

**THE EFFECT OF AGING ON THE IMMUNE RESPONSE TO VACCINATION
IN THE HORSE**

A Thesis

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ABSTRACT

Vaccination programs are designed to protect an animal from infection, however, depending upon the age and health of the animal vaccination may not stimulate a protective humoral response. It is possible that, as in the human and mouse models, geriatric equines may be less responsive than their younger counterparts to current vaccination protocols. The purpose of this study was to identify an age related diminution in the primary and secondary immune responses of geriatric horses in response to vaccination. Two groups of horses were sampled. The first group consisted of an open herd of 39 privately owned horses, varying in age from 2-27 years of age. The second group consisted of a closed herd of 24 horses ranging in age from 7-over 30 years of age. Each group was vaccinated twice intramuscularly with a commercial equine influenza virus vaccine (Ft Dodge, A/Eq/KY/97). Additionally, one group was vaccinated with keyhole limpet hemocyanin (KLH) in order to study a primary immune response to a novel antigen. The other group was vaccinated with ovalbumin (OVA) for the same purpose. Blood samples were obtained via jugular venipuncture prior to vaccination and monthly thereafter for 5 months. All sera samples were analyzed for antigen-specific antibodies using an ELISA assay. Our results show that all horses responded to primary vaccination with either KLH or OVA, irrespective of age. In contrast, when vaccinated with influenza the middle aged and older horses showed significant differences in the their response between both the herds and the age groups. Thus we were able to demonstrate that age was not a factor in the generation of a primary immune response but was a factor in the generation of a memory immune response. Future research should focus on whether increased frequency of vaccination does in fact increase or maintain

vaccine responses and whether antibody responses measured in vitro actually correlate with protection in vivo.

INTRODUCTION

Either following vaccination or primary infection the immune system utilizes a complicated and elaborate strategy to prevent re-infection involving both cellular and humoral responses. Thus initial stimulation of the immune system leads to the production of antigen-specific memory T & B-cells that will provide a rapid response to future encounters with the same agent (1-3). Additionally, plasma cells are produced and stimulated to secrete antibody both in the short and long term following vaccination or infection (4, 5). This secretion of antibody will allow for a first line of defense in the case of future contact with the pathogen. Circulating antibody has the ability to neutralize or opsonize invading organisms thereby presenting them to phagocytes or CD8+ cells before they can infect the host (6).

Extensive research has been conducted regarding the structure and function of antibodies in both the human and mouse model. The antibody molecule is composed of two light chains and one heavy chain connected by a disulfide bridge at a hinge region and together form a Y-shaped molecule. The antigen binding region (Fab) is composed of two light chains and two heavy chains that together form a conserved domain and a variable amino terminal domain. The constant region (Fc) contains heavy chains and is highly conserved. There are five major classes of Fc regions associated with immunoglobulin isotypes that have been defined by anodal electrophoretic mobility: alpha (α), delta (δ), epsilon (ϵ), gamma (γ), and mu (μ). It is from these distinct classes that isotype names are derived: IgA, IgD, IgE, IgG, and IgM. The Fab and Fc regions together confer bifunctional properties to the antibody molecule. The Fab region binds antigen and the Fc region determines the particular isotype and function of the

molecule(7, 8). The hinge region also confers functionality to the molecule depending upon its hinge-length and flexibility. (Figure 1) Increased flexibility may result in reduced steric-hinderance by the Fab and also allow for greater avidity of the antibody molecule (7, 9).

Antibody class and structure is highly conserved among different mammals, thus it can be inferred that similar functions exist between the isotypes of differing species.

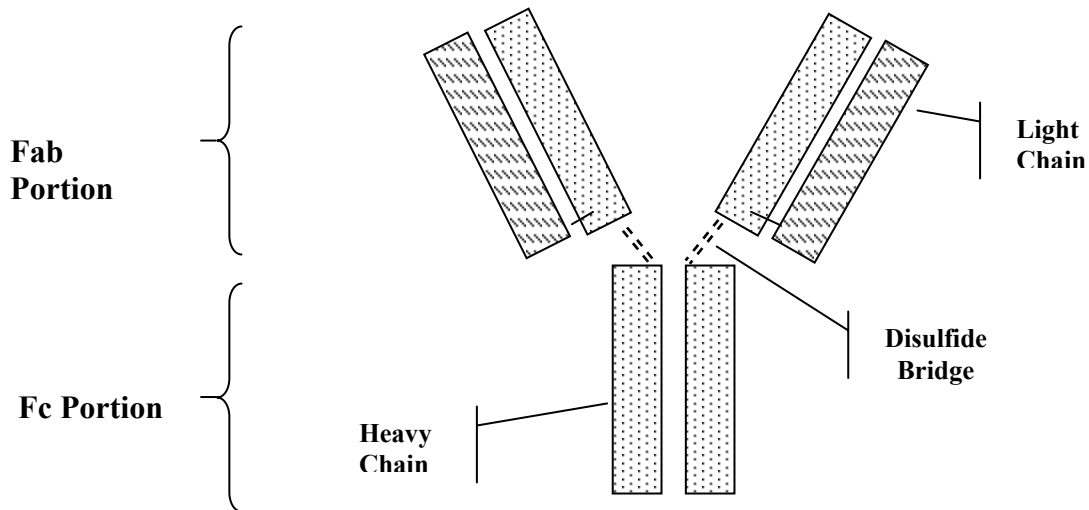


Figure 1: Structure of Immunoglobulin

IgM: IgM is the first isotype expressed during B-cell development and during a primary immune response to infection. IgM is a polymeric antibody with five heavy chain domains. Its unique pentamer shape gives it a high avidity for antigen and a greatly

increased ability to fix complement. IgM makes up 5-10 % of the total serum Ig is also a major antibody of the mucosal immune response, second only to IgA.

IgA: IgA exists as either a dimer or a monomer and is predominantly found in the mucosa, saliva, tears, and sweat. IgA makes up approximately 10-15% of the total immunoglobulin component in the serum and is present mostly in its monomeric form. Dimeric IgA is considered a first line of defense to any organisms infiltrating through the mucosa, and dimeric IgA binds pathogens, thereby preventing their uptake across the epithelium, and encouraging their removal via mucociliary transport. IgA also opsonizes antigens that are later phagocytised by macrophages located on mucosal surfaces.

IgE: This isotype is found in the lowest concentrations of all the immunoglobulins in sera, has the shortest half-life, is unable to activate complement, and cannot opsonize antigens. This antibody is, however, the primary effector of Type I hypersensitivity responses and is a potent initiator of the inflammatory response. After release by plasma cells, IgE is bound by the Fc ϵ receptor on basophils and mast cells. Once the cross-linking membrane bound IgE antibodies bind antigen, they will induce degranulation of the host cell.

IgG: As the most prevalent immunoglobulin in plasma, this class makes up over 75% of the total Immunoglobulin in the serum. Four subclasses have been identified in human and mouse models, IgG1, IgG2, IgG3, IgG4. In the horse, these subclasses are divided up as follows; IgGa, IgGb, IgGc, IgG(T). Despite the different nomenclature used for different species, it is believed that various subclasses share similar functions. For instance, in the mouse, IgG1 and IgG4 in the human are associated with immune responses to parasites and allergens (6).

The IgG subclasses also are capable of fixing complement, and do so to varying degrees. This variation in the ability of complement fixation may be a result of differences in the hinge region of each subclass, thus resulting in possible steric-hindrance properties (10-12).

Isotype production is indirectly governed by T_h cells, which secrete cytokines that regulate isotype switching in B-cells. In the mouse, production of IgG2a is a result of expression of IL-2 and IFN γ by T_h1 cells. IgG1, however, is produced in response to T_h2 cell expression of IL-4, IL-5, and IL-10 (13). IgA and IgE are also products of a strong T_h2 response. In the human IgG1 and IgG3 are produced in response to cytokine expression by T_h1 cells (14).

Until recently very little has been learned regarding the humoral response and the antibody repertoire in horses. In 1939, the first equine isotypes, IgG and IgM were described followed in 1940 by the identification of a 'T-protein'(15). This 'T-protein' was originally considered to be a homologue of IgA, but structural and antigenic studies conducted by Weir et al. (16, 17), and Widders et al. (17) show it to be a subclass of IgG and was subsequently identified as IgG(T). IgGa, IgGb, and IgGc were identified as IgG subclasses thru the use of electrophoretic anodal mobility (18). Nine equine Ig isotypes have currently been described: IgA, IgM, IgGa, IgGb, IgGc, IgG(T), and IgE(19-25), while recent work done by Overesch et al has isolated six immunoglobulin G heavy chain(γ) genes in the equine haploid genome (γ 1- γ 6) (26).

Of the equine isotypes, IgGb is found in significantly greater concentrations in the serum (>60%) of adult ponies and horses, followed by IgG(T)and IgGa. IgGc and IgA are found in low concentrations in sera (27). In contrast to serum samples, nasal washes

contain predominantly IgA with low concentrations of IgGb and IgGa found. IgGc has not yet been detected in nasal washes of the horse (16, 27).

The development of monoclonal antibodies (mAb) allows for advanced studies of antibody isotypes. Work done by Sheoran et al (24) and Sugruia et al (25) has shown success in the production of hybridomas secreting antibodies specific for six equine isotypes; IgA, IgGa, IgGb, IgGc, IgG(T), and IgM. These MAbs are an invaluable tool in determining Ig concentration in mucosal and serum fluids, determination of antibody half-life, and antibody response to specific types of antigenic presentation to name a few.

Age effects on the immune response

Horses over 20 years of age constitute about 15% of the equine population and many remain actively involved in equestrian sports and reproductive capacities as stallions and brood mares (28). However, advancing age in horses, as with other species, is eventually associated with a decline in body condition and muscle tone as well as an increase in susceptibility to infections (29-31). This increased susceptibility to infectious diseases is due to an overall decline in immune function that results from alterations of many different elements of the immune system. While these changes have been extensively documented in humans and mouse models, little research has been done in the equine system. Vaccination remains the most cost-effective measure for preventing and reducing the severity of infectious diseases in the equine population (32). Little is known, however, regarding the effectiveness of vaccinations in the geriatric equine

In order to elucidate the complex events that are involved in immunosenescence we must first consider how the immune system responds to infection. All animals, upon parturition, have a natural or innate immunity that acts as a limited but rapid response to

infection that does not require prior exposure to the pathogen (33-35). These aspects of the immune response include the chemotactic attraction of phagocytic and cytotoxic cells to the site of the infection and the subsequent production of soluble mediators. Macrophages and neutrophils are the key cells involved in the phagocytosis of invading organisms. Macrophages are also the source of various chemokines that recruit other phagocytic cells, including neutrophils, to the infected area. Chemokines also act on local blood vessels causing vasodilation, increased vascular permeability, and the eventual exudation of plasma and cells to the site of ongoing infections (34). Other soluble mediators involved in this phase of the innate immune response are mannose-binding protein, serum amyloid protein, and complement proteins that bind to prokaryotic carbohydrate structures and facilitate their uptake by the phagocytic cells (36). Natural killer (NK) cells are cytotoxic effector cells that play a central role in the innate immune response to tumors and virus-infected cells (37). The non-specific cytotoxic activity of NK cells can be further enhanced by the cytokines interleukin (IL)-2 and interferon-gamma (IFN γ). These so-called lymphokine activated killer (LAK) cells are capable of lysing tumor cells resistant to NK-mediated lysis. NK cells are also the source of IFN γ and as such can augment macrophage microbicidal and tumoricidal activity (37).

