et al., 1999a). Interestingly, 8 mares that were EHV-1 antibody-positive by the gG ELISA in 1995 were no longer positive in 2000. It is likely in this study, that the 8 mares that became antibody negative between 1995 and 2000 still had some EHV-1 antibody, but that this amount of antibody was below the level in this ELISA system that was considered positive.

While the decreased prevalence of EHV-1 antibody-positive foals in 2000 versus 1995 could be an attribute of the vaccination of the mares reducing the amount of virus in the environment, this may not be the real reason. As can be seen from the above analysis caution must be exercised in the interpretation of these sero-epidemiological data and further monitoring is essential if we are to dissect accurately the factors responsible for this decreased seroprevalence. In further recent analysis outside the immediate research reported here, a 2001 cross sectional study supported the results for 2000. The data obtained in 2001 suggested that changes in the prevalence of EHV-1 antibody-positive foals was not due to reduced shedding of virus by vaccinated mares, but was rather more likely to be due to environmental conditions/management practices that placed the mares under stress which resulted in reactivation of latent infections. On the farm involved in these studies the pregnant mares were kept strictly away from the mares and foals and these animals were kept separate from weanlings and yearlings. Thus, there was no opportunity for foals on this farm to come into contact with older horses, except for mares with foals at foot. Consequently, the virus source for these foals was mares/foals in their pre-weaning groups. Taken together, these cross-sectional analyses of EHV-1 antibody support the findings of previous studies which described the cycle of silent EHV-1 infection in the mare and foal population in the absence of outbreaks of EHV-1 abortion (Gilkerson et al., 1999a). It is clear that there are yearly fluctuations in prevalence of antibody-positive foals at weaning and that management practices other than the current vaccination strategy alone must be addressed in the quest to reduce the number of EHV-1-infected mares that consequently infect foals pre-weaning in the silent cycle of infection.

(2) Responses to whole virus vaccine in the field

Two assays were used to determine the animals' response to vaccination: a gD-specific ELISA assay and an EHV-1 gG ELISA assay. Responses to both assays were interpreted using liberal criteria: that is, an animal needed only to show a single response to vaccination at any time during the vaccination regimen to be considered a "responder" and this response could be detected using either the gD or gG ELISA assay. No animal was required to respond in both assays to be defined as a responder. Using gD and gG ELISA assays and the definition criteria outlined in this study, 13.8% or 28.9% of mares and 42.6% or 46.6% of the foal population responded to the vaccine. It is possible that the difference in the numbers of responders and non-responders was attributable to the definitions. However after analysis of the data and comparison of the numbers of responders using both tests, we believe that these definitions most accurately represent the herd response to vaccination. Glycoprotein D is highly immunogenic and is a principal inducer of antibody during infection (Crabb et al., 1991). The gD ELISA used here is based on EHV-1 gD but unlike the gG EHV-1 ELISA will detect an increase in gD antibodies to EHV-4 as well as EHV-1. Therefore high levels of pre-existing EHV-4 antibody may have inhibited the detection of a response to vaccination using the gD ELISA, which may explain the lower percentages of responding animals classified using the gD ELISA as compared with the gG ELISA.

The gG ELISA has been shown to be highly specific and is considered sufficiently sensitive to detect all horses with antibodies to EHV-1 and to distinguish these from antibodies to EHV-4 (Crabb et al., 1995). In contrast to the results presented here, Yasunaga et al. (2000) did not detect any antibody response in horses inoculated with an inactivated vaccine (Nisseiken Co., Ltd. Japan) using a type-specific gG ELISA. The authors recognised that the amount or nature of antigen in that vaccine was not known, and this may account for the difference in ability to detect responses to vaccination in the present study using a gG ELISA. As in this study also the amount of the respective antigens in the vaccine were not known and optimal detection of responses in the population was desirable, it was considered prudent to assay for more than one antigen. As can be seen from the tables, there was generally good agreement among categories using both assays, especially in the foal population.

