Investigation of anti-BSA IgE production post-vaccination in healthy, reactor and commercial plasma donor horses

by

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Abstract

Background: There is anecdotal evidence to suggest risk associated with the administration of commercial plasma to neonatal foals that have previously ingested commercial powdered colostral supplementation within the first 24 hours of life. Bovine serum albumin (BSA) is present in both commercial powdered colostral supplements and commercial equine vaccines. Previous studies in human and equine medicine have investigated the immune response to non-viral vaccine antigens, including BSA and have documented the presence of anti-BSA IgE following vaccination.

Objectives: To measure anti-BSA immunoglobulin E (IgE) levels post-vaccination in healthy horses, those experiencing vaccine reactions and those utilized for commercial plasma harvesting.

Methods: Serum was collected from 65 healthy horses at day 0, 14, 28, 90, 180, 270 and 365 after vaccination as well as from 26 horses at day 1, 180 or 270 after vaccination that had developed an adverse vaccine reaction. Serum was collected from 4 horses that had never been vaccinated and 10 horses that were part of a commercial plasma donation herd. Anti-BSA IgE concentrations were evaluated by enzyme-linked immunosorbent assay (ELISA).

Results: Anti-BSA IgE levels were not detected in non-vaccinated horses and were present in all vaccinated horses. Younger horses demonstrated a higher fold change in post-vaccination anti-BSA IgE levels compared to older horses. Across all age groups there was not a significant difference between pre- and post-vaccine anti-BSA IgE levels in healthy horses. There was not a significant difference in anti-BSA IgE levels between commercial plasma donor horses and healthy horses. There was not a significant difference in post-vaccination anti-BSA IgE levels between reactor horses and healthy horses at day 180 and 270 post-vaccination.

Main Limitations: There was a small number of reactor horses at day 180 and 270 post-vaccination with most samples being at 24 hours post-vaccination. There were no healthy horse samples available for 24 hours after vaccination; therefore, it was not possible to compare the two groups at this time point. The vaccination history of the commercial plasma donor horses was unknown.

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Chapter 1 - Introduction to Immunity

The immune system is an intricate web of cells, chemical messengers and physical barriers designed to protect the host from foreign antigen invasion. While the immune system in itself is extensive, it can be divided into two main pathways; innate and adaptive. These two pathways are intertwined, and both are utilized in immunological defense mechanisms together.

The innate immune system consists of physical barriers, sentinel cells, receptors, leukocytes and chemical messengers. The main role of the innate immune system is to detect foreign antigen, incite a rapid cellular response towards the antigen and destroy it. There are two subsystems of the innate immunity; cellular and humoral. The cellular subsystem consists of leukocytes and their immediate inflammatory response to foreign antigen. The humoral subsystem consists of defense mechanisms such as the complement pathway and acute-phase proteins. The innate pathway results in acute, non-specific inflammation that does not result in any immunologic memory towards the foreign antigen.

In contrast, the adaptive immune system works with the innate immune system to create immunologic memory towards foreign antigen and promote more effective protection at subsequent exposure. A crucial element of this branch of the immune system is the extensive number of diverse receptors that are able to detect foreign antigens. Lymphocytes are the main cells associated with adaptive immunity, more specifically B and T lymphocytes. There are two subsystems of the adaptive immune system; cell mediated and humoral. The cell mediated subsystem consists of pathways to protect against intracellular antigens. Specialized lymphocytes, T cells, are the main effector cells in cell mediated immunity and communicate with B cells in order to create memory towards antigen. The humoral branch is directed towards extracellular antigens resulting in antibody production by B cells.

In order for adaptive immunity to be effective, there must be an initial (priming) exposure to antigenic proteins. Antigen exposure leads to inflammation, clearance of the antigen, clonal expansions of immune cells, antibody production and antigen specific memory cell development. Development of memory occurs through priming of the adapted immune cells with antigen.

At the time of pathogen exposure, inflammation is induced resulting in damaged cell release leading to host exposure to damage-associated molecular patterns (DAMPs). When an invading microbe is present it will be recognized by host recognition of pathogen specific molecules known as pathogen-associated molecular patterns (PAMPs). Macrophages, dendritic cells and mast cells express pattern recognition receptors (PRRs) which will bind to PAMPs and/or DAMPs leading to cellular activation. These cells respond by cytokine and chemokine synthesis and release, facilitating communication with various immune system cells. With the help of these chemical messengers, other cells can be recruited and activated in order to respond to the inciting inflammatory factor and where appropriate lead to pathogen or altered self- cell elimination.

Lymphocytes are the predominant cell type dedicated to adaptive immune responses. T lymphocytes are required for cell-mediated immune response. B lymphocytes are required for antibody production. These cell types are found within lymphoid tissue throughout the body and within circulation. T cells are originally produced within the thymus and then migrate and reside within the lymph nodes, spleen and Peyer's patches. Circulating lymphocytes are composed of 60-80% of T cells. B cells originate within the bone marrow, mature in the Peyer's patches or bone marrow and then reside within secondary lymphoid organs. These secondary lymphoid organs include lymph nodes, spleen and Peyer's patches.

Both B and T cells express antigen receptors on their surface. These are known as B cell receptors (BCRs) and T cell receptors (TCRs) depending on if they are present on a B or T cell, respectively (Figure 1). B cell receptors are soluble antibody proteins. Both B lymphocytes and T lymphocytes express cytokine receptors to allow for cross talk between cells. T lymphocytes can be further divided into T helper cells, T regulatory (Tregs) or cytotoxic T cells (CTL) based on their receptor expression and function. T helper and Tregs express surface cluster of differentiation (CD) 4 antigen and therefore are categorized as a type of CD4+ T cells. CD4+ T helper cells are further divided into Th1, Th2, Th17 and Tregs. CD8+ T cells are also known as cytotoxic T cells (CTLs).

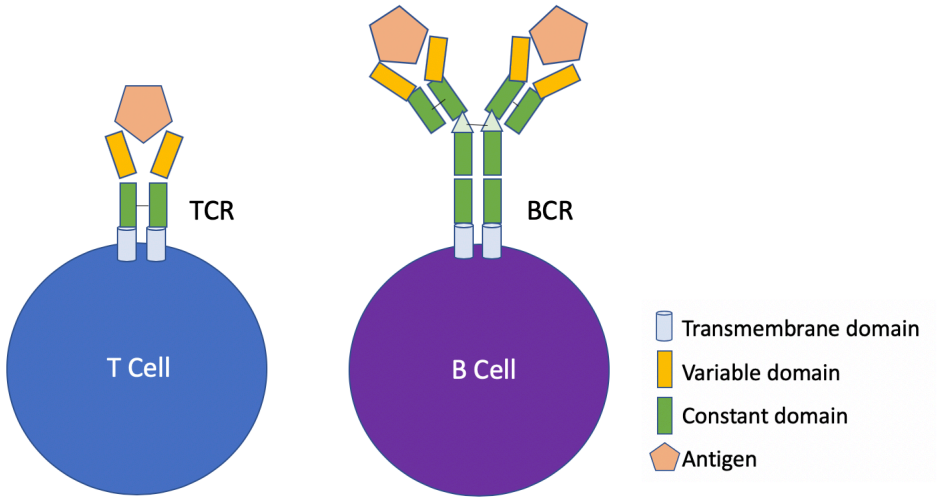


Figure 1: Depiction of a TCR on the surface of a T-cell and a BCR on a B-cell. The BCR consists of 2 pairs of variable domains while the T cell consists of 1 pair.

Antigen presenting cells (APCs) include dendritic cells (DCs), macrophages and B lymphocytes. These cells process antigen and present antigen to helper T cells. Each helper T cell has surface expression of antigen specific TCRs. When the appropriate concentration of TCRs are bound with antigen, the helper T cell will secrete cytokines and begin the process of clonal expansion (Figure 2). It is essential that the appropriate T cell receptors are stimulated in order for the T cell to become fully activated.

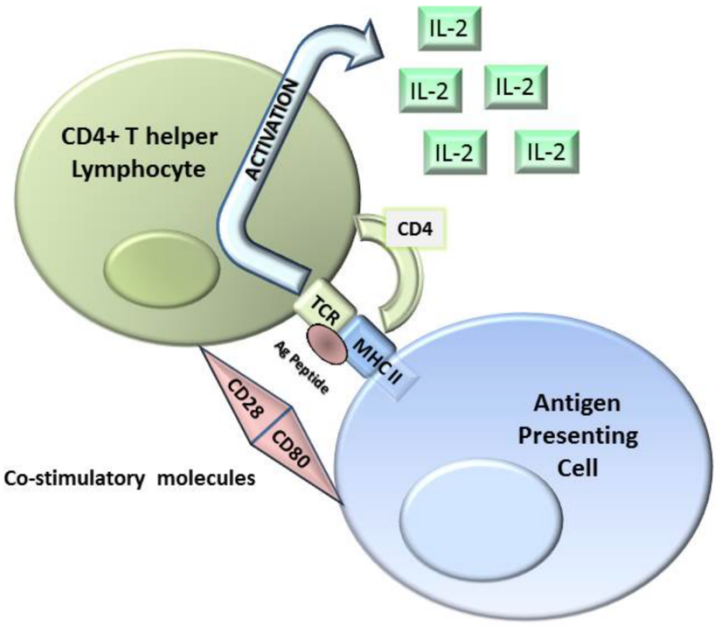


Figure 2: An APC interacting with a T helper cell resulting in activation of the T cell and release of cytokines.

Upon activation, helper T cells (Th1, Th2, Th17, and Treg cells) demonstrate independent responses that are cell line specific. For instance, Th1 cells promote cell-mediated immune responses that lead to macrophage activation and delayed hypersensitivity reactions. Th1 cells are responsible for immunity against intracellular organisms, such as virally infected cells and altered self-cells. Th2 cells predominately respond to antigens presented by macrophages or DCs and contribute to development of humoral immunity. Although B cells do not activate Th2 cells, Th2 cytokines stimulate B cell proliferation and the subsequent immunoglobulin secretion. Unregulated Th2 activation leads to severe allergic responses. The major cytokine of Th17 cells is IL-17 which is involved in neutrophil accumulation and the induction of acute inflammation. These cells respond mostly to extracellular bacteria as well as fungi. Excessive production of IL-17 is involved in the development of some tumors, chronic inflammatory diseases and autoimmune diseases such as rheumatoid arthritis in humans.[12] Optimal immune regulation is achieved when helper T cells differentiate into Th1 and Treg cells.

Treg cells act to suppress both Th1 and Th2 cells in order to regulate inflammatory responses and prevent allergic disease.

In general, T cell populations are short lived as they undergo apoptosis for their elimination. Those that resist apoptosis can further develop into a memory T cell population and are the most abundant type of T cells in adult animals. Many T cells are easier to activate and are more effective once activated. CD8+ memory T cells reside beneath epithelial surfaces. CD4+ memory T cells are found throughout tissues within the body. Both of these cell types live for about 8-15 years in humans without the presence of antigen.[12]

B cell antigen receptors are determined by multiple genetic codes within the variable region and one genetic code for the constant region. Amino acid sequences are very diverse in order to create up to 10^{15} different BCRs. Through gene recombination, rearrangement, mutation and conversion this process results in diversity of the BCRs. These BCRs are soluble, can be released from the B cell into circulation and bind antigen. These soluble BCRs are also known as immunoglobulin or, more commonly, antibodies.

B lymphocytes have the capacity to internalize and process antigen leading to expression in the context of major histocompatibility complex (MHC) II, which is then presented to Th2 lymphocytes (Figure 3). Th2 cells act as co-stimulators along with cytokines in order to facilitate B cell division, differentiation, antibody class switching and survival. If B cells interact with certain PAMPs they may initiate antibody secretion independently, without the aid of a helper T cell. Through this pathway B cells only secrete IgM and the process of differentiation into memory B cells does not occur. Th2 cells trigger B cells to divide into plasma cells and memory B cells. Those B cells that escape apoptosis can develop into long-lasting memory B cells. These memory B cells remain in the lymph node cortex forming germinal centers. Germinal centers are

a vital structure for humoral immunity. B cells divide within the germinal center rapidly creating over several thousand clones within a few days.[12] During cellular division mutation occurs within the BCR genetic code leading to the development of a vast array of different BCRs. Cells then undergo the selection process as they are exposed to antigen within the germinal center. Those cells that survive the selection process are either plasma cells or memory B cells and will leave the germinal center. This entire process allows for the expression of new BCRs.