To date, most research has not shown a significant alteration in innate immune function between young and old individuals, though some subtle alterations may be present. Thus there is a general decline in function of the phagocytic capabilities of human neutrophils (35). Likewise there is also a slight increase in the susceptibility of human neutrophils to apoptosis with advanced age (38). Whether these alterations contribute to increased susceptibility to infection in the elderly remains unknown. By

contrast, the phagocytic and bactericidal activities of pulmonary macrophages and neutrophils from young and aged mice appear comparable (39). Alterations in macrophage cytokine production could also play a role in the increased susceptibility of elderly individuals to endotoxins (40). This impairment of cytokine production could have important impacts on the induction of specific immune responses, as discussed below. Similarly, ageing is associated with a decrease in the cytolytic activity of NK cells in circulation, though this appears to be offset by an increase in the total number of these cells in the circulation (36, 37, 41, 42). This functional decline in NK activity may be the result of decreased responsiveness to IFN γ and IL-2 (36, 41, 42).

A few studies have assessed the effect of ageing on innate immune responses of horses. Overall LAK cell activity in old, Standardbred mares appears equivalent to that seen in younger mares (43), though the possibility of there being fewer cells in circulation was not assessed. In another study there was no differences in the circulating numbers of monocytes or granulocytes between young and aged horses (44). Nor was there an effect of ageing on the respiratory burst activity of equine neutrophils (44). These results are thus in agreement with most other studies indicating that ageing has little effect on the innate immune responses (45).

Acquired or adaptive immunity involves the induction of cellular and humoral immune responses directed against a specific antigen. Adaptive immunity is also characterized by the development of cellular memory that provides a more rapid secondary response upon re-exposure to the antigen. Acquired immunity involves two major cell types: B-lymphocytes and T-lymphocytes.

B cells are responsible for the production and secretion of antibodies. While each individual B cell has its own unique antigen-specificity, the process of gene rearrangement during B cell ontogeny leads to the development of B-cells with specificity for 10^7 different antigens. The product of pluripotential hematopoietic stem cells, the B-cell matures in the bone marrow and then migrates to secondary lymphoid tissues. Upon exposure to its antigen, the B-cell can proliferate and differentiate into an antibody secreting plasma cell, or it may become a memory B-cell. Activation of a memory cell by re-exposure to a specific antigen leads to a rapid antibody response.

Ageing markedly reduces antibody production in both humans and mice (46). Though the total number of B-cells in circulation does not appear to be reduced in aged mice, there is a marked decrease in B-cell ontogeny and increased signs of apoptosis (46). There also appears to be decreased migration of plasma cells to effector sites in old animals (47). This latter effect could have significant impact on the development of local immune responses at mucosal surfaces. Advanced age is also associated with an overall shift to antibodies of lower affinity for their specific antigen (48). In young animals, the maturation of the antibody response is associated with an increased affinity of antibodies for their specific antigen. This increased affinity is the result of somatic mutations in the variable region of the immunoglobulin genes. The reduced affinity seen in the elderly can be attributed to the age-related reduction in germinal center reactions and immunoglobulin hypermutation (49).

Lower affinity and reduced B-cell responsiveness to antigen could account for the reduced efficacy of vaccinations in older individuals (50). While current vaccine formulations are highly efficacious in young, healthy recipients (51), their failure rate in

the elderly may be as high as 50% (52). Altered responses, particularly in terms of immunoglobulin isotypic responses, are also seen in elderly recipients (50). Thus elderly patients produce antibodies of a different isotype compared to younger vaccinates (50, 53, 54). Similar results have been obtained in rodent models (55).

Ageing in horses is likewise associated with reduced antibody responses to vaccination (43, 56). In one study, the proportion of horses producing hemagglutination-inhibition (HI) antibodies following equine influenza virus vaccination decreased with advancing age such that no HI antibody responses were seen in horses over 9 years of age (56). When antibody responses were measured by ELISA, a more sensitive measure of total antibody responsiveness, a significant decrease in influenza virus-specific antibody levels was seen in horses greater than 20 years of age following vaccination, as compared to a younger cohort (43). The mechanism of this decline is unknown but appears to be secondary to alterations in T-cell function (43).

While alterations in B-cell ontogeny and function can contribute to decreased humoral responses in the elderly, it is now believed that the dramatic effects of ageing on immune competence are the results of deteriorations in T-cell function (57). T-cells are produced primarily in the thymus as the result of the emigration of T-cell precursors from the bone marrow to this organ. Thymic development involves both the generation and the so-called “education” of T-cells to recognize specific antigen in the context of self major histocompatibility complex (MHC) antigens. Thymic selection gives rise to the two major subsets of T-cells, CD4⁺ helper T-cells and the CD8⁺ cytotoxic T-cells. The CD8⁺ T-cell recognizes its foreign antigen in association with MHC Class I antigens that are present on the surface of all nucleated cells. These cytotoxic effector cells play a

primary role in the immune response to viruses and tumors lysing their target cells via a variety of mechanisms (58). The CD4⁺ T-cells recognize foreign antigens presented in the context of MHC Class II antigens found on macrophages and other antigen presenting cells. The CD4⁺ T-cell population can be further subdivided into T helper (Th) subsets based on the pattern of cytokines they produce. Th1 cells produce IL-2 and IFN γ , and Th2 cells produce IL-4, IL-5, and IL-13. The significance of this segregation of cytokine production is that Th1 cytokines are associated with immune responses to intracellular pathogens, whereas the Th2 response is directed against extracellular agents (59). Therefore, immunity to viruses and intracellular parasites is mediated by Th1 cells, whereas protection against intestinal helminths involves Th2 responses. Though initially described in murine models, similar patterns of Th subsets have been described in other species, including the horse (60).

One of the most consistent changes noted in immune competency with advancing age is the decrease in the number of naive T-cells with a concomitant increase in T-cells with an activated/memory phenotype (61). While it has long been established that with age there is a significant involution of the thymus and a resulting decline in naïve T-cell production (61-63), increased apoptosis and lower responsiveness of aged lymphoid cells to activation and proliferation signals also contribute to this process (64). Thus T lymphocytes derived from elderly humans are more susceptible to activation-induced cell death than T-cells from young individuals (65). While this increase in apoptosis could be the result of increased FAS antigen (CD95) expression on the T-cell surface (66), alterations in normal signaling pathways via other receptors might also contribute to this effect (67-69). In addition to an increased susceptibility to apoptosis, T-cells from aged

individuals also exhibit an impaired proliferative capacity (61). This reduced proliferative capacity is the result of the diminished production of IL-2 by aged T-cells (61, 70). The reduction in IL-2 production is itself associated with the reduced activation of various intracellular signaling pathways (68, 71-73), particularly those initiated by the T-cell receptor/CD3 complex (74-76). The central role IL-2 plays in age-associated alterations in T-cell function is evident in those studies where supplementation with this cytokine could restore immune responsiveness both *in vitro* and *in vivo* (77, 78).

Ageing in horses is associated with similar changes in the thymus and the circulating T-cell compartment (79, 80). When compared to their neonatal counterparts, MHC II expression is increased on the T lymphocytes of adult horses (80). This is consistent with a shift towards a larger memory T-cell population with age as seen in other species. Ageing in equids is also associated with a decreased proliferative response of their T-cells to *in vitro* stimulation with mitogens (43, 81). Though there was no difference in the overall expression of the IL-2 receptor on mitogen-stimulated T-cells from young and aged ponies, supplementation of these cultures with recombinant IL-2 failed to restore the proliferative response of the older cells (81). These results indicate that alterations in IL-2 production alone may not play a central role in age-associated immune deviation in the horse.

While the decreased proliferative responses of T lymphocytes from elderly humans and mice is correlated with decreased release of IL-2, the expression of other cytokine genes and their receptors may also be affected (82). Indeed many of the problems associated with an ageing immune response may be due to age-dependent alterations in cytokine gene expression by CD4⁺ T-cells (82-84). While the pattern of

these changes is complex, ageing appears to be generally associated with a decline in Th1 cytokine expression and a concomitant increase in susceptibility to those infections best controlled by this type of response (77, 85). This increased susceptibility to infection is compounded by ageing-associated alterations in CD8⁺ T-cell function. As age increases, there is a resultant decrease in the cytotoxic activity of virus-specific CD8⁺ cells in humans (86). This decreased cytotoxicity is associated with a decline in perforin gene expression (87). A similar decline in cytotoxic activity and IFN γ production is seen in aged mice and is associated with increased susceptibility to viral infection (88).

The central thesis of this review has been a focus on the changes that occur in the immune responses of older individuals. While it is clear that current vaccination strategies may have to be modified to address the unique needs of the older human (51) and equine populations, an important point to consider is the effect of other health conditions on the immune system. There is good evidence that healthy individuals of advanced age can respond to vaccinations as well as younger individuals (89-91). Variations in vaccine responses of the elderly may be most dependent upon individual genetic variations (89), health status (92-94), and other external factors (95). Similar considerations apply when evaluating the effect of ageing on the ability of horses to respond to vaccination. The adverse effect of parasitism on vaccine responses in the horse has been recently reported (96). While adult horses tend to have lower levels of parasitism than younger animals, aged individuals may also have elevated levels despite the active use of anthelmintics (97-99). The effect of various stressors on immune responses in both humans (100, 101) and horses is also well recognized (102, 103). Dietary and nutritional status are widely viewed as important factors affecting immune

responsiveness of the aged population (104-107). All of these factors must be considered when vaccinating both aged and young horses.

In order to assess the effectiveness of vaccination on aged horses, we vaccinated two populations of privately-owned, mixed aged horses to novel and recall antigens and assessed their antibody response to these vaccines by ELISA. This study required dividing both herds into three age groups, young, middle, and old. The three age groups were selected in an attempt to maintain a normal distribution of horses between both populations. The age groups were divided as follows: Young, 1-10 years, middle, 11-19 years, and old, 20+.

MATERIALS AND METHODS

Horses. A group of 36 horses maintained at the BREC Farr Park Horse Activity Center (Baton Rouge, LA) that have been vaccinated annually for equine influenza using commercially available inactivated virus products. A second group of 24 horses maintained at the Marydale Girl Scout Camp (Table 1) were not routinely vaccinated for equine influenza virus prior to inclusion in this study. Each group was of mixed sex, breed and age. The BREC horses were kept in stalls during the week and put out to pasture on weekends. While stalled they were fed a pelleted ration and sweet feed (Purina Stable Star, St Louis, MO). The Marydale horses were kept primarily on pasture and brought in daily to be given a pelleted feed. The BREC herd was considered an “open herd” as horses routinely entered and left the facility. The Marydale horses were a closed population with no new additions during the course of this study (Table 1).