The percentage of "persistently seropositive mares" using the gG ELISA in this study is similar to the percentage of EHV-1 positive mares reported in other studies in Australia (Crabb et al., 1995; Gilkerson et al., 1999a) and worldwide (Nordengrahn et al., 1999) and may indeed reflect the underlying experience of the general population to EHV-1 infection. Vaccination responses were seen only in the group of mares

with the lower mean ELISA absorbances at the time of initial vaccination during the study period. The failure of mares with pre-existing antibody to respond to vaccination was particularly evident using the gG ELISA assay, although using the criterion of Gilkerson et al. (1997b) for a positive antibody absorbance for EHV-1, neither the responder nor the non-responder group would have been considered to have experienced an active infection with EHV-1 immediately prior to vaccination. The much higher mean ELISA absorbances in the gD assay in both responder and non-responder groups compared with the gG assay absorbances may thus reflect a high cross-reactive EHV-4 antibody absorbance. The inability of the gD ELISA to distinguish antibodies to EHV-1 from antibodies to EHV-4 may have contributed to the lower percentage of responder mares identified using this assay.

There was a significant difference between the mean gD ELISA antibody absorbances of responder and non-responder foals at week 0. These foals had low mean gG ELISA absorbances (less < OD 0.129) and interestingly, lower mean gD ELISA absorbances when compared with both responder and non-responder mares ($P < 0.01$; data not shown). Therefore, it is likely that these differences contributed, at least in part, to the higher numbers of foals that responded to vaccination.

Responder foals were more likely to have been born to responder mares than to other mares. This may reflect in part the low level of antibody passed to the foal from the mare in colostrum. In this context, it is of considerable interest that mares with consistently high EHV-1 antibody levels did not respond to vaccination. One possible explanation for these observations in persistently seropositive mares is the immunogenicity of the EHV-1 antigens in the vaccine was reduced by the high levels of EHV-1 antibody (gG ELISA mean 0.357, Table 2) present at the time of vaccination preventing an active response by the mare. Non-responder foals had high pre-existing antibody levels using the gD ELISA (Table 4) however there was no corresponding difference between non-responders and responders using the EHV-1-specific gG ELISA. This suggests that high levels of pre-existing antibody to EHV-4 may interfere with active immunisation to EHV-1. There was no association observed between the response of mares to vaccination and the number of previous vaccinations. The manufacturer recommends mares be vaccinated in their 5^{th}, 7^{th} and 9^{th} month of gestation every year. This is a significant cost for the industry and identification of these non-responder mares along with persistently seropositive mares, and reassessment of vaccination policies in these groups may represent considerable savings to the industry if management includes the multiple use of such vaccines in pregnant mares.

Age has been reported as a critical factor in determining responses to vaccination in foals. While foals are known to be immunocompetent at birth, the difficulties in obtaining antibody responses to inactivated vaccines against viruses such as equine influenza in the presence of maternal antibody have been reported (Van Oirschot et al., 1991; van Maanen et al.,1991;Conboy et al., 1997; Cullinane et al., 2001). This poor response to vaccination of young foals was supported in this study, with the majority of the responder foals aged four months or older. The presence of high levels of maternal antibody in younger foals to EHV-4 may inhibit a response to vaccination, possibly due to the presence of maternal antibody. Our findings are similar also to those of (Wilson & Rossdale, 1999) who suggested that it may be difficult to effectively vaccinate foals less than five months of age against EHV-1/4 using inactivated whole virus vaccines.

Serological evidence of EHV-1 infection in unweaned foals from 30 days of age as well as in foals after weaning has been previously demonstrated (Gilkerson et al., 1998, Gilkerson et al., 1997b). If interpretation of these serological data is correct and young foals are becoming infected with EHV-1 before the time of recommended vaccination, alternative control strategies may be required in order to protect this group of horses from becoming latently infected persistently seropositive animals. Additionally, if less than half of this population is responding to vaccination, the factors contributing to this need investigation.

It should be noted that the serological response to vaccination reported in this paper is not necessarily a measure of protection induced following vaccination. Despite a considerable body of research over many years, the precise determinants of protective immunity to EHV-1 have not been elucidated (Slater & Hannant, 2000). Nor is it known what proportion of responders is required in a population to minimise spread and provide “herd immunity”. However, as is known with many persistent and latent infectious diseases such as herpesviruses, population susceptibility plays a less critical role than it does in acute infections (Villarreal et al., 2000) and percentage of responders and herd immunity may not be an issue in protection against EHV-1 infection. As well, parent to offspring spread is a feature of many latent infections and EHV-1 infection is no exception as has been supported by sero-epidemiological studies

(Gilkerson et al., 1997b, Gilkerson et al., 1999a, Gilkerson et al., 1999b). Therefore the key to control of EHV-1 would appear to be prevention of infection of very young foals. Whether or not this can be achieved has yet to be demonstrated, but the emergence of a variety of new vaccine technologies that can be targeted to specific components of the immune system may eventually make this goal a reality. In the next section several options using recombinant DNA-based vaccines were explored.