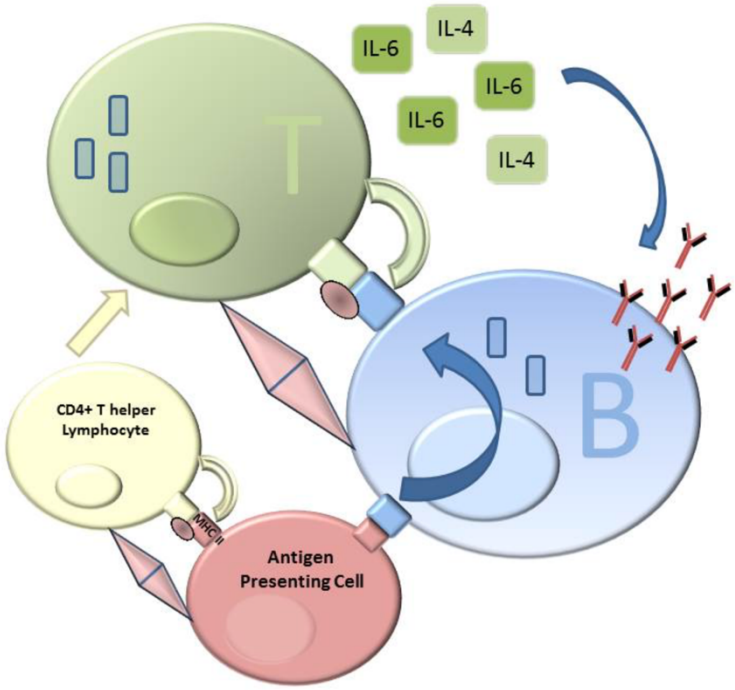


Figure 3: A B cell that has processed an antigen and presented it to a helper T cell. This interaction can occur during a secondary immune response where the B cell acts as an APC.

During B cell division and differentiation plasma cells are exposed to more antigen than daughter cells are exposed to. Plasma cells develop a rough endoplasmic reticulum, have an increased rate of synthesis and secrete large volumes of IgM specific to the original BCR. Secreted immunoglobulins are antigen specific, opsonize foreign material and bind to Fc receptors on phagocytic cells. In a matter of days immunoglobulin isotypes can change through the process of class switching to the production of IgG, IgA or IgE instead of IgM.[12]

Immunoglobulin molecules are made up of 2 heavy chains and 2 light chains forming the letter Y (Figure 4). The portion of the immunoglobulin that attaches to the B cell surface is known as the fraction that is constant (Fc) region. The portion that binds antigen is known as the fraction that binds antibody (Fab) region. The Fab region is a variable region and determines the shape of the antigen-binding site. The Fc region is the constant domain and the number of constant domains per BCR depends on the type of immunoglobulin class. It is the heavy chain that is responsible for transfer of IgG in colostrum and antibody-mediated cellular cytotoxicity. Immunoglobulin class switching occurs after deletion of heavy chain genes. This process is controlled by cytokines released by Th1 and Th2 cells. This change occurs without affecting the antigen specificity. The immunoglobulin isotype is determined by the constant heavy chain region of either mu, gamma, alpha, epsilon or delta genes.

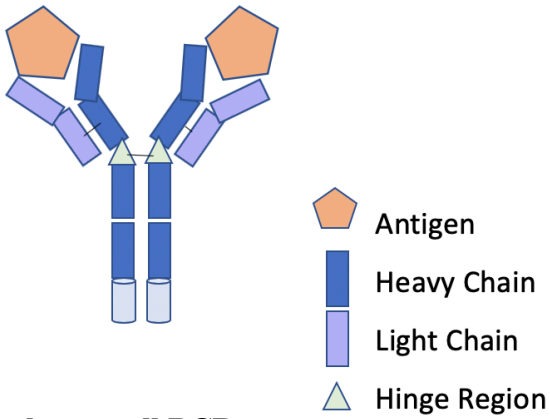


Figure 4: IgG representing the overall BCR structure.

A gamma heavy chain region results in an IgG isotype which can directly neutralize bacteria, viruses and toxins, opsonize organisms for phagocytosis and activate complement. IgG is produced within the spleen, lymph nodes and bone marrow by plasma cells. It has been documented that IgG provides the most significant impact in humoral protection.[2] The highest concentration of IgG is found in circulation; however, IgG is also present within tissues and colostrum. Within tissues, the highest concentrations are found within the urinary tract, lower respiratory tract and lung.[4] IgG is the smallest (180 kDa) of all immunoglobulins and can easily

escape from blood vessels in order to aid in inflammatory processes within tissues. Neonatal Fc receptors allow for active transport of colostral IgG across the intestinal epithelium; however, these receptors are only expressed during the first 24 hours of life.[12]

There are seven different IgG isotypes expressed in the horse which are all dependent on the amino acid sequences within the heavy chain constant region. This is highest number of IgG constant region genes of any mammalian species examined to date.[7] The most abundant of these are $IgG_{4/7}$ (IgGb), $IgG_{3/5}$ (IgGT), IgG_1 (IgGa) and IgG_6 (IgGc). Colostrum is high in $IgG_{4/7}$ and is also made up of $IgG_{3/5}$. Equine nasal wash samples are high in IgG_1 and $IgG_{4/7}$.[7] It is believed that vaccines which elicit a response towards $IgG_{1, 3, 4 \text{ and } 7}$ are more effective due to their complement-mediated elimination mechanisms.[7]

An epsilon heavy chain region creates an IgE isotype (Figure 5). Plasma cells present within lymphatic tissues lead to the production of IgE. IgE is present in circulation and tissues where it binds to mast cells. There is an extremely small amount of IgE in general circulation. IgE has the shortest half-life of only 5-8 hours when unbound in serum and about 10 days when bound to mast cells.[2] IgE acts as a signal-transducing molecule for mast cells when it binds antigen. Some IgG classes, such as $IgG_{3/5}$ can also bind to mast cell receptors, however, at a much lower affinity when compared to IgE.[7]

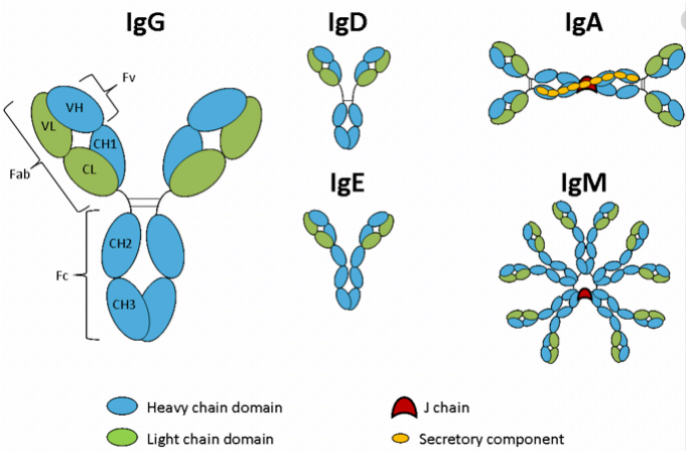


Figure 5: Immunoglobulin isotypes.

Immunoglobulin class switching to IgE is induced by cytokines IL-4 and IL-13 while it is inhibited by the cytokine interferon gamma. Lipopolysaccharide (LPS), an important constituent of endotoxin can induce class switching to IgE. RNA processing also plays a major role in immunoglobulin class switching. There is evidence that the pre-mRNA of an integral sequence locus specific for IgE synthesis is poorly processed in horses resulting in low membrane IgE expression.[1] This limits the number of cells able to secrete IgE and subsequently limits the magnitude of an IgE-mediated immune response.[1] This mechanism along with negative feedback regulation results in a more than 1000-fold lower IgE expression when compared to IgG expression in horses.[1]

Mast cells express PRRs which recognize and bind PAMPs and DAMPs just beneath the body surface epithelium within lymphoid tissue. This interaction leads to the release of inflammatory molecules. The highest concentrations of mast cells are found just beneath the skin and within the intestinal wall and airways. Mast cell degranulation occurs when the surface bound IgE binds an antigen. The release of mast cell proinflammatory molecules regulates blood flow, promotes cellular migration and induces the inflammatory cascade. Mast cells express proinflammatory molecules that include histamine, dopamine, prostaglandin, leukotrienes, cytokines and chemokines.

Principles of vaccination

Vaccination is an important method of disease control and prevention and is an essential aspect of maintenance of equine host health. At the time of vaccine administration, inflammation is induced at the injection site. Inflammation results in APC recruitment and cellular activation, which occurs following interaction with the pathogen expressed PAMPs. Activated APCs localize to lymph nodes to activate B and T cells. B cell receptors recognize antigen and

subsequently secrete antigen specific IgM antibody proteins. Some of these B cells will immediately become plasma cells and secrete these antibodies. Other B cells will develop a germinal center and over the course of a few days will secrete different antigen specific antibody isotypes (IgG, IgE, IgA).

There are known risks associated with the administration of vaccines in all species, including horses. The United States Center for Disease Control and Prevention (CDC) categorizes vaccine-associated adverse events into four groups.[12] First, a **vaccine-induced adverse event** can be attributed to the properties of the vaccine. An example being vaccine-associated strangles after receiving a modified live *Streptococcus equi* subspecies *equi* vaccination. Second, a **vaccine-potentiated reaction** that may have eventually occurred without vaccination, however, was precipitated by vaccination. An example being a predisposed individual developing purpura hemorrhagica post-vaccination. Third, an **iatrogenic reaction** occurs due to a technical error such as improper storage, preparation or administration. An example would be sepsis secondary to improper surface cleansing prior to vaccination. Lastly, a **coincidental event** is described as those events occurring by chance post-vaccination. An example being a horse developing signs of colic immediately following vaccination.[12]

While rare, a serious allergic reaction post-vaccination can develop which may be life threatening. The most common cause of vaccine associated allergic reactions involves IgE production against vaccine antigens, other than the immunizing antigen. The source of these sensitizing antigens commonly involves adjuvant components such as egg or tissue culture cells. See Chapter 2 for further discussion on hypersensitivity reactions.

A systematic data collection program for vaccine-induced adverse events is lacking in equine medicine. Voluntary reporting occurs through the USDA Center for Veterinary

Biologics; however, it lacks a robust sample / subject recovery number. Many equine veterinarians and owners report adverse events directly to the vaccine manufacturer. However, there is no enforcement for the manufacturers to share this data with the public.

Chapter 2 - Challenges of Humoral Immune Activation

Hypersensitivity reactions

Hypersensitivity reactions are the result of an exaggerated or inappropriate immune response. A type I hypersensitivity reaction, also known as immediate hypersensitivity, occurs when severe and life-threatening immune reactivity develops. Immediate hypersensitivity reactions are mediated by mast cells, basophils and eosinophils, specifically when mast cells are bound with antigen-specific surface IgE, which is then cross-linked with the inciting antigen. At the time of a hypersensitivity reaction IgE concentrations may be as high as one thousand times the healthy baseline level.[2] Previous antigenic exposure is required in order to stimulate an anaphylactic response. Medications, vaccines, blood products, insect bites and food can all act as an antigen stimulating an immediate hypersensitivity reaction. These hypersensitivity reactions are considered immediate and can be either local or systemic and life-threatening.

The respiratory and intestinal tracts are classified as shock organs in the horse. Equine patients suffering from systemic anaphylaxis demonstrate clinical manifestation of a type I hypersensitivity response including tachypnea, cough, pulmonary edema, urticaria, pruritis, colic and diarrhea. Asphyxia and vasculogenic shock can occur leading to pulmonary edema and hypotension. Two phases of a hypersensitivity reaction include antigen sensitization and antigen re-exposure. Antigen sensitization begins with antigen presenting cells presenting novel antigen to T helper cells, which, in turn, activate antigen-specific B cells through the expression of interleukin-4 (IL-4). B cells secrete antigen-specific IgE which binds mast cells and basophils resulting in sensitization. With subsequent exposure to the same antigen comes the potential for a hypersensitivity response. Antigen binds to the Fab fragment of IgE leading to cross-linkage,

activation and degranulation of mast cells (Figure 6). Chemical mediator release from mast cells results in enhanced vascular permeability, smooth muscle contraction, vasodilation, leukocyte diapedesis, thrombopathy (altered platelet function) and activation of both the complement and coagulation cascades. Classic chemical mediators of anaphylaxis in horses are histamine and serotonin; however, many other cytokines and chemokines are involved.[12]

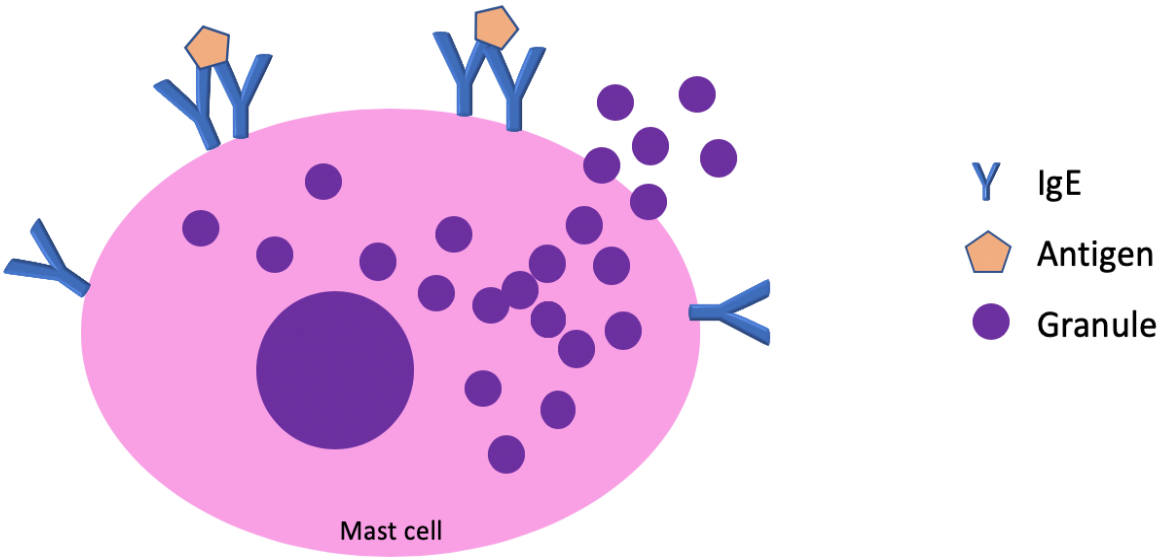


Figure 6: After a subsequent exposure to the same antigen, the antigen binds to mast cell receptors cross-linking two IgE molecules leading to degranulation.