Table 1: BREC and Marydale horses

<u>BREC</u>				<u>Marydale</u>				
NAME	AGE	NAME	AGE		NAME	AGE	NAME	AGE
Roxanne	2	Missy	13		Nevada	7	Dallas	17
Molly	4	Star	14		Molley	8	Pretty	17
Ari	5	Beaux	14		Babydoll	11	Kneehigh	18
Zack	5	Blue	16		Sassy	11	Little Rick	19
Debbie	6	Angel	16		Spider	12	Puddin	19
Sweetpea	6	Dolly	16		Buckshot	13	Roman	19
Prince	7	Daisy	17		Eagle	13	Chester	20
Maya	9	Hannah	17		Penny	14	Chief	20
M&M	9	Trojan	19		Apache	15	Mickey	21
Chic	10	Teddy	19		Ariel	16	Scout	22
Leroy	10	Rudy	20		Patches	16	Taylor	22
Donna	10	Pal	20		Peggy	16	Pj	30
Jack	10	Love	23					
Casey	11	Zayid	24					
Pooh	11	Wiggins	25					
Patience	11	Amigo	26					
Skip	11	Doc	27					
Blaze	13							

Vaccination. Both populations of horses were vaccinated with a deep intramuscular injection on the right side of the neck into the serratus cervicis with 1 mL of a commercially-available, formalin-inactivated equine influenza virus vaccine (Fluvac+, Fort Dodge Laboratories Inc., Fort Dodge, IA) that contained the A/Equine/Kentucky/91 strain of influenza virus. The BREC horses also received, immediately after the flu vaccination, a 1 mg dose of ovalbumin (OVA, Sigma, St Louis, MO) in alum adjuvant (OVA, Sigma, St Louis, MO). Shortly after a 1 ml vaccination of inactivated influenza virus, the Marydale horses were vaccinated intramuscularly with 1 mg of Keyhole Limpet Hemocyanin (KLH, Sigma, St. Louis, MO) also in an alum adjuvant.

Serum collection. Blood was collected aseptically into vacutainer tubes (Becton Dickinson & Co., Franklin, NJ) via jugular venipuncture one month before vaccination and at monthly intervals post vaccination for seven months. The blood was clotted and subsequently centrifuged for 10 minutes at 1000 rpm to separate the serum. The serum was then withdrawn using a slow pipetting technique and stored in 2mL micro tubes (Sarstedt, Numbrecht, Germany) at -20° C until later use.

Isotype quantitation. Antigen-specific equine antibody isotypes in the sera were quantitated using a capture ELISA assay(108), as adapted for equine antibodies (109). The first two rows of replicate 96-well plates were coated (50 μ L per well at 5 μ g of antibody/mL) overnight with affinity purified polyclonal antibodies specific for an equine immunoglobulin isotype (Bethyl Laboratories, Montgomery, TX). Additionally, the remaining wells were coated with specific antigen (50 μ L per well at 5 μ g of antibody/mL). The plates were washed and subsequently blocked with 2% fish gelatin

(Sigma, St. Louis, MO) in PBS. A standard curve was prepared on each plate by applying an equine reference serum (Bethyl laboratories, Montgomery, TX) containing known concentrations of antibody for each isotype to the wells coated with the affinity purified antibody at a starting dilution of 1 $\mu\text{g}/\text{mL}$ and serially diluted two-fold out to a final concentration of .00781 $\mu\text{g}/\text{mL}$. Test serum samples, initially diluted 1/100 and then two-fold to a final dilution of 1/400, were added in duplicate to the wells coated with the specific antigen. The plates were then incubated for 60 minutes at 37° C. Following incubation, the plates were washed and equine isotype-specific monoclonal antibody was added at a dilution that produced an absorbance at the midpoint of the standard curve. Equine isotype-specific murine monoclonal antibodies (mAbs) to IgA, IgGb (CVS 39), IgGa (CVS 45), and anti-IgG(T) (CVS 38) were kindly provided by Dr. Paul Lunn (Madison, WI). Each was used as a hybridoma generated supernate. Following a 60-minute incubation, the plates were washed with an ELISA wash consisting of PBS, 25% Tween-20, and deionized water. Subsequently, an HRP-conjugated goat-anti-mouse IgG antibody (BD Biosciences, San Diego, CA) was added at a 1:2400 dilution. The plates were again incubated for 60 minutes and washed with ELISA wash. A TMB1-component (3,3',5,5'-tetramethylbenzidine) peroxidase-substrate solution (75 μl per well) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added to each well. This caused a color-change (blue) to occur that was allowed to develop for 5 minutes. Then the reaction was stopped with a 1% HCL solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD). The plates were read at 450nm using the MRX microplate reader (Dynex Technologies, Chantilly, VA). The results generated by the reference curve allowed sample results to be expressed in $\mu\text{g}/\text{mL}$.

Cross-reactivity ELISA. Four plates were coated with IgA, IgGa, IgGb, and IgG(T) polyclonal antibodies such that each isotype was applied across each plate in two rows at a concentration of 5µg/ml. The plates were then stored overnight at 4° C. The plates were then washed with ELISA wash and blocked with a 2% Fish gel and PBS solution for 60 minutes at room temperature. Reference serum was added to each plate at the concentration specific to each isotype: IgGa 2.2 mg/ml, IgGb 9.55 mg/ml, IgG(T) 10.6 mg/ml, IgA 1.15 mg/ml, IgM 1.5 mg/ml. After a 60 minute incubation at 37° C and a subsequent ELISA wash an isotype-specific monoclonal antibody (anti-equine IgGa, anti-equine IgGb, anti-equine IgA, anti-equine IgG(T)) was added at a 1:10 dilution to all of the wells on a plate such that all four plates each received a different monoclonal per plate. Following 60 minutes incubation the plates were washed and a goat-anti-mouse IgG-HRP antibody was added at a 1:2400 dilution. The plates were again incubated for 60 minutes and then washed. Plates were developed with TMB1-component peroxidase-substrate (75µl per well) and stopped, as previously described. The plates were read at 450nm with the MRX microplate reader [Dynex Technologies, Chantilly, VA].

Monoclonal antibody specificity assay. One plate was coated overnight with 5µg/ml of purified IgG(T) antibody. After washing and blocking the plate, mAb, of each isotype, was added to the plate starting at a 1:10 dilution in a two-fold series. The plate was then incubated for 60 minutes at 37° C and subsequently washed. HRP-conjugated anti-mouse IgG antibody was added at a 1:2400 dilution to the wells and the plates incubated and washed, as before. The plate was developed with TMB1-component peroxidase-substrate solution and read at 450nm on the MRX microplate reader.

Monoclonal antibody assay. One plate was coated overnight at 5µg/ml with the equine reference serum (Bethyl Laboratories). After washing and blocking the plate with a 2% fish gel solution, isotype specific monoclonal antibody was added to the wells such that each isotype was tested in duplicate. After a 60-minute incubation at 37° C, the plate was washed and HRP-conjugated, anti-mouse IgG antibody was added at a 1:2400 dilution to the plate. After a final incubation and wash, the plate was developed with TMB1-component peroxidase-substrate solution, and subsequently stopped with the 1% HCl solution. Lastly, the plate was read at 450nm by the MRX microplate reader.

Reference serum radial immunodiffusion assay. The equine reference serum and sera samples from three randomly selected horses were pipetted, undiluted, at 5µl per well in a VET-RID radial immunodiffusion plate (Bethyl Laboratories) specific for IgG(T). Following 18 hours incubation at room temperature, the precipitin rings were measured to the nearest tenth of a millimeter using a light microscope equipped with an ocular scale. A standard curve was established by plotting the ring diameter of each standard onto a logarithmic scale. The IgG(T) concentration of each sample was determined by plotting its ring diameter on the standard curve.

Monoclonal antibody radial immunodiffusion assay. The PermaRID Radial Immunodiffusion Kit (QED Biosciences, San Diego) was used to quantify mAb concentrations. Mouse IgG standards of 1000, 500, 250, & 62.4 µg/ml were pipetted into wells on the gel at 20µl. Neat equine monoclonal antibody samples were pipetted into wells at 20 µl per well in duplicate. The gel was then allowed to incubate at room temperature for 48 hours. After incubation the gel was washed in phosphate buffered solution (PBS) and subsequently blotted and rinsed with blotting paper and double-

distilled water to remove excess indicator dye. Lastly, the gel was allowed to dry and the precipitate was measured and plotted against a standard curve and the relative concentrations were extrapolated from the corresponding numbers on the curve.

Polyclonal antibody specificity assay. Two plates was coated overnight with 5 μ g/ml of purified IgG(T) antibody or purified IgA antibody. After washing and blocking the plate, affinity-purified polyclonal goat anti-equine IgG(T) and affinity-purified polyclonal sheep anti-equine IgA was added at 1 μ g/ml and then incubated for 60 minutes at 37° C. Once the plate was washed, a biotinylated mouse anti-goat antibody was added at a 1:50 dilution, titrated-out in a two-fold series, then incubated again for 60 minutes. After another wash, streptavidin was added at a 1:1000 dilution. Again, the plate was incubated for 60 minutes and then washed, at which point the plate was developed with TMB1-component peroxidase-substrate solution at 75 μ l per well and subsequently the reaction was stopped and the plate read at 450nm by the MRX microplate reader.

Competition ELISA. A plate was coated overnight with 5 μ g/ml of a goat anti-horse IgG(T) polyclonal antibody per well in 50 ul volume. After blocking the plate, four rows of wells receive 1 μ g/ml of reference serum and the remaining four rows received 1 μ g/ml of purified equine IgG(T) (Bethyl laboratories). The plate was incubated for 60 minutes at 37° C and subsequently washed. Next a monoclonal IgG(T) antibody was added to two of the rows containing the reference serum and to two of the rows containing the purified IgG(T). An HRP-conjugated goat anti-equine IgG antibody (Bethyl Laboratories, Montgomery, TX) was added to the remaining two rows in the reference serum and the purified IgG(T) wells. After a 60 minute incubation and wash,

HRP-conjugated IgG antibody was added at a 1:2400 dilution to the wells that had previously received the monoclonal antibody. The wells that had previously received the HRP-goat anti-horse antibody, received an addition of 50µl per well of PBS in order to prevent the wells from drying out. Lastly, after a 60-minute incubation and wash cycle the plates were developed with TMB1-component peroxidase-substrate solution, stopped as previously described, and read at 450nm with the MRX microplate reader (Fig. 2).