(3) Comparative vaccine study

This study compared the ability of the envelope glycoprotein D of equine herpesvirus 1 (EHV-1 gD), delivered either as protein (gD sububnit vaccine), as DNA, or as DNA followed by protein, to elicit antibody responses in horses. The recombinant EHV-1 gD, used here in the gD subunit vaccine, had been shown previously to induce protective responses in a mouse model of respiratory disease (Tewari et al., 1994) but had not been investigated in horses. The studies described here demonstrate that i.m. injection of this EHV-1 gD produced a specific antibody response in over 90% of horses tested and that this response was associated with an increase in the level of virus neutralising antibody in serum. The EHV-1 gD formulations were also compared with antibody responses to a whole virus vaccine containing both EHV-1 and equine herpesvirus 4 (EHV-4) antigen. The responses elicited by the gD subunit vaccine were similar to those obtained for the inactivated whole virus vaccine, which although showing more variable ELISA antibody levels, elicited virus neutralizing antibody in all horses. In contrast, only one horse responded to a single dose of EHV-1 gD DNA, while two horses responded after a second dose. In this DNA-only group there was no significant increase in mean virus neutralizing antibody after two inoculations. However, in the prime-boost group, six out of seven gD DNA-primed horses showed increases in ELISA absorbance following boosting with gD subunit protein, and increases in virus neutralizing antibody.

All vaccine formulations or strategies generated a similar IgG isotype profile, with elevated IgGa and IgGb relative to IgGc, IgG(T) and IgGA antibody. Hence although only a single antigen, the EHV-1 gD homologue, delivered as a recombinant protein or in a DNA-prime/protein-boost protocol, generated comparable antibody responses to those of an inactivated whole virus vaccine. The results support previous conclusions from small animal models that EHV-1 gD is a suitable antigen for use in a subunit vaccine.

The experiments were conducted on horses that are representative of horse populations worldwide in that they had pre-existing antibody to either or both EHV-1 and EHV-4. Such populations are the standard target for vaccination programs, and to facilitate statistical treatment of the data, the horses were allocated into groups randomized according to their antibody levels prior to any inoculations. The use of the gD ELISA enabled the measurement of responses to recombinant gD, to gD DNA and to the gD component of the whole virus vaccine, while the gG ELISA measured antibody due to natural infection by either EHV-1 or EHV-4 and antibody responses to the gG component of the whole virus vaccine. In animals inoculated with either gD subunit vaccine, gD DNA, or by prime boost an increase in anti-gD antibodies in the absence of an increase in anti-gG antibodies, and supported by an increased virus neutralization titre, was assumed to be a result of vaccination. Neither of the two ELISAs were able to distinguish between responses to the whole virus vaccine responses to infection. In this group of horses (ie inoculated with the whole virus vaccine) it was assumed that all increases in antibody levels resulted from vaccination, bearing in mind that there were only a few instances of infection in the other groups, and that this did not statistically affect the overall conclusions.

The reliable induction of ELISA and neutralising antibody by the gD subunit vaccine was not reflected in the responses to DNA encoding the same gene product. Nonetheless the two horses in this group that had positive EHV-1 gG ELISA absorbances prior to and throughout the sampling period could be classified as responders to vaccination. This supports the work of (Ruitenberg et al., 2000a) who that showed that gD DNA inoculation was associated with antibody responses in horses with pre-existing antibody. However in that study approximately 50% of horses responded to gD DNA, and the reason for the lower numbers in this present study is not known.