Hypersensitivity reactions in horses have been reported following whole blood and blood product transfusions, antibiotic, anthelmintic, vitamin, muscle relaxant, vaccine and anesthetic administration. The overall prevalence of anaphylactic reactions in horses is unknown[2]; however, in 2007, Hurcombe *et al* reported that 16% (7/44) of blood transfusions in adult horses resulted in adverse reactions varying from local urticaria to anaphylactic shock.[6] In 2012, Gershwin *et al* reported that vaccine-induced hypersensitivity reactions may be related to IgE responses towards non-target antigens such as vaccine adjuvants.[4]

Plasma transfusions in foals

Foals are born essentially agammaglobulinemic and therefore require ingestion of maternal colostrum for adequate absorption of IgG which provides adequate circulating

immunoglobulin protection early in post-natal life. Colostral ingestion and absorption of intact immunoglobulin must occur within the first few hours of life, prior to gut closure. Trypsin inhibitors in colostrum provide protection to colostral proteins to prevent degradation by proteases within the stomach and small intestine. Neonatal Fc receptors (FcRn) are expressed on the brush border of neonatal enterocytes which bind colostral immunoglobulin molecules. These Fc receptors mediate pinocytosis of colostrally-derived immunoglobulin into the small intestine enterocyte, which cross the enterocyte to enter lacteals, ultimately entering general circulation. Immunoglobulins then reach the bloodstream through the capillaries, classifying passive transfer of immunity as being complete (Figure 7). Neonatal equine enterocytes are selective to the class of immunoglobulin(s) that are absorbed. IgG and IgM are preferentially absorbed while IgA is retained within the intestinal lumen.[2] There is a limited timeframe where immunoglobulin absorption is possible and is short-lived due to replacement of FcRn-bearing enterocytes with enterocytes lacking this receptor expression.[10] After just 3 hours of life, absorption rates of IgG drop to only 22% in horses.[2] IgG is unable to be absorbed through the gut after 12 hours.[2] Peak concentration of serum IgG concentration occurs between hours 18 and 24.[12] For this reason, plasma IgG concentrations should be measured at no later than 24 hours of life.[2] Total failure of passive transfer occurs when serum IgG concentrations are less than 400 mg/dL at 24 hours of life.[2] Partial failure occurs when serum IgG concentrations are between 400 and 800 mg/dL.[2] When failure of passive transfer is diagnosed after gut closure has occurred, the only effective

treatment is transfusion of equine plasma. There are two options for sources of equine plasma, commercial or non-commercial.

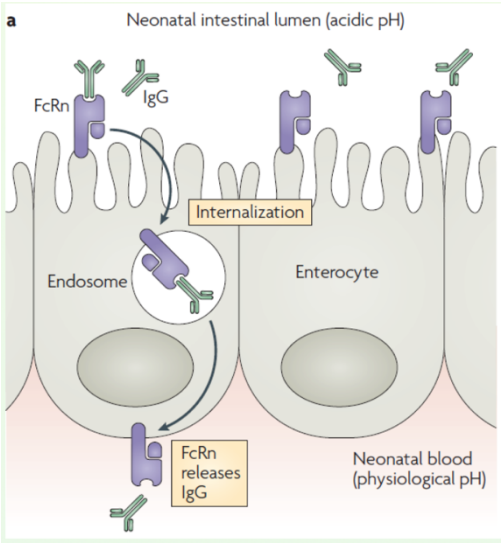


Figure 7: FcRn binding to maternal IgG, facilitating pinocytosis into the endosome within the enterocyte and then across the basal membrane into the neonatal bloodstream.[10]

Before 2009, there had been no documented studies investigating the incidence of adverse reactions in horses receiving plasma transfusions. The initial report by Wilson *et al* (2009) documented the incidence of transfusion reactions in horses receiving non-commercial plasma where plasma was collected from donor horses and was subsequently administered to hospitalized equine patients.[14] Ten donor horses provided plasma for 50 hospitalized horses. Each horse received between 15-25 ml/kg of plasma from an individual donor. Five out of 50 horses (10%) developed clinical signs of a mild adverse reaction, and none of these reactions were fatal. These mild reactions included urticaria, tachycardia, pyrexia, tachypnea, severe pruritis and chemosis. In a subsequent study, Hardefeldt *et al* (2010) investigated the incidence of adverse reactions following transfusion with commercial equine plasma in adult horses and foals.[5] An individual commercial vendor (Equiplas) was used for all plasma transfusions in this study. Among the horses studied, foals received hypergammaglobulin-containing plasma while adults received a commercial product that contained a lower IgG concentration. The overall

incidence of adverse reactions in this study was much lower than the previous non-commercial plasma transfusion study among adult horses. Of 38 adult horses that received a plasma transfusion, 0% developed adverse transfusion reactions to the commercial plasma. However, 9.7% of foals developed adverse transfusion reactions to the commercial plasma and all of these foals were less than 7 days of age. Adverse transfusion reactions were mild, including fever, tachycardia, tachypnea, colic and muscle fasciculations; and all affected foals survived.[5]

In 2017, Freccero Francesca *et al* from the University of Bologna investigated the efficacy and safety of a different commercial hyperimmune plasma in foals suffering from failure of passive transfer.[3] Sixty-two foals were included in this study where 34 had complete failure of passive transfer and 28 had partial failure of passive transfer. No foal experienced adverse reactions during or after the transfusion with PlasmaLife commercial hyperimmune plasma. Based on the available literature, it appears to be relatively safe to administer commercial plasma to both foals and adult horses.

Case example

A colt of unknown gestational age was born to a malnourished dam with little to no udder development. The foal received commercial powdered bovine/multi-species colostrum supplement (MannaPro) within the first 2 hours of life. The colostrum supplement was manufactured from bovine colostrum and was labeled for use in calves, foals, kids, lambs, crias, piglets, kittens and puppies. At 12 hours of age an IgG SNAP ELISA (IDEXX) test was performed resulting in a diagnosis of complete failure of passive transfer (0 mg/dL IgG). The owners elected to continue supplementing the foal at home with the powdered colostrum with plans to recheck the IgG concentration the following morning after a full 24 hours had elapsed

despite communications that it was unlikely for adequate transfer of passive immunity to occur via this plan.

The following morning an IgG SNAP ELISA (IDEXX) test was repeated, which identified an IgG level of 400 mg/dL IgG consistent with ongoing partial failure of passive transfer. At that time the owners elected to perform a plasma transfusion with 1L (22ml/kg) of commercial high globulin equine plasma (Lake Immunogenics HiGamm). An intravenous catheter was placed in the left jugular vein while the plasma was thawed aseptically according to the label instructions. An initial physical examination was performed which was within normal limits. Plasma was administered at a very slow rate (1 drop/2-3 seconds) (0.05ml/kg/minute). Temperature, heart rate and respiratory rate were evaluated at 3-5 minute intervals. Signs of colic, dyspnea and urticaria were monitored for continuously. Since no signs of an adverse reaction were evident, the administration rate was slowly increased to 1 drop/second (0.15 ml/kg/minute) over the subsequent 5-10 minutes. Vital parameters remained within normal limits and there continued to be no evidence of colic, dyspnea or urticaria. After 15 minutes the plasma rate was increased to approximately 2-3 drops/second (0.38 ml/kg/minute) and the foal quickly began to show signs of colic. The plasma infusion was stopped, and vitals were evaluated. Crackles were auscultated in all lung fields, and pink-tinged foamy nasal discharge became evident bilaterally suggestive of pulmonary edema. The foal became apneic after one minute. Intravenous epinephrine (0.02 mg/kg) was administered along with furosemide (1 mg/kg) and dexamethasone (0.15 mg/kg). The foal did not respond to emergency medications or attempts at field cardiopulmonary resuscitation and died within 3 minutes following discontinuation of the plasma.

Through collaborative conversations with equine veterinarian specialists, that included anecdotes of similar cases of fatal plasma reactions in neonatal foals after receiving powered colostrum supplementation, further investigation into this issue was prompted.

Bovine serum albumin

Bovine serum albumin (BSA) has been used during the manufacturing process of vaccines for decades. Bovine serum albumin is an integral part of cell line cultivation during viral vaccine production. Previous work has demonstrated that BSA is associated with both milk and beef allergies in people.[9] There is strong evidence to demonstrate that vaccine reactions in people occur secondary to BSA hypersensitivities.[8] One example involved two 13-year-old girls. One was known to be allergic to cow's milk and the other was allergic to beef and pork; unfortunately both died of anaphylaxis after receiving a vaccination for rubella in Sri Lanka.[11] This specific vaccine was known to use BSA during the manufacturing process. Subsequent to this and other reports and because of the concern for life-threatening anaphylaxis, the World Health Organization requires BSA concentrations to be less than 50 ng per human vaccine dose.[8] Bovine serum albumin continues to be used during the manufacturing process of some vaccines for animal use without restrictions.

Rajiva de Silva *et al* (2017) investigated BSA as a possible cause of allergic reactions to vaccines.[11] The Sri Lanka cases provided important information for this study. Serum was collected from patients who developed immediate hypersensitivity reactions after vaccination. A total serum IgE as well as antigen specific IgE to cow's milk, beef, beta lactoglobulin, BSA, casein and gelatin were analyzed and quantified. Total serum IgE was measured by ELISA while the specific IgE concentrations were measured using ImmunoCAP. ImmunoCAP is a quantitative test designed as a sandwich ELISA. Depending on the allergen being tested

sensitivity and specificity for the ImmunoCAP assay ranges between 84-95% and 85-94% respectively.[16] Anti-BSA IgE was present in 57.1% of patients who developed immediate hypersensitivity to vaccines.[11] When investigating those with allergic reactions and age matched controls, 73.3% of patients had anti-BSA IgE.[11] This study concluded that while the presence of anti-BSA IgE alone does not appear to be a prerequisite for developing vaccine reactions, those who demonstrated a vaccine reaction were more likely to have antigen specific anti-BSA IgE expression.[11] This study supported the hypothesis that sensitization to BSA could possibly be playing a role in allergic reactions to vaccines.[11]

In 2012, Laurel J. Gershwin *et al* investigated equine IgE responses to non-viral vaccine components in horses.[4] This study followed a group of 52 horses over 5 years monitoring their IgE, IgGa, and IgGc responses to BSA by ELISA. The positive control in this study was a pool of serum from horses that developed anaphylactic reactivity to vaccination with tissue culture grown viral vaccines. The negative control was a pool of serum from horses that had not been vaccinated for several years. A microplate reader was used to read optical densities (OD). These results for IgE were converted to a percentage of the positive control and determined as either positive or negative. The horses were then grouped into three different categories: "(1) pre-vaccine and/or post-vaccine level >60% of positive control, (2) pre-vaccine level <60%, but post-vaccine level at least twofold greater than pre-vaccine level, and (3) pre- or post-vaccine level >40% and <60%." The IgG ELISA results were determined positive by whether the OD values were greater than the mean of the negative control plus three standard deviations. It was determined that horses that were positive for anti-BSA IgE were also positive for anti-BSA IgGa. However, some horses that were negative for anti-BSA IgE were positive for anti-BSA IgGa. Some horses were negative for both anti-BSA IgE and IgGa. This study demonstrated that some

horses with IgE response to BSA do not show signs of adverse reactions post-vaccination. There were horses that were highly positive for anti-BSA IgE that responded to vaccination with mild to severe signs of hypersensitivity. However, some horses that demonstrated post-vaccination adverse reactions did not test positive for anti-BSA IgE. Over the five-year period of monitoring there did appear to be an increase in anti-BSA IgE levels. The data from this study supported that annual or semi-annual vaccination of horses with certain vaccines could enhance the likelihood that an immune response to non-target proteins could occur.[4]

A theory of BSA hypersensitivity reactions in foals consuming powdered colostrum followed by IV commercial plasma infusion was hypothesized. BSA is a major component of commercial powered colostrum. Commercial plasma is harvested from hyperimmunized horses. It is known that humans develop anti-BSA IgE post-vaccination[11], then it is also possible for horses to develop anti-BSA IgE post-vaccination, particularly horses that are hyperimmunized. It has been documented that continuous vaccination could enhance the production of anti-BSA IgE.[4] A hyperimmunized horse is exposed to BSA at a much higher rate than a typical client-owned horse. Therefore, it is possible that some hyperimmunized plasma donor horses are producing high levels of anti-BSA IgE which could be present in commercial plasma. If the foals that ingest BSA prior to gut closure are then exposed to high levels of anti-BSA IgE through commercial plasma, a severe plasma-associated hypersensitivity reaction could occur. This could be the reason why veterinarians are documenting fatal plasma reactions in neonatal foals that have received powdered colostral supplementation during the first 24 hours of life.