Epitope Competition Assay

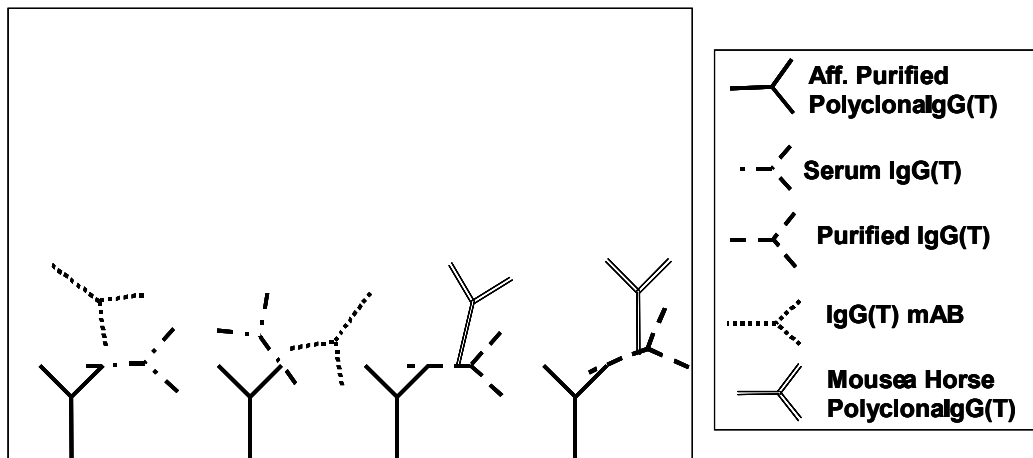


Figure. 2: Epitope Competition Assay

Statistics. All data were analyzed using SAS (110). Comparisons of vaccine response in all horses over time were evaluated by analyzing differences of the means of each time period using a Tukeys Studentized test. In order to determine if there was a difference in vaccine response in each age group per period (pre-vx. 1 mo, 3 mo, 5 mo, 6 mo, 7mo) a split plot design was used such that age group and individual horse within age group were on the main plot, period and age group by period interaction on the

subplot. The least squares means were then tested using ANOVA. Results were determined significant at $P \leq 0.05$.

RESULTS

As expected three of the capture ELISA assays (IgA, IgGa, and IgGb) showed excellent sensitivity and little cross reactivity for their respective isotypes (Figures 3-6). However, the IgG(T) capture ELISA failed to detect IgG(T) antibody in the reference serum and appears to cross-react with IgA.

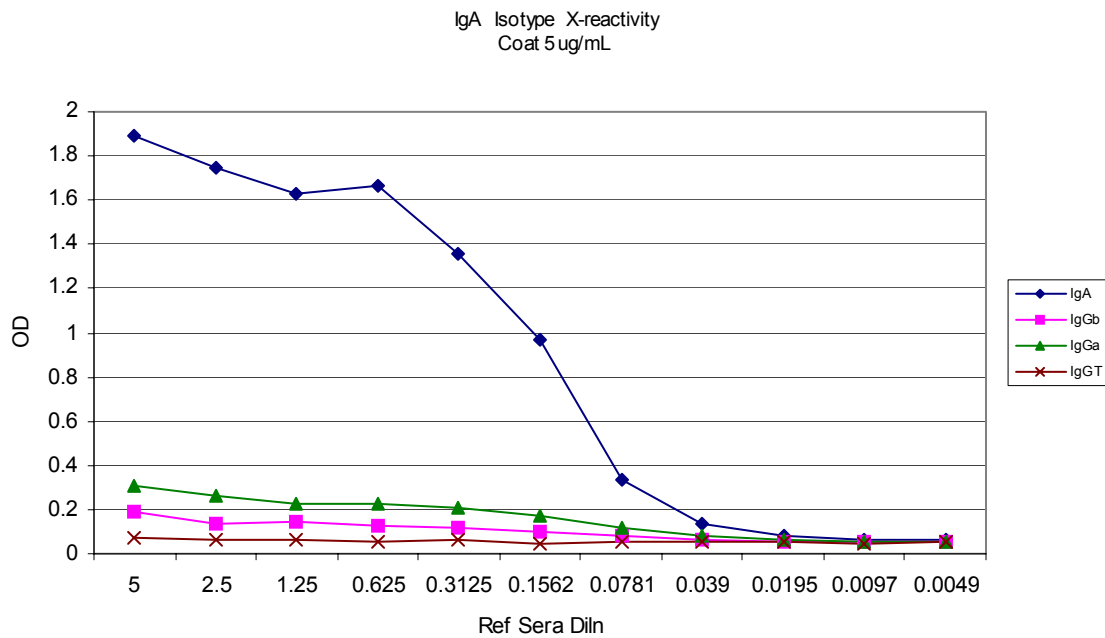


Figure 3: Crossreactivity of monoclonal antibodies with sheep anti-horse IgA polyclonal antibody

To better characterize the specificity and apparent cross-reactivity of the anti-IgG(T) and anti-IgA monoclonal antibodies, purified IgG(T) and IgA were used to coat ELISA plates. Both of the monoclonal antibodies recognized their respective purified antibody when bound to the ELISA plate directly (Table 2). Neither monoclonal antibody exhibited cross reactivity, nor did any of the other mAbs cross react with either purified protein (Table 2).

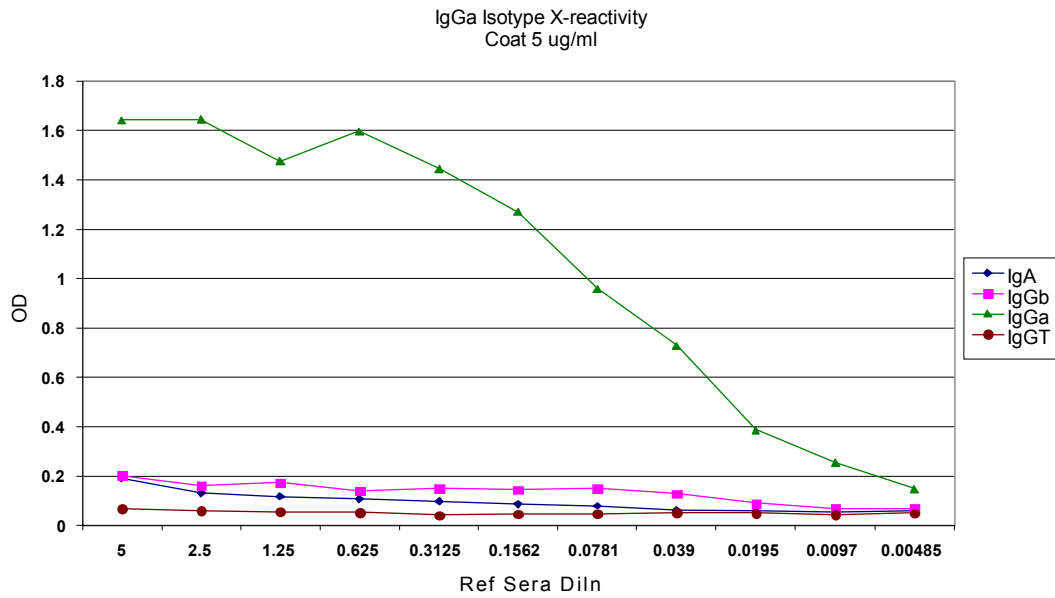


Figure 4: Crossreactivity of monoclonal antibodies with sheep anti-horse IgGa polyclonal antibody.

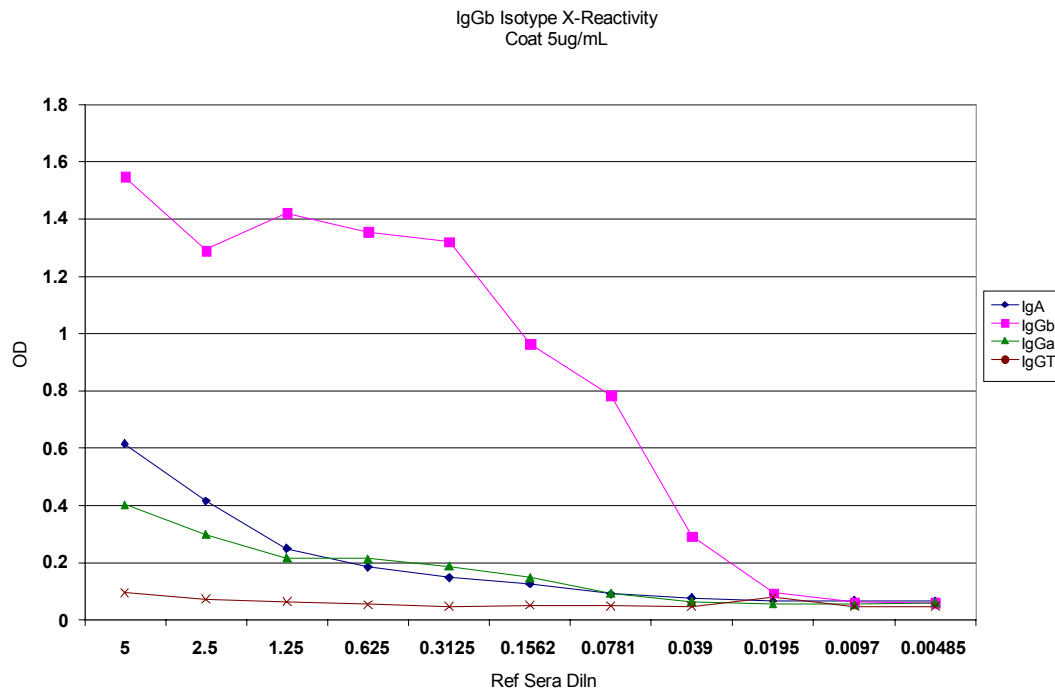


Figure 5: Crossreactivity of monoclonal antibodies with sheep anti-horse IgGb polyclonal antibody

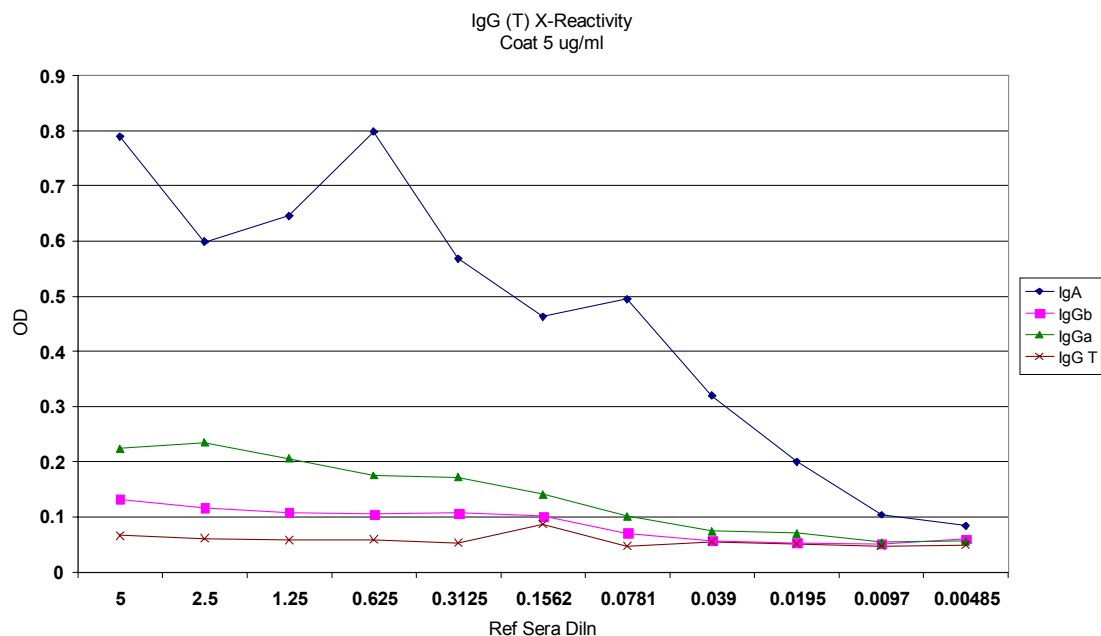


Figure 6: Crossreactivity of monoclonal antibodies with sheep anti-horse IgG(T) polyclonal antibody

Table 2: Specificity of IgA and IgG(T) monoclonal antibodies

Purified IgG(T)	mAb	Diln	1:10	1:20	1:40	1:80	1:160	NEG
	Anti-IgA	OD	0.048	0.052	0.048	0.047	0.050	0.057
	Anti-IgGb	OD	0.059	0.048	0.049	0.052	0.050	0.054
	Anti-IgGa	OD	0.051	0.046	0.047	0.071	0.050	0.057
	Anti-IgG(T)	OD	0.894	0.709	0.640	0.395	0.316	0.056
Purified IgA	mAb	Diln	1:10	1:20	1:40	1:80	1:160	NEG
	Anti-IgA	OD	1.192	1.155	1.062	1.087	1.061	0.058
	Anti-IgGb	OD	0.047	0.044	0.045	0.051	0.054	0.053
	Anti-IgGa	OD	0.046	0.046	0.050	0.054	0.047	0.056
	Anti-IgG(T)	OD	0.044	0.045	0.049	0.047	0.044	0.054

To rule out the unlikely possibility that the reference serum contained less IgG(T) than specified by the manufacturer, radial immunodiffusion (RID) analysis of the serum was performed. Results from the RID indicated that the reference serum concentration of

IgG(T) was equivalent to that specified by the manufacturer and similar to that of three randomly chosen serum samples (Table 3).