Consistent with the DNA-only results, horses that received the gD DNA as the initial inoculation in the prime-boost strategy did not demonstrate a significant increase in antibody levels until after the gD subunit boost. Since the gD subunit vaccine alone generated a similar response, it was not possible to determine whether a genuine prime-boost effect had occurred. It could be postulated that since the horses had been exposed to infection with either or both EHV-1 and EHV-4, that in effect all horses were already primed by

natural infection. Hence any of the vaccine strategies used here are really 'prime-boost'. The DNA alone may not express as much antigen as is delivered in the subunit or whole vaccine, which along with adjuvants may elicite a stronger and more rapid response. It is possible that over time the gD DNA may be effective as it continues to express its gene product in the animal. The potential of DNA vaccine strategies has yet to be fully realized for large domesticated animals (Littel-van den Hurk et al., 2000). Immune responses to DNA may be enhanced by the inclusion of immunostimulatory CpG motifs, and in this context it has been shown recently that the synthetic oligonucleotide GTCGTT can stimulate proliferation of cells of seven 'veterinary' species including the horse (Rankin et al., 2001).

The percentage of horses in this study (over 90%) that responded to vaccination with either the gD subunit vaccine or the whole virus vaccine was much higher than the percentages determined in the analysis of responses to the whole virus vaccine on a large stud farm (study 2, above). That study classified 14% or 29% of mares and 43% or 47% of foals using the gD or EHV-1 gG ELISA respectively as responders following vaccination as per farm protocol. The difference in the response to vaccination may have been be due to the amount of circulating virus in the herd, as measured by the number of animals that have consistently elevated positive EHV-1 gG ELISA absorbances. On the stud farm the percentage of antibody positive mares ranged between 23% and 32% during three separate seasons, but only 16% of horses in the immunogenicity study and 20% in the comparative vaccine study. Additionally, it should be noted that the animals in our comparative vaccine study were ponies of mixed breeds, compared to the Thoroughbred mares and foals previously mentioned. It is yet to be determined if breed type has an effect on the response to vaccination.

In this study we were only able to measure serum antibody, which provides a picture of one component of the immune response. Nonetheless elevated virus neutralizing antibody is an indicator of the immunogenicity of a vaccine, is likely to be important in limiting the systemic spread of EHV-1, and if present in the upper respiratory tract would target incoming virus. Some indication of the balance of immune responses can be obtained by analysis of immunoglobulin isotypes. All the vaccine protocols and formulations used here appeared to be associated with a similar isotype profile, which was similar to that obtained following natural infection (Sugiura et al., 1994; Sugiura et al., 1999), and to some previous vaccination studies in horses (Lunn et al., 1999; Ruitenberg et al., 2000a). The gD subunit and the whole virus vaccine, which are essentially inactivated preparations, each had an adjuvant in their formulation. The Iscomatrix in the gD subunit vaccine was aimed at influencing the immune system towards a Th1 or equivalent response (Morein & Bengtsson, 1999). Earlier experiments in a hamster model of EHV-1 infection described protection against lethal challenge following immunisation with an ISCOMS preparation of EHV-1 glycoproteins from whole virus (Cook et al., 1990). A Th1-biased response is also a feature of gD DNA i.m. inoculation in mice even after a protein boost (Ruitenberg et al., 2000b). An assessment of cell-mediated responses in horses is now possible using several approaches, including a CD8+ CTL assay (Allen et al., 1995). Primers are available for the application of Real-Time PCR methods to quantify cytokines that are associated with expression of different arms of the immune response (Giguere & Prescott, 1999).

The vaccine potential of gD homologues for herpesvirus diseases of large animals has been shown also in a BHV1 challenge trial, where purified BHV-1 gD was more effective in reducing clinical and virological parameters after challenge than two killed or modified whole virus vaccines (Littelvandenhurk et al., 1997). This protection was associated with higher neutralizing antibody titres in serum and nasal mucosa. Likewise in order to further assess the potential of the EHV-1 gD subunit vaccine it will be important to conduct EHV-1 challenge experiments in horses. In related studies we are investigating whether this vaccine can elicit responses in the face of maternal antibody in young foals, with the aim of limiting the level of infection early in life and perhaps interfering with the cycle of silent infection by EHV-1. Although there are existing whole virus vaccines available and various genetically attenuated whole virus vaccines may also become available, one generation of animal vaccines is likely to be based on subunit preparations for reasons of safety, flexibility in generating cocktails of vaccine components, ability to distinguish vaccine from natural infection, and production without generating large amounts of infectious virus. The ability of the EHV-1 gD to evoke similar neutralizing antibody responses to the whole virus vaccine indicates that this is an excellent candidate for inclusion in a new subunit vaccine against EHV-1. Furthermore, since antibody to EHV-1 gD also neutralizes EHV-4 (Love et al., 1993) it is likely that any protective responses induced by the gD subunit vaccine against EHV-1 will also act against EHV-4.