Chapter 3 - Experimental Study

Introduction

Maintenance of optimal host health involves the administration of routine vaccines. At a minimum, horses should initially begin on a series of vaccines during the first year of life, aimed at protection against serious pathogens that include eastern and western equine encephalomyelitis, West Nile virus, tetanus and rabies. Vaccines against these pathogens have been listed as core vaccines by the American Association of Equine Practitioners.[15] Core vaccinations are defined as those "that protect from diseases that are endemic to a region, those with potential public health significance, required by law, virulent/highly infectious and/or those posing a risk of severe disease. Core vaccines have clearly demonstrated efficacy and safety, and thus exhibit a high enough level of patient benefit and low enough level of risk to justify their use in the majority of patients."[15] In addition to core vaccination, risk-based vaccines may be administered for various respiratory pathogens or other agents of particular concern depending on the geographic region.

Vaccination is not without risk, there are potential complications that may accompany vaccination. Complications may include immediate hypersensitivity reactions or delayed reactions. In some instances, local sepsis may occur following vaccination leading to abscess formation or severe myonecrosis if the inciting agent is a clostridial organism. In instances where hypersensitivity is a concern, identification of the inciting allergen can be beneficial to avoid future allergic reactions through the process of antigenic avoidance.

For the reasons listed, routine vaccination is a requirement for the maintenance of equine health. In instances where adverse allergic type events have occurred determination of the inciting antigen is important. The primary objective of this investigation was to determine the

humoral response to the vaccine component bovine serum albumin (BSA) in horses following annual vaccination. Horses that were included in this study were established to be healthy and previously vaccinated. It was the aim of this study to determine the kinetics of an amnestic antibody response at the time of annual booster vaccination towards BSA. A second objective of this study was to compare concentrations of anti-BSA IgE in horses that had developed an immediate hypersensitivity response after vaccination to those horses that had not developed this type of response. A third objective of this study was to compare concentrations of anti-BSA IgE in hyperimmunized horses utilized for commercial plasma harvest to healthy horses. The hypothesis was that a clinically healthy horse that had never experienced an adverse vaccine reaction would have a lower concentration of anti-BSA IgE compared to those horses that had developed this type of response as well as hyperimmunized horses.

The null hypothesis was that a clinically healthy horses that had never experienced an adverse post-vaccine reaction would not demonstrate an increase in anti-BSA IgE after vaccination.

Horses were selected based on health status and historical information that they had been on an AAEP-approved vaccine protocol for annual vaccinations. The aim was to measure anti-BSA IgG and IgE over the course of the year. Vaccination records over the course of previous years were analyzed determining an average of about 200 vaccinations administered per year by one of the investigators (CB). Power analysis determined an ideal subject number of 50 horses. In order to investigate whether or not horses were mounting an immune response to BSA, an experimental study was designed.

Materials and Methods

Healthy Horses

All procedures performed on horses during the study were approved by Kansas State University Institutional Animal Care and Use Committee (IACUC #4003). Sixty-five horses were enrolled as either client-owned with client consent or part of the Kansas State University College of Veterinary Medicine (KSU-CVM) teaching herd horses. Healthy horses were comprised of Quarter horses (n=39), Thoroughbreds (n=9), Warmbloods (n=5), Appaloosas (n=3), Paints (n=3), Morgan (n=1), Miniature (n=1), Mustang (n=1), Draft (n=1), Haflinger (n=1), and Tennessee Walker (n=1). All horses were between 3 and 32 years of age. Thirty-three were mares, 31 geldings and 1 stallion. A convenience sample of client-owned horses and all KSU-CVM horses that were due for their annual booster vaccinations during the months of March through July were enrolled into the study. Enrollment criteria included that the owners reported routine annual vaccines were administered and no previous adverse vaccine reactions via a client survey. All horses were deemed to be healthy and able to receive vaccination after a physical examination was performed by a veterinarian (CB).

Horses were grouped according to age: 3-9 (n=12), 10-14 (n=24), 15-19 (n=12) and 20-32 (n=17) years of age. All horses received the same type of vaccine, and these vaccines were administered by the same veterinarian (CB). Vaccines included a combination for EEE/WEE/influenza/EHV-1/4/tetanus/WNV (Vetera Gold – Boehringer Ingelheim) and a separate vaccine for rabies (EquiRab - Merck). All vaccines were administered intramuscularly following label instructions.

For sample collection, after topical cleansing of the haired skin surface with 70% isopropyl alcohol, 20 mL of blood was collected through an 18 g vacutainer needle directly into a red top tube via jugular venipuncture. Blood collection was performed on days 0, 14, 30, 90, and 180 after vaccination. A small subset of horses also had blood collected on days 270 and/or

360 after vaccination. Vaccines were administered immediately following blood collection on day 0. Blood samples were stored at 4°C immediately after collection for less than 48 hours prior to be being centrifuged at 534 gravitation force (g) for 15 minutes. Serum was collected by routine aspiration with a pipette and was placed in a second red top tube and frozen at -18°C for a maximum of 14 days. Serum was thawed at 4°C for 24 hours, after which it was aliquoted into 1.5 mL per 2 mL plastic collection tubes and stored at -80°C. Serum samples were tested in batches and were only thawed once on the morning of testing to minimize freeze thaw cycles from degrading protein integrity.

Reactor Horses

A group of 21 horses were enrolled into the study that had experienced an adverse reaction post-vaccination either previously or during the course of this study. A questionnaire was completed either by the owner or veterinarian (appendix). Survey questions included length of ownership, vaccine administration frequency, whether adverse post-vaccination reaction had occurred in the past, vaccine type that was administered prior to the reaction, the date of that reaction, if any medications had been administered and the date of the blood draw. Blood was collected and processed as previously described. Fourteen horses had blood drawn within 24 hours post-vaccination. Two horses had blood drawn 180 days post-vaccination. Three horses had blood drawn 270 days post-vaccination. Two horses had blood drawn at an unknown timepoint post-vaccination. Horses ranged from 2 to 15 years of age and age was not documented for 3 horses. There were 13 Warmbloods, 4 Quarter horses and 3 horses did not have their breed documented. Thirteen were geldings and 8 were mares.

Commercial Plasma Donor Horses

A group of 10 horses were utilized in the study from the plasma donor herd at Lake Immunogenics, Inc. Horses were chosen by Lake Immunogenics personnel. Two 10 mL plasma samples from each of donor horse were collected and shipped to Kansas State University. These samples were aliquoted into 2 mL vials and stored in a -80ºC freezer. No information on the plasma donors was provided by Lake Immunogenics other than they were standard blood donors that they use for commercial blood product collection.

Non-Vaccinated Horses

A group of 4 horses were utilized in the study that had never been vaccinated before. These were Quarter horses between 6 months and 1 year of age. Blood was collected at a single time-point, processed and stored as described above.

Neonate with Fatal Plasma Reaction

A single plasma sample was obtained from a one-day old neonatal colt that did not receive maternal colostrum. The foal received an all-species commercial powdered colostral supplementation (MannaPro) during the first 24 hours of life. Whole blood and serum were collected around 12 hours of life. Partial failure of passive transfer was diagnosed (IgG 400 mg/dL) and he was administered commercial HiGamm plasma (Lake Immunogenics) at approximately 24 hours of life. The foal experienced an apparent anaphylactic reaction within the first 15 minutes of plasma administration, did not respond to emergency therapy and died. The serum sample that was collected during the first 12 hours of life was stored at -80ºC prior to analysis.

Commercial Plasma Samples

Two plasma samples were obtained from commercial plasma sources. An aliquot of the Lake Immunogenics HiGamm plasma that had been administered to the foal described

previously which resulted in a fatal plasma reaction was available for analysis. This sample was aliquoted into 10 mL and 2 mL vials and stored at -80ºC. A second commercial plasma sample included Immunoglo equine plasma from MG Biologics. Although this was not used in the subject patient, this sample was used for testing purposes as a comparison commercial plasma product. This commercial plasma was thawed as according to label instructions, aliquoted into 10 mL and 2 mL vials and stored at -80ºC.

Anti-BSA IgG and IgE Analysis

Flow cytometry, Western Blot and ELISA testing were utilized throughout different phases of this study. The initial plan was to develop a multiplex flow cytometric assay to quantify anti-BSA IgE and IgG in apparently healthy horses, reactor horses and commercial plasma samples. Western Blot was performed with samples from apparently healthy horses, reactor horses and commercial plasma samples to confirm the specificity of IgG against BSA. This qualitative test was used to support the results from the quantitative flow cytometry assay. An ELISA was then utilized as a semi-quantitative test to measure anti-BSA IgE in healthy horses, reactor horses and commercial plasma samples.

Flow Cytometry

Two samples were used for testing in each horse, an isotype (iso) and an antibody (ab). Carboxylated microspheres (beads) were covalently coupled with BSA according to the manufacturer's protocol.[17] BSA-coated beads (10^6) and serum (1:500 in PBS) were combined and incubated together for 30 minutes at room temperature to allow binding of anti-BSA immunoglobulin to the beads. Samples were rinsed with PBS. Rabbit IgG isotype antibody conjugated to FITC (diluted 1:100 in PBS) was added to the isotype test tubes and incubated at 4ºC for 15 minutes. Rabbit anti-horse IgG-FITC and mouse anti-horse IgE primary antibody

(diluted 1:100 in PBS) were combined with the Ab test tubes and incubated at 4ºC for 15 minutes. Both samples were rinsed with PBS. Secondary antibody, goat anti-mouse IgE conjugated with APC (diluted 1:100 in PBS) was then combined with both the Ab and Iso test tubes, incubated as described previously and rinsed with PBS. Beads were analyzed by flow cytometry on a BD LSR Fortessa X-20 flow cytometer for the presence of anti-BSA IgG and IgE. Isotype controls were used to establish background fluorescence and determine the cutoff for negative or positive events (beads). Fluorescence that exceeded the cutoff point were considered to be positive for BSA reactivity. Data were represented as a percentage of the total number of BSA coated beads which expressed positive FITC fluorescence indicated the presence of BSA-specific antibody in the serum sample.

Western Blot

Western blot analysis was also performed to detect the presence of anti-BSA IgG in serum samples. The protocol was first performed on the positive control sample (HiGamm Lake Immunogenics) identified in the flow cytometry assay to test whether the anti-BSA IgG was present and able to be identified by Western blot. The positive control sample was tested at four different dilutions (1:10, 1:50, 1:100, 1:500) in order to aid in determining the optimal dilution (1:50) to be used on the test samples. It was evident that the Western Blot protocol was able to identify the presence of the anti-BSA IgG, and the protocol was then performed on the healthy horse samples.

For this experiment a 10-well gel was utilized. Purified BSA (5 µL) was added to sample buffer (5 µL) with PBS (10 µL) and heated at to 95ºF for 5 minutes to create a BSA sample. Wells were loaded with purified BSA (5 wells) alternating with molecular weight markers (5 wells). The gel was prepared in running buffer. Running buffer was made from Tris Base (30 g),

glycine (144 g) and SDS (10 g) dissolved in distilled water (900 mL) to a final volume of 1 L. The voltage was set at 50V for 5-10 minutes, followed by an additional 60 minutes at 150V. The gel was then soaked in transfer buffer for 10 minutes. Transfer buffer was made with tris base (30 g) and glycine (144 g) dissolved in distilled water (800 mL) and was then diluted with 20% methanol (200 mL). The transfer apparatus was set for 1 hour at 100 V. Blocking buffer of 0.1% tris-buffered saline with tween (TBSt) and 5% powdered milk was added to the filter paper and placed in an orbital shaker for 2 hours at room temperature. The filter paper was then placed in 4^{o}C overnight. The filter paper was cut, and placed in the following solutions and then placed on the orbital shaker for 1 hour. Anti-BSA antibody at 1:2,000 was created with blocking buffer (5 mL) and mouse anti-BSA (2.5 µL). Anti-BSA antibody was applied to one filter paper strip. Horse serum samples were diluted to 1:50 with blocking buffer and applied to the remaining 4 filter paper strips. The filter paper was then added to TBSt and washed for 5 minutes 3 separate times. A secondary antibody, goat anti-horse IgG horseradish peroxidase (HRP), was diluted to 1:5,000 with blocking buffer. The horse serum samples were covered with 5 mL of goat anti-horse IgG HRP for 30 minutes. All were then washed with TBSt for 5 minutes 3 separate times. The filter paper was then covered with a mixture of water (27 mL), opti4CN diluent (3 mL) and substrate (0.6 mL) and allowed to sit for 5 minutes. The filter paper was then rinsed with water. Bands appeared on all samples corresponding to BSA.