Table 3: RID Assay: IgG(T) concentration in reference sera and random sera .

Samples	Calc Conc. (mg/dl)
Ref Sera	830
Ariel	830
Chief	1900
Dallas	1050

The amount of monoclonal antibody specific to each equine isotype in each hybridoma supernate was determined using a mouse immunoglobulin specific radial immunodiffusion assay. The anti- IgA, IgGa, and IgG(T) were all within the specified range of the standard curve, except the anti-IgGb which was below detection levels (Table 4).

Table 4: RID Assay: Anti-Equine antibody concentrations per supernate.

Linear Trendline			
Sample	Dil	Diameter	ug/ml
anti Eq IgG(a)	1	43	66.126
anti IgG(b)	1	38	-14.684
anti IgA	1	44	82.288
anti IgG(T)	1	45	98.45

As our results indicate, mAbs to equine IgG(T) and IgA showed excellent specificity and sensitivity for their respective isotypes. We subsequently tested the anti-IgG(T) and anti-IgA polyclonal antibodies in a similar manner. Both polyclonal

antibodies were effective in the ability to recognize and bind to their respected purified antigens when bound to an ELISA plate (Table 5). Additionally these two polyclonal showed no cross-reactivity towards each other.

Table 5: Recognition of purified antigen by polyclonal anti-IgG(T) and IgA antibodies.

Polyclonal	1:50	1:100	1:200	1:400	NEG
IgG(T) OD	0.64	0.512	0.566	0.554	0.042
IgA OD	0.28	0.239	0.152	0.151	0.042

In order to determine whether the IgG(T) monoclonal antibody was being blocked by the polyclonal antibody, we tested whether a different antibody could detect the captured IgG(T). Our results show that an HRP-conjugated polyclonal antibody is able to bind the IgG(T) in the reference serum and purified IgG(T) captured in the ELISA but that the monoclonal antibody to IgG(T) fails to bind either serum IgG(T) or the purified antigen in the same assay (Table 6).

Table 6. Monoclonal anti-IgG(T) fails to detect the captured IgG(T) but a polyclonal antibody does.

	Detect	.5 ug/ml	.25 ug/ml	.125 ug/ml	.0625 ug/ml	.0312 ug/ml	.0156 ug/ml	NEG
Ref Sera	mAb	-0.007	-0.014	-0.013	-0.013	-0.013	-0.014	-0.009
	Polyclonal	0.639	0.55	0.413	0.408	0.211	0.13	0.013
Purified IgG(T)	mAb	-0.002	-0.014	-0.015	-0.014	-0.014	-0.016	-0.01
	Polyclonal	0.636	0.476	0.415	0.235	0.148	0.057	0.016

Both the BREC and Marydale horses were vaccinated with a commercially available vaccine. Post-vaccination total influenza virus specific Ig levels in the BREC herd were significantly elevated compared to pre-vaccination at 1, 3, and 5 months and

returned to pre-vaccination levels by 6 months post (Figure 7). The post-vaccination IgG(T) response to influenza vaccination was significantly different from pre-vaccination levels at 1 and 3 months. The IgG(T) response to the flu vaccine began to fall off and return to pre-vaccination by 5 months post. The influenza specific IgGb post-vaccination levels were significantly higher only at 1 month post-vaccination. The return to pre-vaccination levels occurred rapidly after 1 month post-vaccination. There was no significant difference in the post-vaccination IgA antibody levels, however, despite a lack of statistical significance there was a general increase in flu specific IgA levels accompanied by a quick return to pre-vaccination levels. As noted above, in each analysis, the duration of the vaccine response was relatively short-lived and returned to near or below pre-vaccination levels within 3-5 months post-vaccination in the BREC herd.

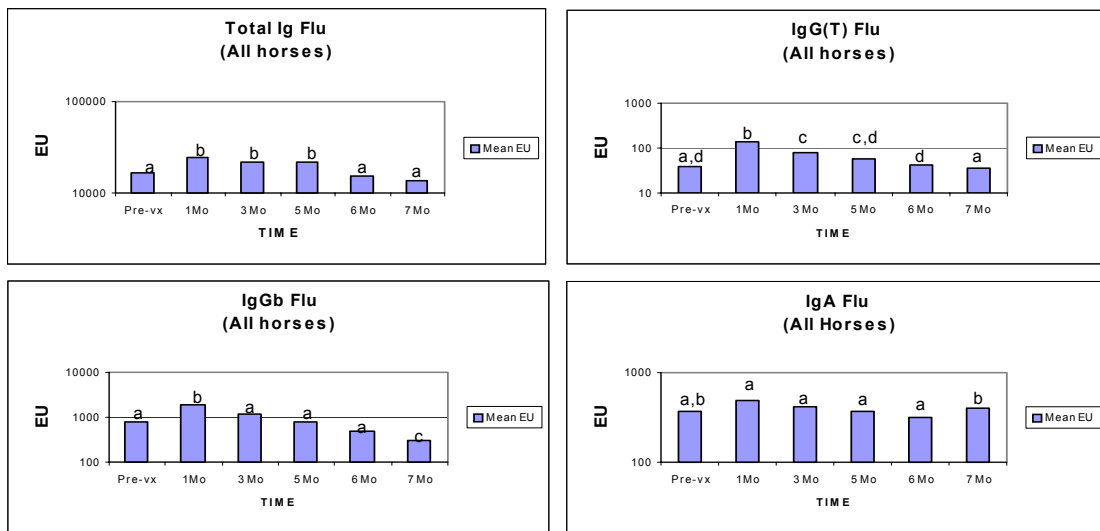


Figure 7: BREC total Ig and isotypic antibody responses to Flu vaccine. Time periods with letters in common are not significantly different by Tukeys studentized range (HSD) Test ($P \leq 0.05$).

The Marydale herd established a similar pattern in the overall response to the flu vaccine with the total Ig post-vaccination levels being significantly different from pre-vaccination values at all time points (Figure 7). The sub-isotype IgGb to the flu vaccine was significantly higher than pre-vaccination at all post-vaccination time points. The intensity of the IgGb response began to decrease after 1 month. There was no significant difference in the influenza virus specific IgG(T) or IgA isotypes post-vaccination.

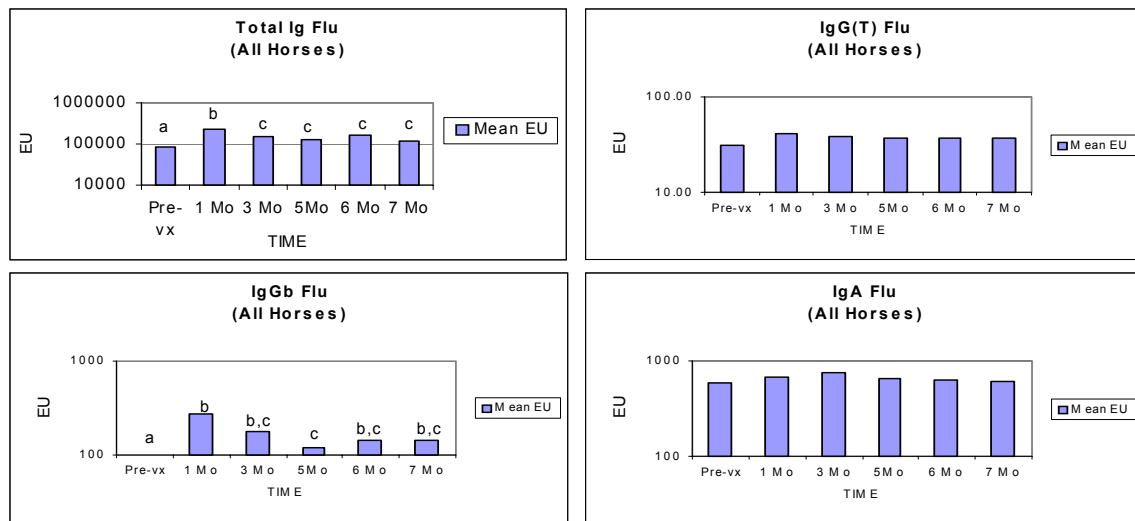


Figure 8: Marydale total Ig and isotypic antibody responses to Flu vaccine. Time periods with letters in common are not significantly different by Tukeys studentized range (HSD) Test ($P \leq 0.05$).

To assess the effect of ageing on the primary immune response to vaccination both herds were vaccinated with of a novel antigen vaccine in an alum adjuvant. The BREC herd received 1 mg of OVA in an alum adjuvant. The anti-OVA total Ig response established classical signs of a primary immune response to vaccination with low pre-vaccination levels and a significant increase at all time points post-vaccination (Figure 9). The duration of this response lasted 6 months. Similarly, the anti-OVA IgG(T) response

reflected a classical primary immune response at all time points post-vaccination, though antibody levels began to decline after 3 months. The OVA-specific IgGb levels were even less long-lived than IgG(T) with significant differences from pre-vaccination levels seen only at 1 month and 3 months post-vx. The IgA response increased significantly at 3 and 5 months with a return to pre-vaccination levels by 6 months.