6. Implications and Recommendations

The first component of the project provided a picture of the status of EHV-1 in a vaccinated population of horses on a large stud farm, where all vaccine regimens had been carefully adhered to, as per manufacturer's recommendations. The use of an EHV-1 specific test allowed the detection of the prevalence of EHV-1 antibody, as distinct from EHV-4 antibody, in mares and foals. The serological data indicated that these viruses are continuing to circulate and infect foals at a young age. This has been supported by other recent detection by PCR of EHV-1 and EHV-4 DNA in very young foals (C. Foote, personal communication). The virus source for these foals is mares or foals in their pre-weaning groups. Taken together, our analyses support the findings of previous studies which described the cycle of silent EHV-1 infection in the mare and foal population in the absence of outbreaks of EHV-1 abortion. This is continuing even in the face of widespread vaccination of the population.

Therefore management practices other than the current vaccination strategy alone must be addressed in order to reduce the number of EHV-1-infected mares that are a source for infection of foals pre-weaning in the silent cycle of infection. This could include careful attention to eliminating practices that may facilitate transmission of viruses between animals during procedures in the crush.

The second part of the project used two antibody tests to assess what percentages of horses were responding to the currently used vaccine in the field. The data indicated that under the liberal criteria used to define a responding animal, at most only 30% of mares and 50% of foals were responding to the vaccine used as per manufacturer's instructions. This was much less than the responders seen in the experimental vaccination in part (3) of the project. In general the horses that showed responses had higher levels of pre-existing EHV-1 antibody at the time of vaccination, supporting the ability of antibody to remove antigen, and reduce the amount of antigen available as a target for the immune system. There was no association observed between the response of mares to vaccination and the number of previous vaccinations. The manufacturer recommends mares be vaccinated in their 5^{th}, 7^{th} and 9^{th} month of gestation every year, which is a significant cost for the industry.

Identification of non-responder mares along with persistently seropositive mares using antibody testing may allow a more selective vaccination policy to be adopted, as those animals do not respond to vaccination, either initial or subsequent to previous vaccinations. In addition the poor response to vaccination of young foals was supported in this study, with the majority of the responder foals aged four months or older. Taken together the data on non-responding mares and foals suggest that a reassessment of vaccine strategies and regimens could provide considerable savings to the industry, especially where management presently includes multiple vaccinations of pregnant mares.

The third component of the research was to investigate the potential of new vaccines based on recombinant DNA technology as alternatives to the existing whole virus vaccine. The envelope glycoprotein D (gD) of EHV-1 was selected as a trial antigen for this research. The results showed that at least by the tests used, the ELISA and virus neutralizing antibody responses to gD delivered as a protein subunit was highly comparable to those elicited by the whole virus vaccine. The data illustrate the potential of this technology to identify vaccine antigens that may provide equivalent or greater protection against EHV diseases than available whole virus vaccines. As stated above, such subunit vaccines have a range of one generation of animal vaccines is likely to be based on subunit preparations for reasons of safety, flexibility in generating cocktails of vaccine components, ability to distinguish vaccine from natural infection, and production without generating large amounts of infectious virus. The study also indicated that at least at this stage the use of direct DNA as a vaccine requires further research before application to the horse. The ability of the EHV-1 gD subunit vaccine to evoke similar neutralizing antibody responses to the whole virus vaccine indicates that this is an excellent candidate for inclusion in a new vaccine against EHV-1. Furthermore, since antibody to EHV-1 gD also neutralizes EHV-4 it is likely that any protective responses induced by the gD subunit vaccine against EHV-1 will also act against EHV-4.

The results from the project warrant further assessment of this subunit formulation and in particular it would be important to carry out challenge experiments to determine whether the immune responses elicited correlate with protection against EHV-1 disease in horses. This could be addressed in the first instance by vaccinating weanling foals and then infecting via the respiratory

route. A reduction in clinical signs, virus shedding and other parameters would support the development of the gD subunit formulation as a useful vaccine against EHV-1. Similar challenge experiments could be carried out with EHV-4.

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