Seven apparently healthy teaching horse samples and 2 reactor horse samples that were all previously evaluated via flow cytometry were analyzed. The samples were from different time points after vaccination (Table 1).

Horse	Group	Time point after vaccination
Reyetta	Reactor	270 days
Peppy	Reactor	270 days
Jana	Healthy	90 days
Reba	Healthy	90 days
Daisy	Healthy	90 days
Toby	Healthy	90 days
Fuel	Healthy	90 days
Jana	Healthy	180 days
Reba	Healthy	180 days

Table 1: Samples used in the Western Blot analysis.

ELISA

This protocol was adapted from Gershwin 2012[4] with modifications. ELISA plates were coated with 1 ug/well BSA in bicarbonate-carbonate buffer pH 9.6 overnight. ELISA wells were blocked with 0.5% rabbit serum albumin (RSA) in coating buffer. Each well in a 96 well plate was filled with 200 µL of 0.5% RSA for blocking. The plates were incubated at 4ºC overnight. After incubation concluded, the wells were washed with 300 µL of PBSt (PBS + 0.1% Tween-20) by an electronic plate washer 3 times. Commercial plasma (HiGamm) was used as a positive control. Unvaccinated horse plasma was used as a negative control. PBS was used as a blank. Two wells were designated as the positive control, two for the negative control and two for the blank. Undiluted serum samples (100 µL) were added to each remaining well in duplicate and incubated at room temperature for 2 hours (Figure 8). A standard curve was not available, and it was cost prohibited to purify IgE in order to create a standard curve. For this reason, OD values were used in order to analyze the data.

	Positive control	Positive control	Blank	Blank	Negative control	Negative control	Blank	Blank	Blank	Blank	Blank	Blank
Horse A	0	0	14	14	30	30	90	90	180	180	360	360
Horse B	0	0	14	14	30	30	90	90	180	180	360	360
Horse C	0	0	14	14	30	30	90	90	180	180	360	360
Horse D	0	0	14	14	30	30	90	90	180	180	360	360
Horse E	0	0	14	14	30	30	90	90	180	180	360	360
Horse F	0	0	14	14	30	30	90	90	180	180	360	360
Horse G	0	0	14	14	30	30	90	90	180	180	360	360

Figure 8: 96 well ELISA plate layout where the samples of 7 horses were plated in duplicate. Numbers 0, 14, 30, 90, 180 and 360 represent the days after vaccination for the horse in that row. All ELISA plates utilized the same positive control and negative control samples in duplicate.

Plates were washed as described above. Anti-horse IgE horse radish peroxidase (HRP) was diluted out to 1:500 with wash buffer, added to each well (100 µL) and incubated for 1 hour at room temperature. The plates were washed again. Tetramethylbenzidine (TMB) (100 µL) was added to each well and left to incubate for 30 minutes at room temperature protected from the light. TMB is a chromogenic substrate which facilitates a color change reaction. After 30 minutes 100 µL of sulfuric acid (2 M SO2H4) was added to each well to terminate the reaction. Plates were read with an electronic plate reader at 450 nm and recorded to obtain the optical density.

All horse samples including 0, 14, 30, 90, 180, 270, and 365 day post-vaccination, if available, were tested by way of ELISA for the presence of anti-BSA IgE. All horses classified as a reactor were tested by ELISA. One MG Biologics (Immunoglo) was tested by ELISA. Plasma from the plasma donor samples from Lake Immunogenics were all tested by ELISA. All plates contained the same positive control, negative control and blanks. The OD value for the blank wells was averaged for each plate and then subtracted from each sample well to correct for background absorbance. The adjusted duplicate sample OD values were then averaged together for each horse.

Statistical analysis

Prism (v.8) was used to perform statistical analysis on the anti-BSA IgE ELISA data. Samples were performed in duplicate and were averaged together to provide one value per sample. Horses in the apparently healthy group had their subsequent serum samples compared to their 0 day sample. Means were determined at each timepoint for each age group. There was a near normal distribution of data within each horse's data set and differences in the means were compared using paired two-tailed T-tests. Significance was defined as a p-value <0.05. Data was then compiled in Microsoft Excel as a spreadsheet function.

Results

Healthy Horses

Sixty-five horses were enrolled into the study. Sixteen of these horses were part of the KSU-CVM teaching herd while 49 of these horses were client owned. All 65 horses had blood samples obtained on days 0, 14, 30, 90 and 180 after vaccination. One time point from this timeframe was missing from a total of 6 of these horses. Nineteen of the 65 horses also had blood samples obtained on day 270 after vaccination. Twenty-one of the 65 horses also had blood samples obtained on day 365 after vaccination. Eight of the 65 horses had blood samples collected at both 270 and 360 days after vaccination in addition to the 0 -180 day time points.

Horses Experiencing Adverse Vaccine Reactions

Twenty-one horses were enrolled as vaccine reactors. Fourteen horses had blood drawn within 24 hours after the vaccination was administered. Clinical signs in these 14 horses included swelling at the injection site, fever, colic and stiff neck. Two horses had blood drawn 180 days after the vaccination reaction occurred which included fever, lameness, increased digital pulses and injection site swelling. Three horses had blood drawn 270 days after the

vaccination reaction occurred which included fever, colic, injection site swelling, neck soreness, muscle tremors and lameness. Two horses had blood drawn at an unknown timepoint after the vaccination reaction.

Commercial Plasma Donor Horses

Ten horses were enrolled from Lake Immunogenics with 2 plasma samples collected from each horse on the same day.

Non-Vaccinated Horses

Four horses aged 6 months to 1 year were enrolled as negative controls. Two of these samples were used for a majority of the tests. One of these samples was used for every ELISA plate.

Neonate with Fatal Plasma Reaction

One plasma sample from 12 hours of life was analyzed.

Commercial Plasma Samples

The sample from Lake Immunogenics (HiGamm) was used for every ELISA plate as the positive control. Both the Lake Immunogenics sample and the MG Biologics samples were analyzed by Flow cytometry, ELISA and Western Blot analysis.

Flow Cytometry IgG and IgE

An increase in post-vaccination anti-BSA IgE compared to pre-vaccination anti-BSA IgE was identified. Anti-BSA IgE increased following vaccination. All horses that had been previously vaccinated demonstrated a low basal level of antibody against BSA (Figures 9 and 10). The HiGamm Lake Immunogenics plasma sample displayed a higher level of antibody against BSA (73.1%) compared to all healthy horse samples (Figure 11). The flow cytometry assay failed precision and accuracy testing that was performed over the course of 5 separate

days. Results were fairly similar when ran in duplicate over the course of the same day; however, day to day the results similar enough to be considered accurate. These values were fairly precise; however, overall very unreliable in terms of accuracy (Table 2).

BSA IgG Accuracy/Precision																																									
	Tuesday Group 1					Tuesday Group 2				Wednesday Group 1				Wednesday Group 2					Thursday Group 1					Thursday Group 2					Friday Group 1					Friday Group 2					Tuesday Group 1				Tuesday Group 2			
Toby	42	41	41	40	39	47	43	44	43	41	35	40	36	60	59	45	44	39	24	25	30	30	23	2	2	3	5	7	22	27	26	25	25	4	4	5	6	6	4	2	3	2	5	5	5	5
Reba	44	44	47	43	39	45	43	47	48	83	78	78	73	79	79	76	75	71	84	84	81	78	78	84	80	72	69	59	94	93	92	92	92	85	78	74	68	64	78	65	61	67	57	54	55	55
Snip	35	35	35	34	33	44	40	40	39	2	3	6	6	4	4	4	5	7	1	2	2	3	5	1	4	12	12	12	1	2	6	6	11	1	1	2	2	3	1	1	1	2	1	1	2	2
Plasma	86	87	82	82	78	84	87	83	82	87	83	82	81	76	75	73	72	71	95	93	91	90	88	73	70	67	61	57	94	93	91	90	87	86	86	83	82	80	87	88	87	88	85	84	83	83

Table 2: Flow cytometry anti-BSA IgG accuracy and precision testing results performed on 3 healthy horses and the positive control in duplicate over the course of 5 separate days.

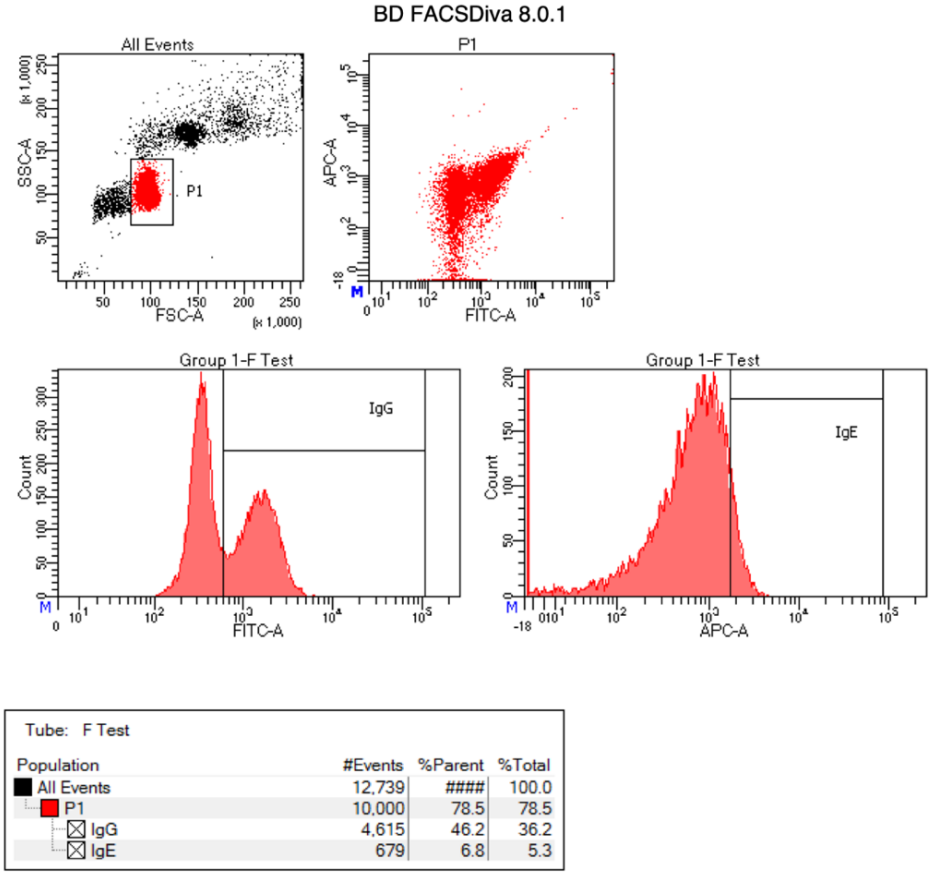
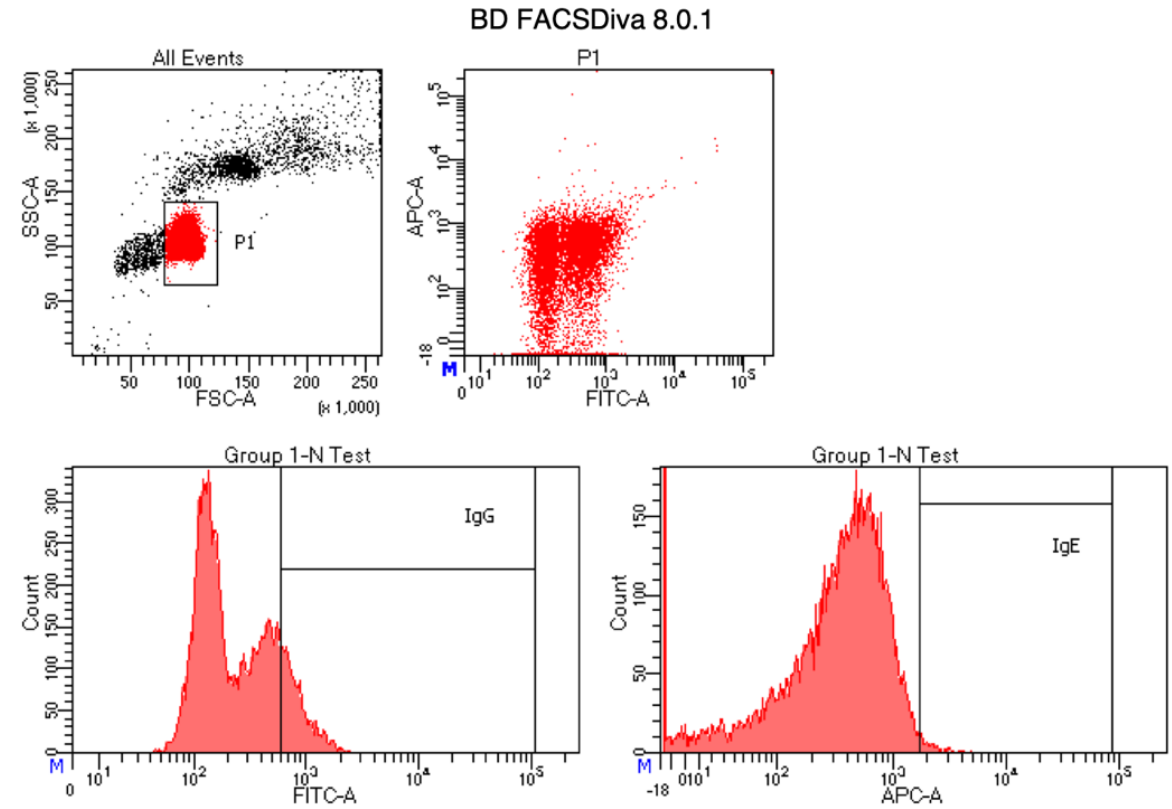