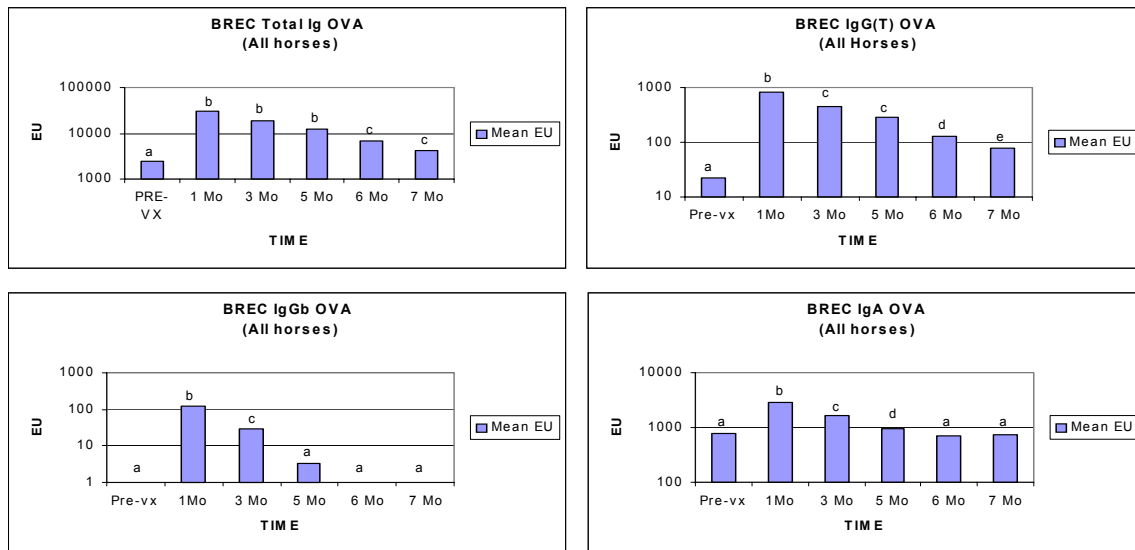


Figure 9: Total Ig and isotypic antibody responses to OVA vaccine. Time periods with letters in common are not significantly different by Tukeys studentized range (HSD) Test ($P \leq 0.05$).

Similarly, the Marydale horses were given 1 mg of an experimental KLH vaccine in an alum adjuvant. The total Ig response to KLH was quite vigorous with 1 month post-vaccination EU levels exceeding that of either Flu or OVA antibody levels (Figure 10). Likewise, the antibody levels at all other time points were significantly different from pre-vaccination for the 7 months of the experiment. The KLH-specific IgG(T) was significantly increased at 1 month and 3 months post-vaccination with the greatest difference being at 3 months, after which, the anti-KLH IgG(T) levels rapidly returned to

pre-vaccination levels. Anti-KLH IgGb increased significantly from pre-vx levels at 1 and 3 months. As with the other isotypes, the duration of the IgGb response was limited to 3 months. The IgA response to KLH exhibited a significant increase at 1 month and 3 months post-vaccination.

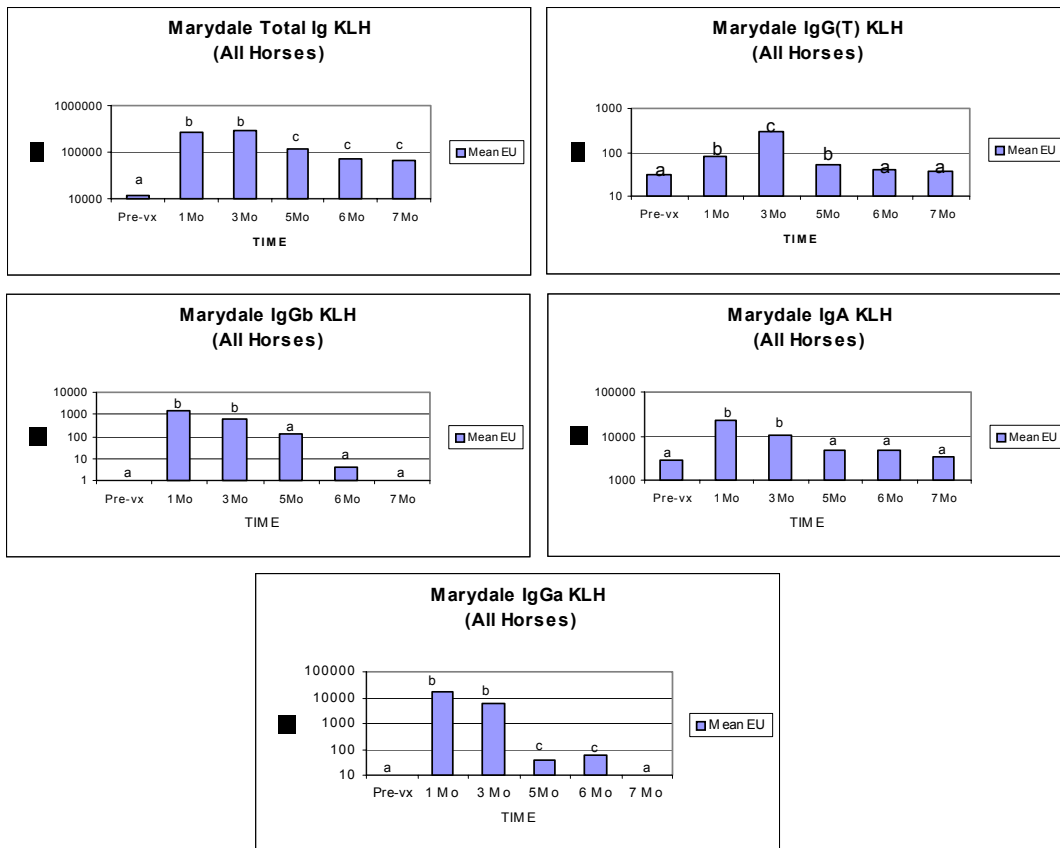


Figure 10: Total Ig and isotypic antibody responses to KLH vaccine. Time periods with letters in common are not significantly different by Tukeys studentized range (HSD) Test ($P \leq 0.05$).

Within the BREC herd, old horses showed no significant difference in their total Ig response to influenza vaccine at any time post-vaccination, while young and middle-aged horses showed significant vaccine-induced increase in their post-vaccination

antibody levels (Figure 11). Both middle-aged and old horses showed significantly higher pre-vaccination antibody levels than their younger counterparts. Additionally, middle-aged horse total Ig anti-influenza antibodies were significantly elevated at 5 months compared to young horses. All age groups showed significant increases in their influenza specific IgG(T) levels and there was no significant difference between the age groups and specific time periods. At 1 month post-vaccination, all age groups had significant increases in their IgGb levels, which were followed by a rapid return to pre-vaccination titers. While young and old horses maintained similar levels of antibody at all times middle aged horses had much higher levels of IgGb. Post-vaccination influenza virus specific IgA levels were not significantly different from pre-vx levels in either middle-aged or old horses. Young horses, however, did show a significant increase in post-vaccination influenza virus specific antibody levels which lasted for 5 months post-vx. Both middle aged and old horses did have higher levels of influenza-virus specific IgA antibody when compared to the younger horses and were not significantly different from each other. Old horses were also significantly different from young horses at 1 month post-vx while the middle aged horses were not.

The Marydale herd did not respond as well to influenza vaccination as the BREC herd (Figure 12). The total Ig response showed significant differences mainly within the middle age group. There was a significant increase in anti-influenza antibody in the young group at 6 months and at 7 months in the old age group, but overall were generally nonresponsive to vaccination. Concomitant with this lack of responsiveness is the fact that none of the Marydale horses showed any IgG(T) response to the influenza vaccine at any time period, nor were there any differences between age group. IgGb-specific

response to the vaccine in middle-aged horses was seen at 1, 3, and 5 months post-vaccination while old horses similarly showed significant increases at 1 and 3 months post-vaccination, as such, the middle and old aged horses demonstrated significant differences in influenza virus-specific IgGb antibody from there younger counterparts at all time points. Young horses did not exhibit any IgGb-specific response to vaccination. IgA levels remained relatively unchanged in the young horses. Middle-aged horses showed a significant increase from pre-vaccination levels at all time points. In the old age group, IgA levels actually declined following vaccination with a significant difference from pre-vaccination antibody levels seen at 3 months post-vaccination. No significant differences between the age groups were detected at any time point.

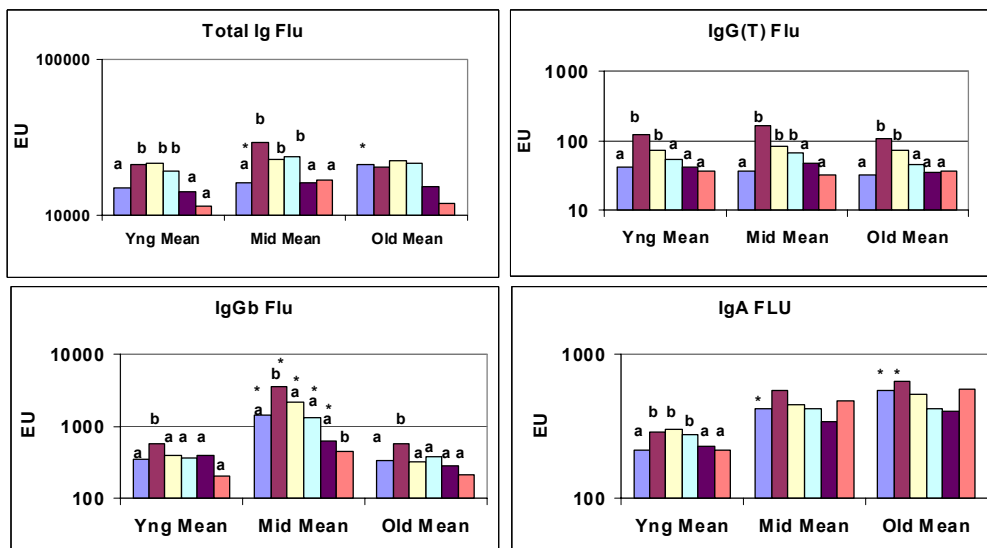


Figure 11: BREC herd antibody response to Flu-vx within age group and between age group by period interaction. Letters denote significant differences from pre-vx levels within age group over time. Asterisk denotes significant differences from the young age group of antibody levels at a specific time point. Test conducted by least squares means analysis ($P \leq 0.05$).

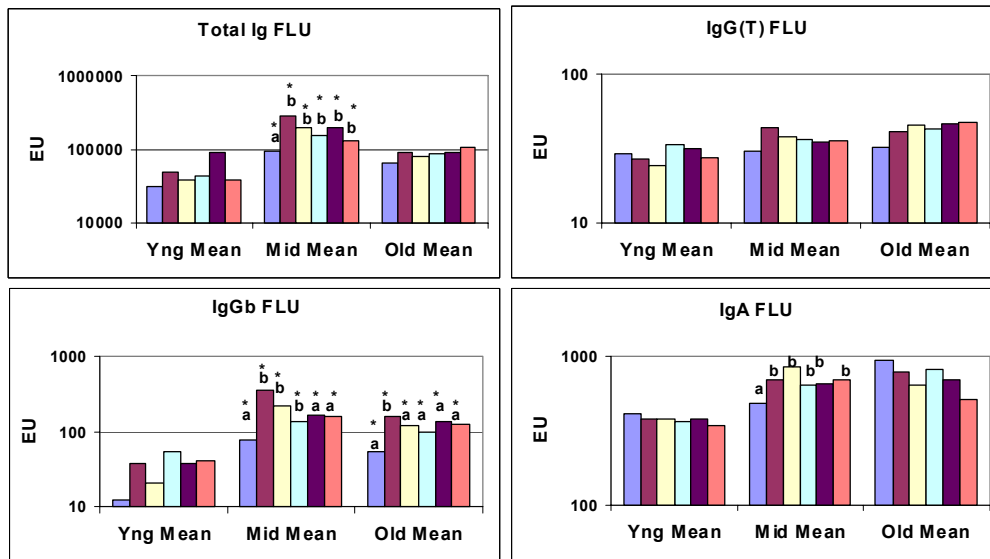


Figure 12: Marydale herd antibody response to Flu-vx within age group and between age group by period interaction. Letters denote significant differences from pre-vx levels within age group over time. Asterisk denotes significant differences from the young age group of antibody levels at a specific time point. Test conducted by least squares means analysis ($P \leq 0.05$).