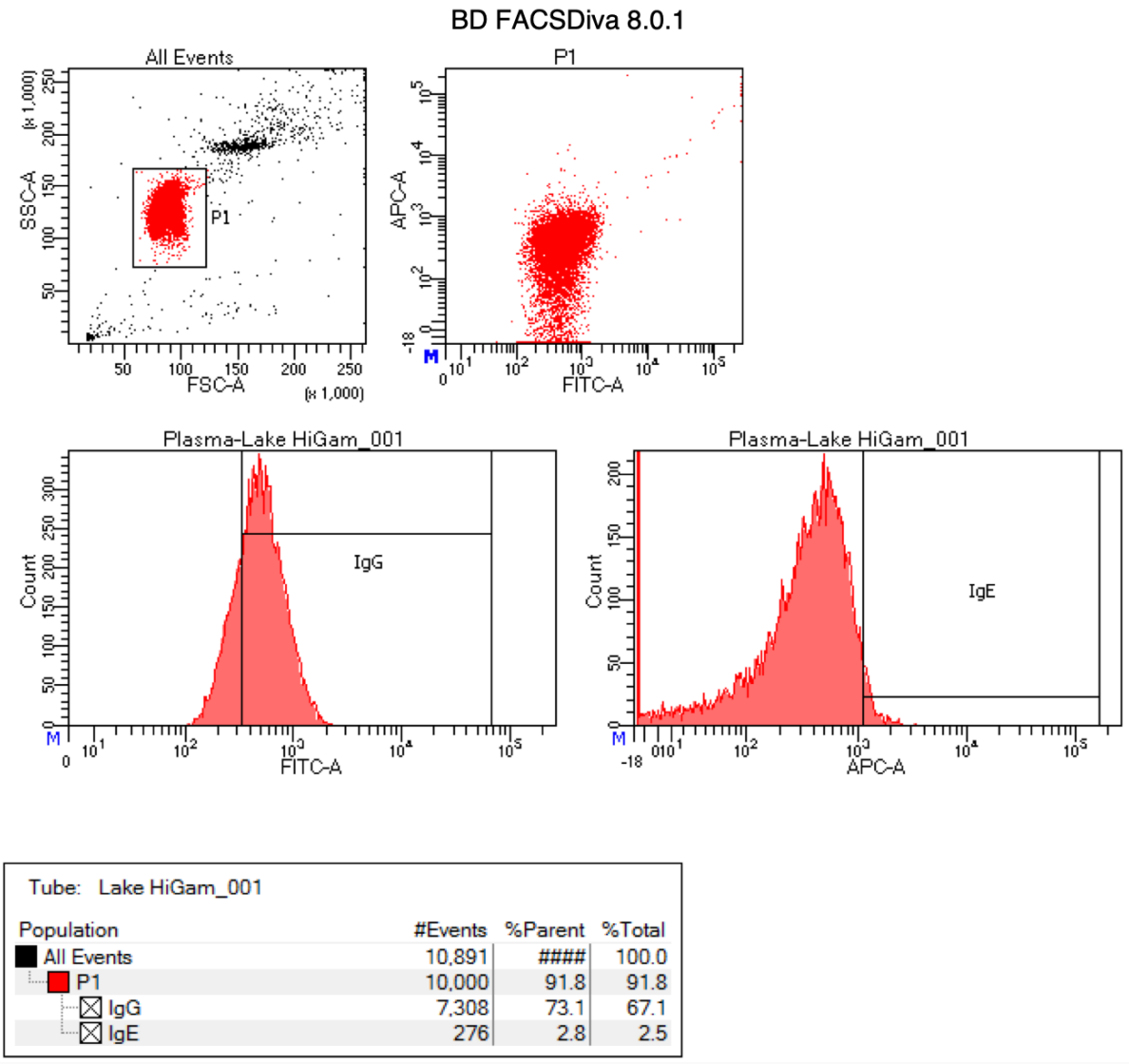
Figure 9: Flow cytometry results from a heathy teaching horse 90 days after vaccination resulting in 46.2% of BSA coated beads expressing positive FITC fluorescence indicating the presence of BSA-specific IgG in the serum sample. Histogram is representative of multiple horses.



Tube: N Test

Population	#Events	%Parent	%Total
All Events	12,498	####	100.0
P1	10,000	80.0	80.0
IgG	1,473	14.7	11.8
IgE	95	1.0	0.8

Figure 10: Flow cytometry results from a heathy teaching horse 14 days after vaccination resulting in 14.7% of BSA coated beads expressing positive FITC fluorescence indicating the presence of BSA-specific IgG in the serum sample. Histogram is representative of multiple horses.



Tube: Lake HiGam_001

Population	#Events	%Parent	%Total
All Events	10,891	####	100.0
P1	10,000	91.8	91.8
IgG	7,308	73.1	67.1
IgE	276	2.8	2.5

Figure 11: Flow cytometry results from the Lake Immunogenics HiGamm plasma administered to the foal resulting in a fatal anaphylactic plasma reaction. A total of 73.1% of BSA coated beads expressing positive FITC fluorescence indicating the presence of BSA-specific IgG in the serum sample.

Western Blot Anti-BSA IgG

Anti-BSA IgG was found in the positive control commercial plasma (HiGamm, Lake Immunogenics). Anti-BSA IgG was also found in all 9 tested serum samples described above (Table 1 and Figure 12). These tested samples included 2 reactor and 7 healthy horses serum samples. This information supported the hypothesis that horses were mounting an immune response (IgG) against BSA.

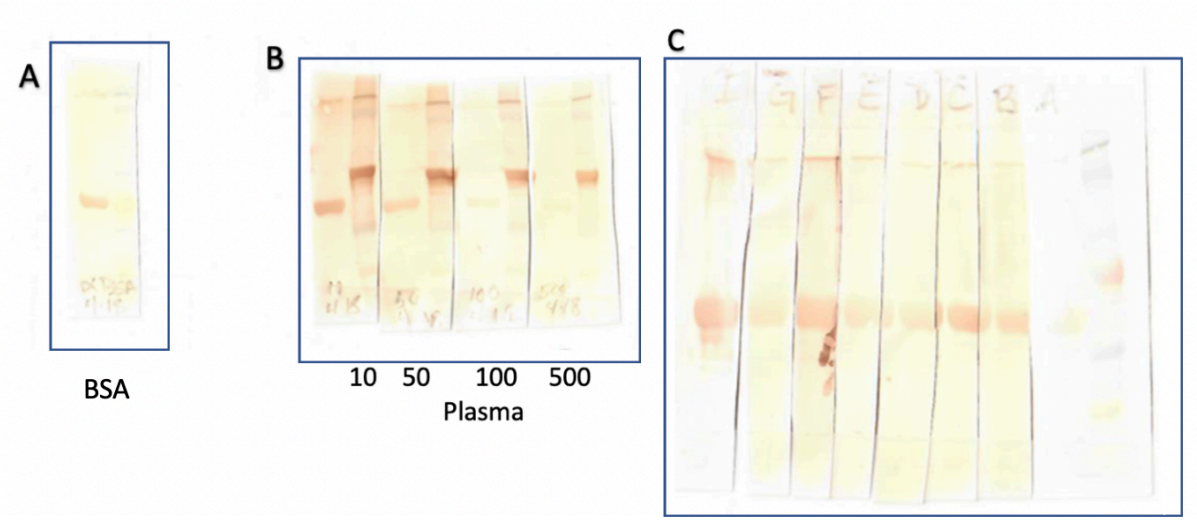


Figure 12: Panel A shows the band associated with the presence of anti-BSA within the sample (control). Panel B show the band associated with the presence of anti-BSA IgG within the control plasma (HiGamm) sample throughout serial dilutions. Panel C shows the band associated with the presence of anti-BSA IgG within the serum samples collected from different study horses

ELISA anti-BSA IgE

The OD value for the positive control mean across all plates was 0.340. The OD value for the negative control mean across all plates was 0.062. The OD value for the blank control mean across all plates was 0.071. The OD value for the positive control mean was significantly different from the negative control mean (p. value = 0.0016 E-6). The OD value for the negative control mean was significantly different from the OD value blank control mean (p. value = 0.008). Assay precision was assessed by calculating the coefficient of variation (CV%). There was an inter-assay precision of 7.375% CV% and an intra-assay precision of 7.391% overall (range 7.042 to 7.849%) for the positive control. There was an inter-assay precision of 14.084% CV% and an intra-assay precision of 14.301% overall (range 12.27 to 19.23%) for the blank control. There was an inter-assay precision of 12.903% CV% and an intra-assay precision of 13.677% overall (range 11.392 to 16.211%) for the negative control. A cut-off value of 0.200 was applied when analyzing the reactor horses in comparison to the healthy horses. This value

was chosen by analyzing the reactor samples obtained at 24 hours post-vaccination and noting that 85.7% of these samples were greater than 0.200.

Healthy Horses

The mean OD value for day 0 anti-BSA IgE in all healthy horses (0.108) was not significantly different when compared to the mean OD value for 14 days post-vaccination anti-BSA IgE in all healthy horses (0.124) (p value = 0.43) (Figure 13). The mean OD value for pre-vaccination anti-BSA IgE in all healthy horses (0.108) was significantly higher when compared to the mean OD value for 180 days post-vaccination anti-BSA IgE in all healthy horses (0.070) (p value = 0.007).

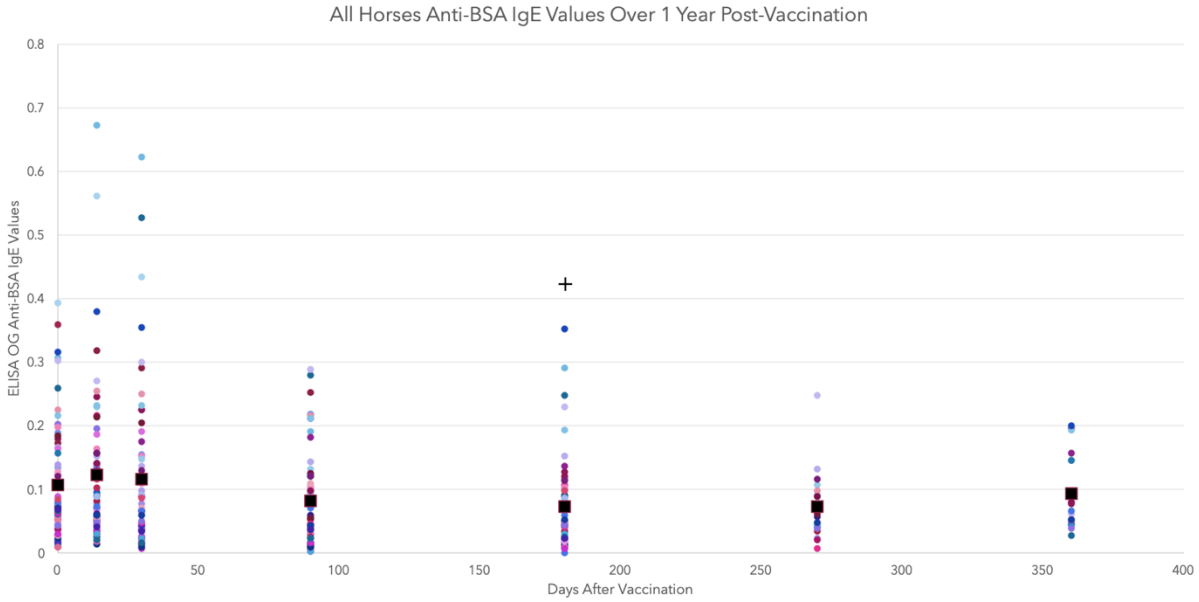


Figure 13: ELISA OD Anti-BSA IgE values for all healthy horses over the course of the 1-year sampling period. The day of vaccination was day 0. The mean of all horses at each time point is represented by a black square. Each individual horse is represented by a different color circle. The + depicts time points in which the mean was significantly different than day 0.

When comparing the fold change between pre-vaccination anti-BSA IgE OD values and 14 days post-vaccination anti-BSA IgE OD values, 63% of horses between the ages of 3 and 9 years had a fold increase greater than 1.5 (Figure 14). The percentage of horses displaying a fold

change greater than 1.5 decreased with age across age groups. Only 29% displayed a fold change greater than 1.5 in the 10-14 year old age group (Figure 15), 0% in the 15-19 year old age group (Figure 16) and 5.6% in the 20 -32 year old age group (Figure 17) (Table 3).

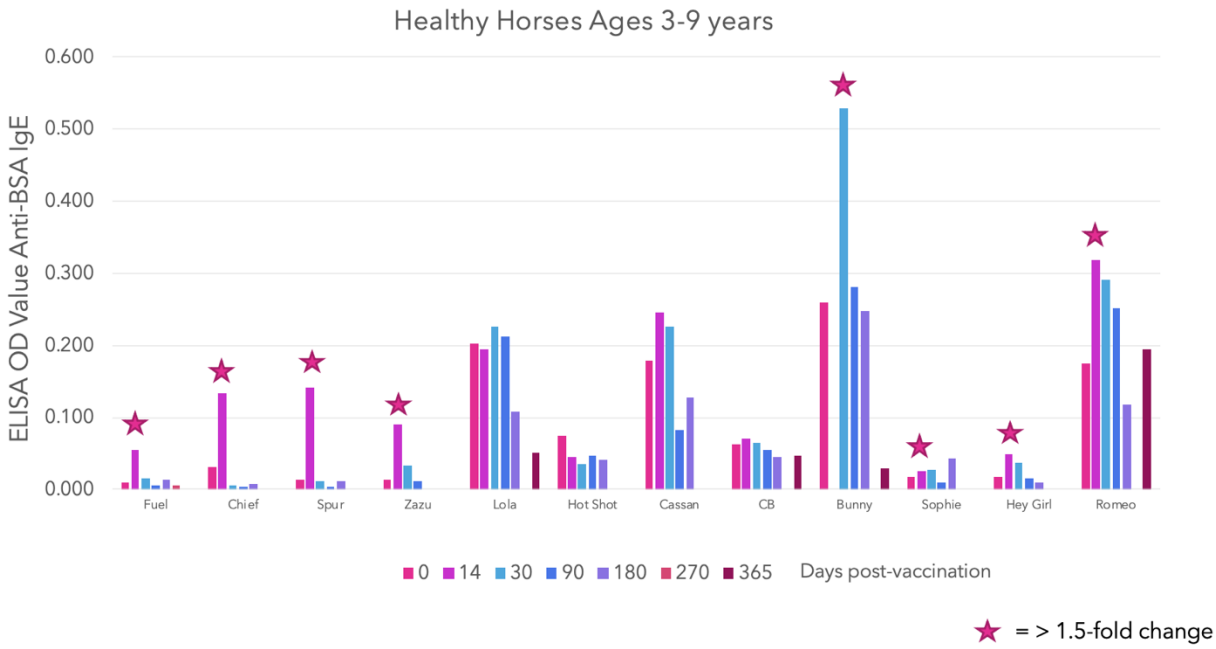


Figure 14: ELISA OD Anti-BSA IgE values for healthy horses' ages 3-9 years. The sample time points for each horse are represented by the different colored bars. A star labels the first post-vaccination time point where the fold change in anti-BSA IgE was greater than 1.5 when compared to day 0.

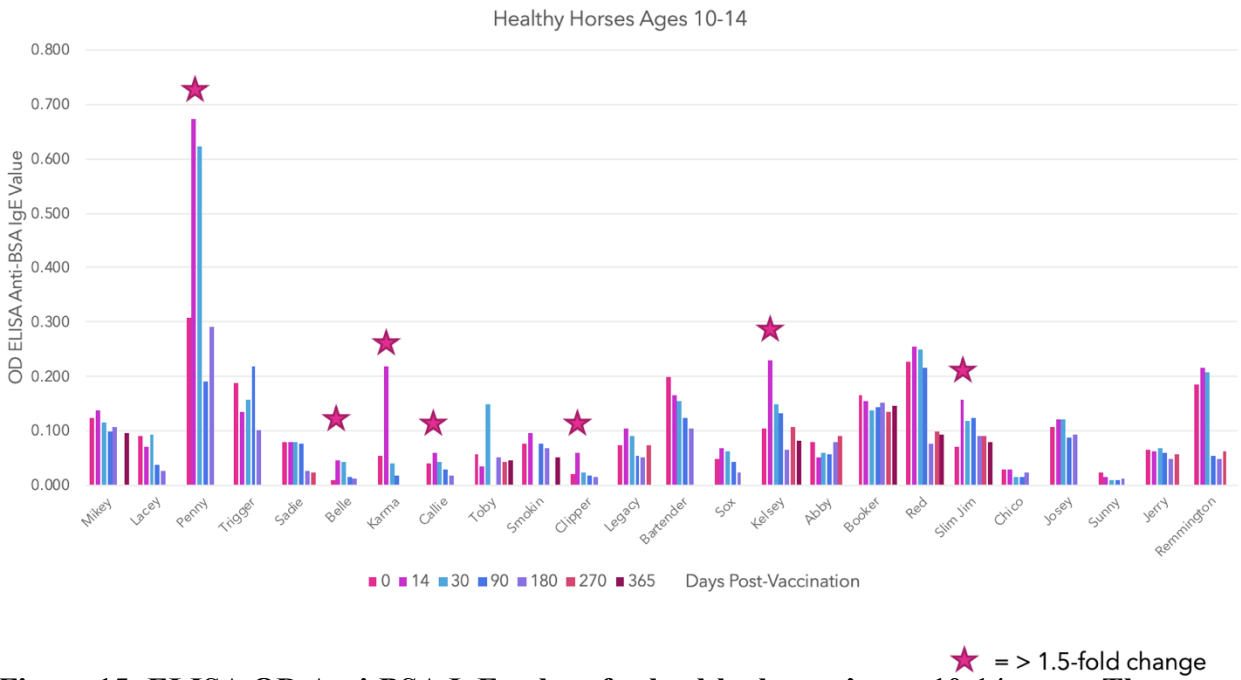


Figure 15: ELISA OD Anti-BSA IgE values for healthy horses' ages 10-14 years. The sample time points for each horse are represented by the different colored bars. A star labels each 14 day post-vaccination time point where the fold change in anti-BSA IgE was greater than 1.5 when compared to day 0.

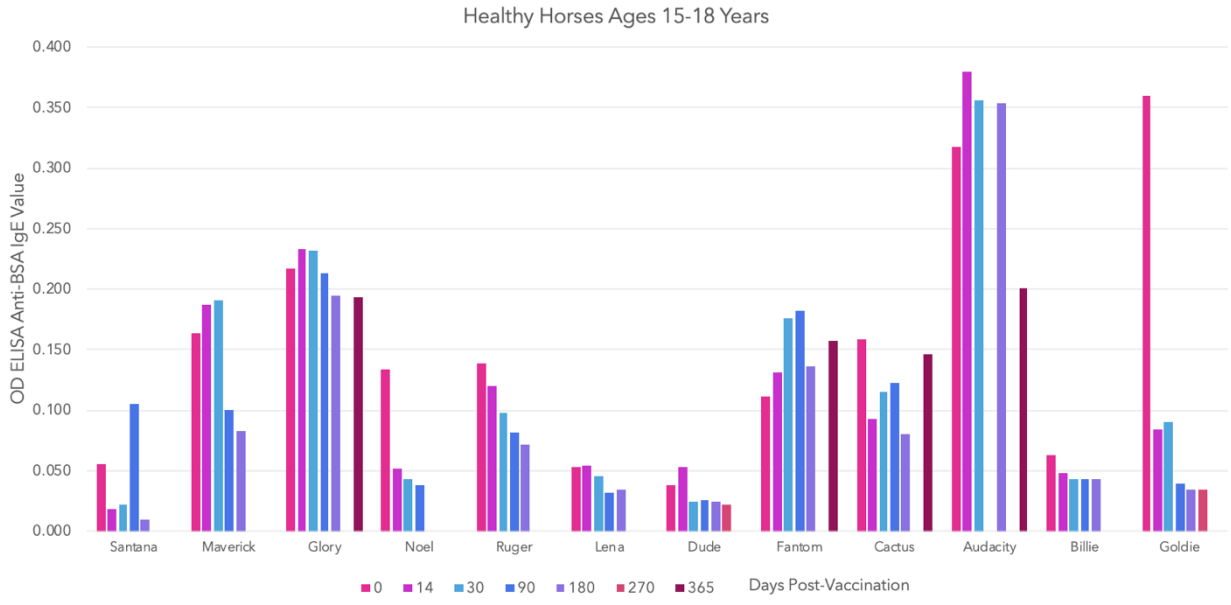


Figure 16: ELISA OD Anti-BSA IgE values for healthy horses' ages 15-18 years. The sample time points for each horse are represented by the different colored bars. There are no time points where the fold change in anti-BSA IgE was greater than 1.5 when compared to day 0.

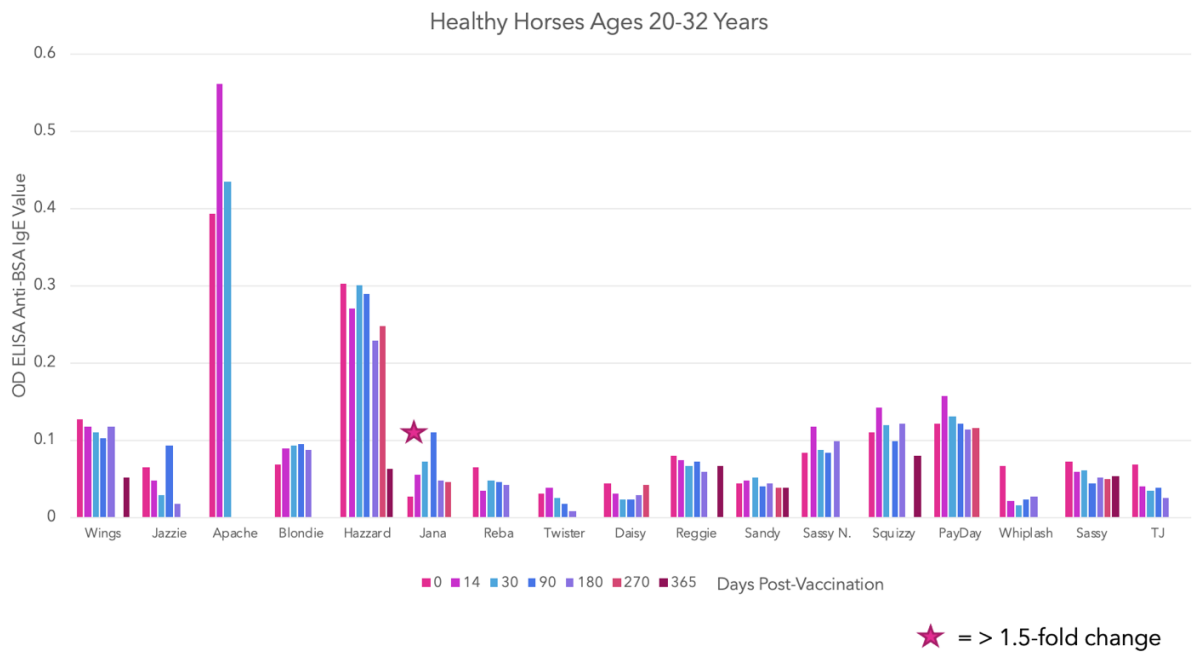


Figure 17: ELISA OD Anti-BSA IgE values for healthy horses' ages 20-32 years. The sample time points for each horse are represented by the different colored bars. A star labels each 14 day post-vaccination time point where the fold change in anti-BSA IgE was greater than 1.5 when compared to day 0.

Age groups (years)	Horses (n=)	0 – 14 day fold change mean	% with fold change >1.5	0 – 180 day fold change mean
3-9	12	3.359	7/11 (63.6%)	0.482
10-14	24	1.511	7/24 (29.2%)	0.615
15-19	12	0.845	0/12 (0%)	0.691
20-32	17	1.027	1/18 (5.56%)	0.836

Table 3: Horses are organized by age group. Within each age group the average fold change (pre vaccine compared to 2 weeks post-vaccine and compared to 6 months post-vaccine) is provided. The percentage of horses in each age group that displayed a fold change greater than 1.5 (pre-vaccine compared to 2 weeks post-vaccine) is also listed.

The mean anti-BSA IgE OD values at time points 0, 14, 30, 90, 180, 270 and 365 days after vaccination were compared across age groups. The mean anti-BSA IgE OD value for horses 3-9 years of age at day 0 was 0.072 (Figure 18). The mean anti-BSA IgE OD value for this group of horses increased to 0.125 after 14 days post-vaccination. These two timepoints of day 0 and 14 were significantly different (p-value = 0.013). The mean anti-BSA IgE OD value decreased to

0.048 at 180 days post-vaccination. These two timepoints of day 0 and 180 were significantly different (p-value = 0.035). All other timepoints compared to day 0 within this age group were not significantly different. When comparing all other age groups' mean anti-BSA IgE OD values at 14 days after vaccination and day 0, the differences were not significant. When comparing all other age groups' mean anti-BSA IgE OD values at 180 days after vaccination and day 0, the differences were significant for age groups 10-14 years (p value = 0.001) and 20-32 years (p value = 0.028) (Figures 19 and 21). In all of these age groups, the mean anti-BSA IgE OD values decreased at 180 days post-vaccination compared to time 0 and 14 days post vaccination (Table 4). No other time points in any age group were significantly different when compared to day 0 (Figure 20).