In order to compare the difference in the primary antibody response to vaccination in the aged horse from younger horses the BREC horses were each vaccinated with 1 mg of an experimental OVA vaccine in an alum adjuvant. All age groups in the BREC herd exhibited robust total Ig responses to OVA (Figure 13). The OVA-specific total Ig responses of the middle aged and old horses were significantly higher than the younger horses at 1 month and 3 months post-vaccination. Additionally, old horses also showed significantly higher levels at 6 months when compared to young horses. All three age-groups exhibited a long duration of elevated antibody levels which continued over the course of the 7 month experiment. As with the total Ig, the post-vaccination mean OVA-specific IgG(T) levels were significantly higher in all age groups when compared to their respective pre-vaccination levels. No significant differences were observed between age

groups at any time period. The OVA-specific IgG(T) response was long-lived, remaining elevated over the 7 month course. All age groups did show an IgGb response to OVA with significant increases seen at only 1 month post-vx. The greatest variability among the age groups in BREC was with the IgA levels. The middle-aged horses had much higher mean OVA-specific IgA levels pre-vaccination than the young and old horses. All three groups did demonstrate significant increases in OVA-specific IgA antibody following vaccination. The duration of the response in all three groups was 3 months before returning to pre-vaccination levels. The middle-aged horses IgA response was greater than that of younger horses at all time points. The old horses showed a significant difference from the young horses at 1 month post-vx.

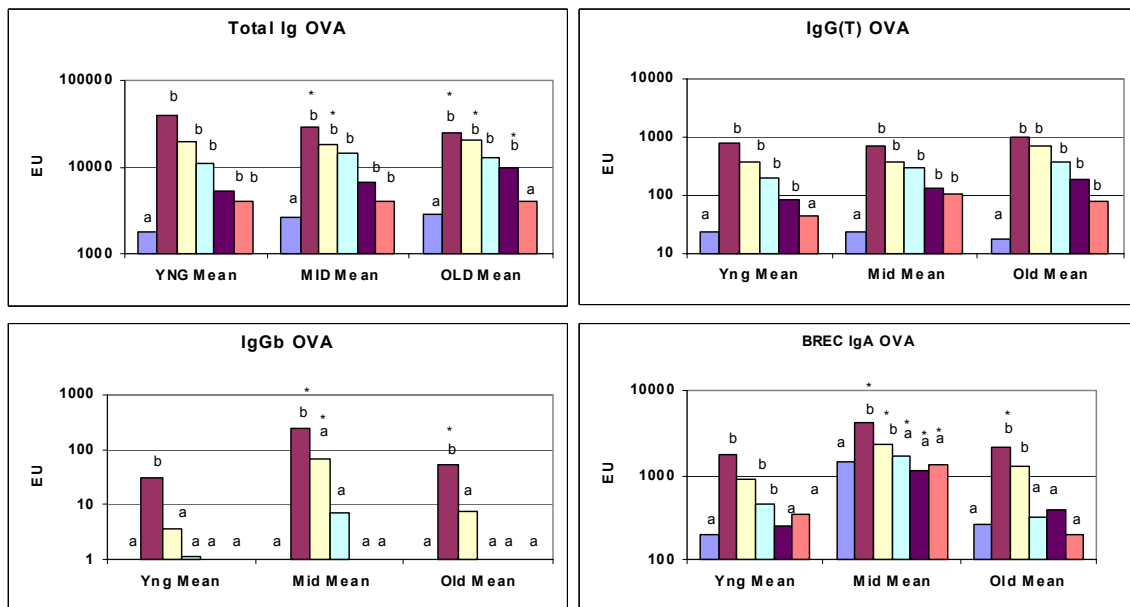


Figure 13: BREC herd antibody response to experimental OVA-vx within age group and between age group by period interaction. Letters denote significant differences from pre-vx levels within age group over time. Asterisk denotes significant differences from the young age group of antibody levels at a specific time point. Test conducted by least squares means analysis ($P \leq 0.05$).

To assess the primary immune response of the Marydale herd, horses were vaccinated with 1 mg of an experimental KLH vaccine. Each age group responded to vaccination demonstrating significant increases in the anti-KLH total Ig, and remaining elevated for the 7 month study, however the middle-aged horses had significantly lower levels of KLH-specific total Ig than either the young or old horses (Figure 14). The IgG(T) response was very similar between the age groups. Each of the three age groups demonstrated a significant increase in mean anti-KLH specific IgGb antibody at 1 and 3 months post-vaccination, and, there were no observed differences between the age groups. Old horses additionally exhibited a greater anti-KLH specific IgGb response when compared to the young and middle-aged horses, and maintained elevated IgGb levels for 5 months before returning to pre-vaccination levels. The IgA response to KLH vaccination was minimal with only the middle-aged horses exhibiting a significant increase of anti-KLH IgA antibody at 1 month post-vaccination, however the middle-aged and old horses maintained elevated levels of IgA for a longer duration with significant differences from young horses at 7 months post-vx.

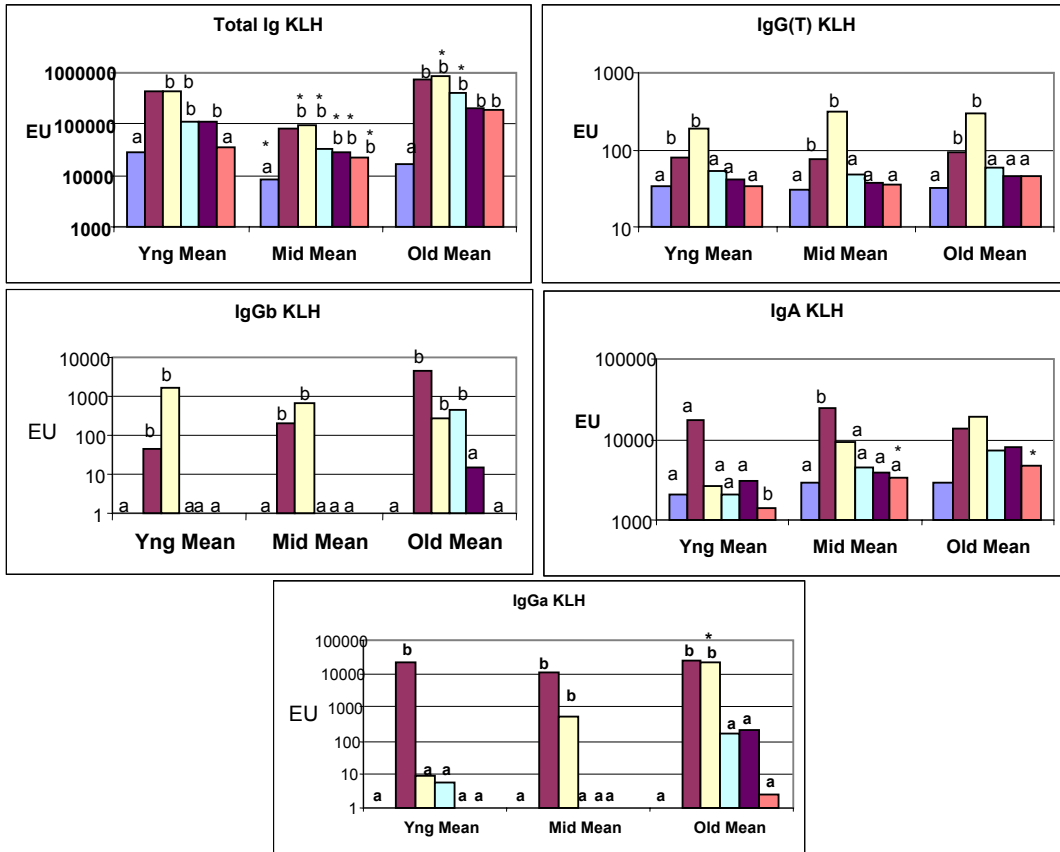


Figure 14: Marydale herd antibody response to experimental KLH-vx within age group and between age group by period interaction. Letters denote significant differences from pre-vx levels within age group over time. Asterisk denotes significant differences from the young age group of antibody levels at a specific time point. Test conducted by least squares means analysis ($P \leq 0.05$).

DISCUSSION

Immunoglobulin isotype expression plays a central role in determining the ultimate functionality of the humoral immune response. This is particularly true regarding the use vaccination as a means of achieving protection against infectious agents. Failure to induce the proper isotypic response may reduce the effectiveness of a vaccine even if it contains the protective antigens. The functionality and molecular regulation of isotype expression is well described for humans and mice, however, less is known regarding this response in the horse. There are isotypic-specific reagents available for studying equine isotypes and sub-isotypes and a recent paper by Breathnach et al (109), described a capture ELISA procedure using these reagents to quantitate equine isotype and sub-isotype expression in an equine herpes virus model. Here we have examined the issue of cross-reactivity and specificity of these reagents using a similar procedure. While our results indicate that this method does discriminate between the equine immunoglobulin isotypes and sub-isotypes with a fair degree of sensitivity, the notable exception is an apparent cross-reactivity between IgA and IgG(T). Somewhat surprisingly, we also discovered that in some horses the IgG(T) capture ELISA failed to work, presumably as a result of steric-hindrance.

Capture ELISAs are widely used to quantitate both specific antigen and antibodies (111). While a number of protocols for capture ELISAs have been described, the basic assay consists of an initial capture step typically involving a polyclonal antisera followed by subsequent detection using a monoclonal antibody. Variations include different combinations of monoclonal or polyclonal antibodies for the capture or detection phase. The specificity of the assay is determined by the affinity and the avidity

of the two antibodies. While capture ELISAs composed of two monoclonal antibodies have greater specificity for their respective antigen, they may be relatively insensitive due to low affinity or avidity for the target antigen (111). Polyclonal antisera are often preferred for the capture phase of the assay due to the greater affinity and avidity associated with a population of antibodies, however, there is also a greater likelihood of non-specific cross-reactivity occurring when polyclonal antibodies are used (111). Cross-reactivity is defined as the ability of antibody to bind to ligands other than the specific antigen. In the case of the equine IgG isotype specific reagents investigated during this study, only low levels of cross-reactivity were detected for most of the isotypes. The one exception to this was the apparent recognition of polyconally-captured IgA in the reference sera by the anti-IgG(T) monoclonal antibody. Neither reagent showed cross-reactivity against purified IgA or IgG(T). IgG(T) was originally considered to be a homologue of IgA, however structural and antigenic studies suggested it was more likely a subclass of IgG (16, 17). While there have been previous reports of a lack of cross-reactivity of the monoclonal anti-IgG(T) antibody for IgA (24, 112), these were performed using IEP and SDS-PAGE analysis and not capture ELISAs as in the current study. As the anti-IgG(T) monoclonal failed to recognize purified IgA, it is possible that the crossreactivity with the reference serum was the result of a unique cross-reactive epitope. Though IgG(T) is the predominant isotype present in the equine reference sera, we failed to initially detect it using the capture ELISA. Our RID results confirmed that there were sufficient amounts of this isotype in the reference sera to be detected using the capture ELISA. Both reagents were independently shown to detect the IgG(T) in the reference sera as well as purified IgG(T). Nevertheless, they failed to work

together in the capture ELISA when the reference sera was used. They did work together when sera from other horses were used in place of the reference sera (data not shown). Together these results indicate that steric-hindrance or epitope competition between polyclonal antibody, sera, and the monoclonal antibody prevented their use in a capture ELISA. Our ability to detect the IgG(T) from some horses, but never the reference sera is best explained by there being allotypic differences in IgG(T) between the horses. Possible causes of binding interference may include: (1) the polyclonal and the mAb may bind to the same epitope with either similar or different affinities, thus the polyclonal being bound first blocks the mAb from binding. (2) The two reagents may bind separate epitopes that overlap. The polyclonal being bound first would block part of the mAbs epitope, thereby blocking it from binding. (3) Both reagents bind separate epitopes, but the binding of the polyclonal to its epitope may cause a conformational change at a distant site, thereby indirectly inhibiting the mAb from binding to its now conformationally changed epitope (24). Since higher concentrations of IgG(T) mAb were required to provide equivalent detection as the other monoclonals, it appears that the IgG(T) mAb also has a relatively low affinity for its target. This would also be consistent with the notion that the antibody is detecting an epitope with a high degree of allotypic variation.

The goal of this study was to characterize both the memory and primary immune response to vaccination in geriatric horses. There are numerous reports in the literature describing age-related changes in immune function, particularly the immune response to vaccination. Studies of age-related antibody responses in both the human and mouse model have rendered conflicting results. In one study an increase in the serum IgG

concentration with age was reported (113), while another study reported no change (114). McFarlane et al have shown that advanced age in horses results in a decrease in peripheral lymphocytes, T-cells, and B-cells which may contribute to an age-related diminution in immunocompetency (115). Recent research in their lab has shown no significant differences between young and old horses regarding serum concentrations of IgM, IgG, and IgG(T) (116). Still very little research has been done regarding the effect of ageing on antibody responses to vaccination in horses. Here we describe the results of a vaccination study of two populations of horses with varying ages of 2-27 years. By vaccinating with an equine influenza vaccine we are able to describe the memory antibody response and the novel or primary response was defined by vaccination with OVA or KLH.

Our initial analysis was of the memory antibody response, irrespective of age, to vaccination in both populations (Figures 6, 7). Both herds elicited a robust antibody response to equine influenza virus vaccination. Additionally, vaccination produced a strong influenza specific IgGb antibody response in both herds, however the EU levels for IgGb in the BREC herd were much higher indicating a more vigorous IgGb response compared to the Marydale horses. The only notable difference in specific isotype responses between the two herds was that the BREC herd showed a flu specific IgG(T) response to vaccination while the Marydale herd did not. The BREC herd is an open population and is more frequently exposed to infection and subsequently vaccinated more often, which may account for the high levels of anti-influenza IgG(T). These results are in concurrence with research done by Nelson et al. (117). They observed a strong IgG(T) response following vaccination with a killed Equine influenza virus. Additionally, their

results indicated strong antibody responses by IgA, IgGa, and IgGb in the serum and nasal mucosa of the naturally infected ponies but not in the vaccinated ponies. Neither herd exhibited any type of IgA or IgGa specific response to influenza vaccination. This is not unexpected since the vaccination was administered intramuscularly and may not have induced an IgA response. Both populations exhibited similarities in the response to influenza vaccination and in the duration of the antibody response.

Within age groups, only middle-aged horses demonstrated a flu specific total Ig response to influenza vaccination in either herd (figures 10 and 11). The total Ig response observed in the BREC herd is likely a result of a longer history of influenza vaccination or exposure to natural infection compared to the Marydale horses. The middle-aged and old BREC horses also had a significantly higher pre-vaccination total antibody levels than the young horses which may possibly be the result of priming due to past exposures to virus. An anti-influenza IgG(T) response was observed within each age group of the BREC herd, while none was seen in any of the Marydale age groups. Again, this may be explained by the more frequent vaccination of the BREC horses than the Marydale horses. No significant differences were detected between any age group to anti-influenza IgG(T) in either herd. All age groups from both herds manifested an anti-influenza IgGb response, except for the young Marydale horses. The fact that only two horses comprised this cohort may have skewed the analysis and may not be an accurate estimation of antibody response in the younger horses. At all time points, middle-aged and old Marydale horses exhibited significantly higher anti-influenza IgGb antibody levels than did the young age group. Within the BREC herd, only middle-aged horses exhibited a significant increase over young horses in their anti-influenza IgGb levels. The old BREC

horses exhibited a very limited IgGb response to vaccination, that was very similar to the response seen in the younger cohort (Figure 10). An anti-IgA antibody response was detected in the middle-aged Marydale horses and in the young BREC horses. It is possible that the middle-aged Marydale horses had a recent natural exposure to virus, however, we cannot explain why neither the young and old age groups failed to respond. There were significant differences in pre-vaccination IgA antibody levels of middle-aged and old horses from their younger counterparts in the BREC herd (Figure 10). The older horses had much higher preexisting anti-influenza IgA titers. The overall lack of an IgA response to the vaccine may be a result of the method of vaccination. IgA secreting plasma cells predominate near mucosal surfaces so it would seem unlikely that intramuscular vaccination would elicit much of an IgA response.

The two populations of horses were also vaccinated with a novel antigen. The BREC horses were vaccinated with 1 mg of OVA in alum adjuvant and the Marydale horses with 1 mg of KLH in alum adjuvant. Both herds demonstrated classical primary immune responses among all isotypes measured (Figure 9). This response was characterized by a low pre-vaccination titer followed by an increase in antigen specific antibody response of a short duration. Interestingly, the antibody response to KLH in the Marydale horses was much higher than that of OVA antibody response in the BREC herd. The Marydale horses demonstrated a strong IgGa response to KLH vaccination as well (Figure 9) which was not detected in the OVA vaccinated horses (Data not shown). The IgGa response to KLH in the Marydale herd was significantly increased in all three age groups with no significant differences detected between them, but the old horses maintained elevated anti-KLH antibody for a longer time than the young or middle-aged

horses. It is possible that the KLH vaccine possesses certain properties which induces an IgG_a response to a primary infection in the horse.

The total Ig response to KLH was significantly increased in each age group, but the middle age group elicited significantly decreased antibody levels in comparison to both younger and older horses. All three groups maintained elevated anti-KLH total Ig for the course of the study. Although the Marydale horses demonstrated an IgG(T) response to KLH, it was short-lived and no differences were observed between age group. The same trend was observed in the anti-KLH IgG_b response, however the response in the old horses was of a longer duration. No significant differences were observed in the IgA response to KLH.

All age groups in the BREC herd produced strong and long lasting anti-OVA total Ig responses. In contrast to the Marydale herd, the BREC horses had a strong anti-OVA IgG(T) response with a long duration. This suggests that the OVA response may be more of a memory response since IgG(T) has been reported to be a strong element of the secondary immune response (117). Ovalbumin is a constituent of many vaccines, and as such may have primed the BREC horses, which may have yielded data that reflects more of a memory than a naïve immune response.

No age related differences were observed with the anti-OVA IgG(T) response. The anti-OVA IgG_b response was similar to the Marydale IgG_b response to KLH, with a significant increase post-vaccination followed by a rapid return to pre-vaccination levels by 3-5 months. Middle-aged and old horses had significantly higher anti-OVA IgG_b levels than the younger horses, which may be a result of being primed more often. IgA

responded to OVA vaccination strongly with significantly higher levels in the middle-aged cohort than in any other group.

CONCLUSIONS

There were a number of limitations that may have impacted our results; both populations were privately maintained, no medical/ vaccination histories were recorded, and we were unable to obtain an adequate number of young horses in the Marydale herd. These limitations may have led to antigenic stimulation by introduction of new horses in the BREC herd, without a medical/vaccination record we were unable to confirm any preexisting conditions that may have had an impact on our results, and with only two young horses in Marydale to use we may not have obtained accurate data regarding primary exposure from that group. Nonetheless, our results suggest that the response to primary infection is not age-dependent, but the response to secondary infection is age-dependent. Differences between open and closed herds have also been observed. Populations with frequent antigenic stimulation produce stonger antibody resposnes to secondary vaccination. We were unable to establish a true correlation between open and closed herds due to the fact that the OVA response may not be a true primary response.

RECOMMENDATIONS

Though vaccine manufacturers make no specific recommendations regarding the vaccination of older horses and ponies, the similarities in age-induced immunological changes between humans and equines suggests that similar vaccination recommendations should be followed. The need for vaccination of the older horse will depend, of course, upon the relative risk of exposure for the individual horse. Particular care should be taken when using attenuated vaccine products as these live agents may pose a unique risk to the older individual. Immunization with inactivated agent vaccines are thus likely to be safer. Although inactivated viruses are safer our results indicate that they may not provide the necessary IgA antibody response to combat respiratory infections. In general, annual vaccination against equine influenza virus infection, tetanus, rabies and encephalomyelitis viruses are warranted. Most older horses will likely have latent herpesvirus infections (118). Reactivation of these latent infections could lead to myeloencephalopathy or serve as a source of infection for susceptible horses (119, 120). As there is no evidence supporting any benefit of vaccination in preventing such occurrences in the older horses, and a possible risk of vaccination-induced reactivation, such practices should be avoided (121). Likewise, there is little support for the use of immunostimulatory or other supplements in the aged horse to boost immune responsiveness. Indeed the potential risk for adverse reactions with such treatments argues against their use until clear benefits have been shown in properly controlled studies. More work, in general, is needed to better understand the immunological needs of this population.

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VITA

Daniel Harel Fermaglich was born on October 20, 1970, in Washington D.C., the son of Dr. Joseph and Mrs. Doreen Fermaglich. He lived in Potomac, Maryland, until he graduated from Winston Churchill High School in June 1988. Daniel then enlisted in the United States Army where he served with distinction as a crewmember on an M1A1 tank. His military career has taken him to Mannheim, Germany, where he served for four years to include service in southwest Asia during Desert Storm. On January 9, 1997, he left the active Army and joined the Louisiana Army National Guard in order to pursue a college education at Louisiana State University.

Daniel pursued and completed his Bachelor of Science with a concentration in animal science while serving in the National Guard as a Tank platoon leader one weekend a month and during the summer as well. His desire to receive an advanced degree lead him to Dr. David Horohov who offered him a position as a graduate student. In May 2003 Daniel graduated from Louisiana State University, Department of Pathobiological Sciences with a Master of Science degree. Following graduation, Daniel received a commission as a Second Lieutenant in the Louisiana Army National Guard and joined the Homeland Defense effort to combat and mediate terrorist incidents involving weapons of mass destruction (WMD). He uses his civilian education to aid in state level responses to WMD incidents. Additionally, he is involved in education and coordination with civilian agencies regarding their preparation WMD incidents.