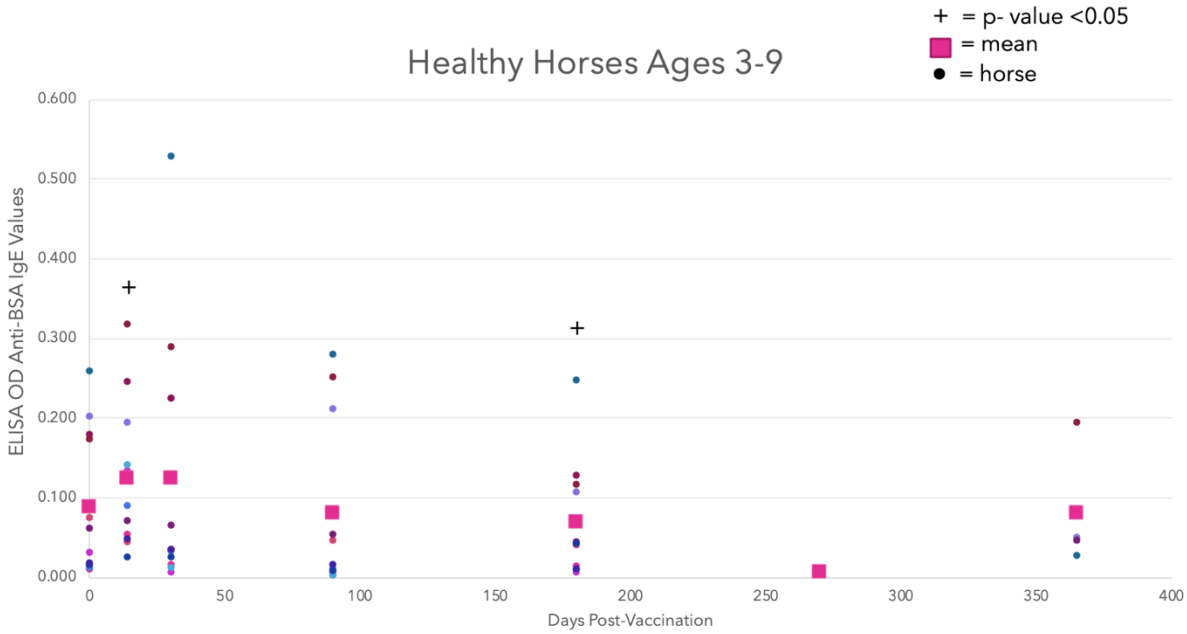


Figure 18: A scatterplot depicting the ELISA OD anti-BSA IgE values for healthy horses between the ages of 3 and 9 years. Time points of collection are described as days post-vaccination. The pink squares represent the mean of each time point. The + depicts time points in which the mean was significantly different than day 0.

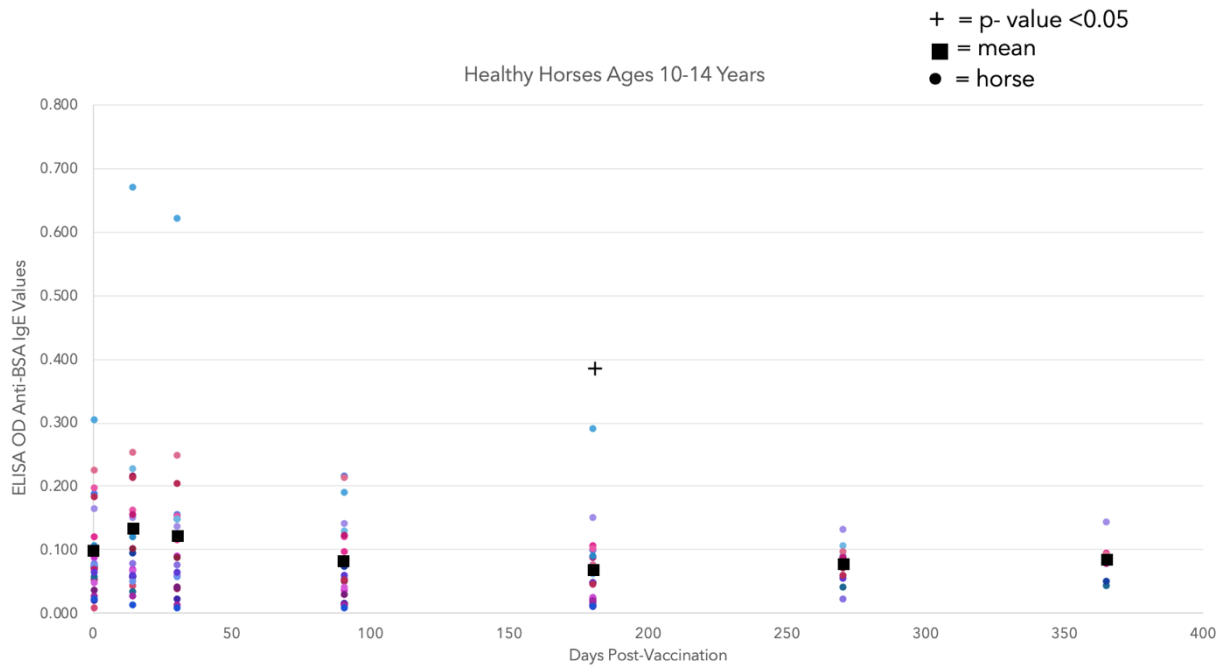


Figure 19: A scatterplot depicting the ELISA OD anti-BSA IgE values for healthy horses between the ages of 10 and 14 years. Time points of collection are described as days post-vaccination. The black squares represent the mean of each time point. The + depicts time points in which the mean was significantly different than day 0.

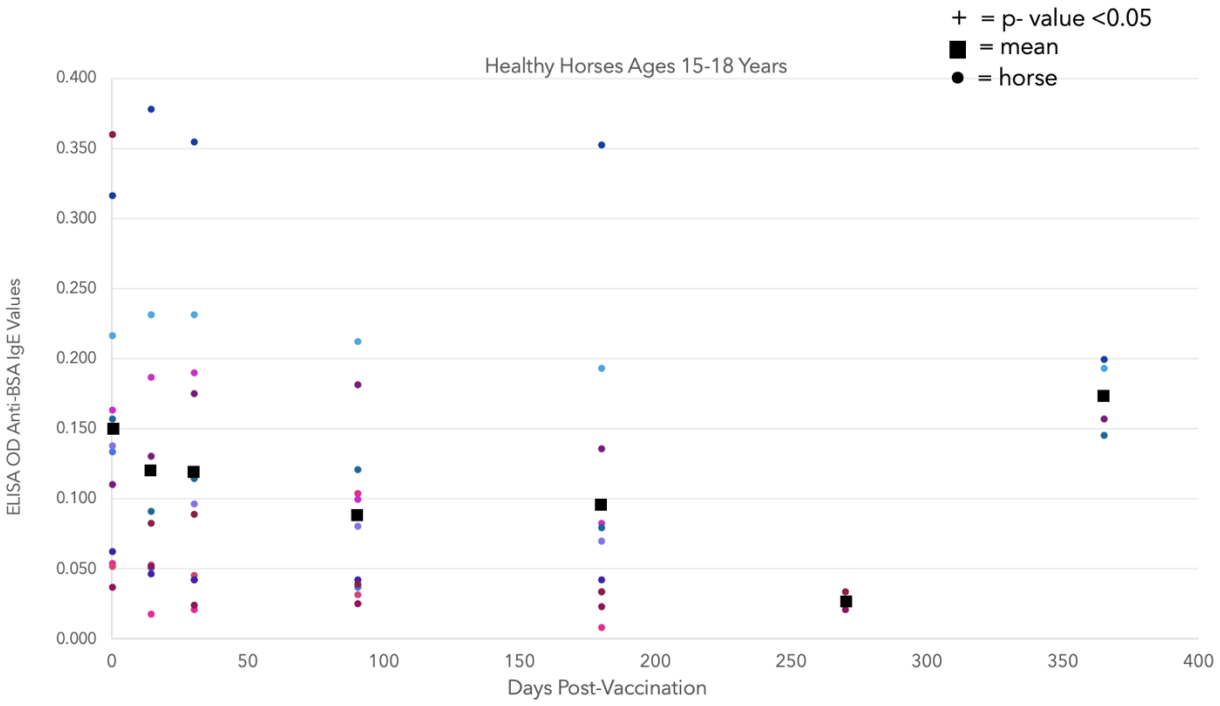


Figure 20: A scatterplot depicting the ELISA OD anti-BSA IgE values for healthy horses between the ages of 15 and 18 years. Time points of collection are described as days post-

vaccination. The black squares represent the mean of each time point. No time points were significantly different when compared to day 0.

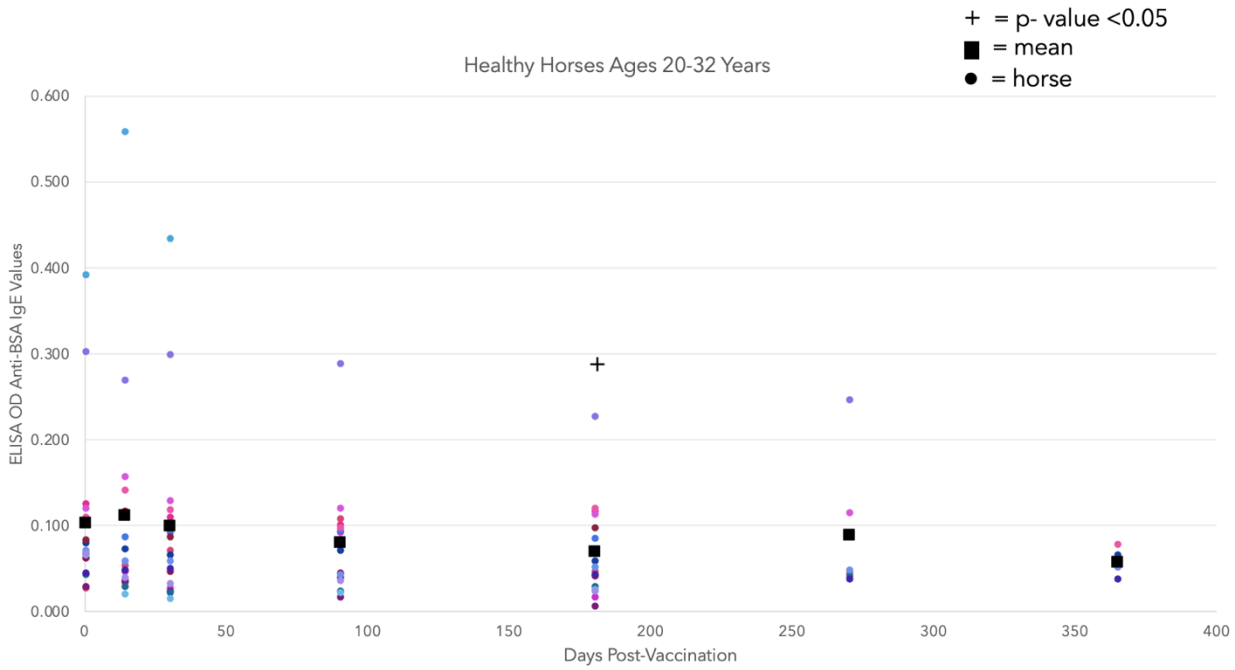


Figure 21: A scatterplot depicting the ELISA OD anti-BSA IgE values for healthy horses between the ages of 20 and 32 years. Time points of collection are described as days post-vaccination. The black squares represent the mean of each time point. The + depicts time point in which the mean is significantly different than day 0.

Age groups (years)	Horses (n=)	Day 0 anti-BSA IgE mean	Day 14 anti-BSA IgE mean	Day 180 anti-BSA IgE mean	Day 0 vs. 14 P value	Day 0 vs. 180 P value
3-9	12	0.072	0.0125	0.048	**0.0125***	**0.0352***
10-14	24	0.101	0.135	0.069	0.0647	**0.0012***
15-19	12	0.151	0.121	0.097	0.2582	0.0866
20-32	17	0.104	0.122	0.0700	0.5018	**0.0276***

Table 4: Horses are divided by age group. The average anti-BSA IgE OD values at time points 0, 14 and 180 days after vaccination are described. The difference between these time points is listed as a p-value.

Reactor Horses

The mean anti-BSA IgE OD value on samples less than 24 hours post-vaccination in those horses that displayed vaccine reactions was 0.286. There were 12 horses that fit this

criterion and the values ranged from 0.142 – 0.409. Out of these 12 horses, 10 of them had anti-BSA IgE OD values greater than 0.200 (83.3%) (Figure 22).

The mean anti-BSA IgE OD value of the 2 reactor horse samples obtained at 180 days post-vaccination was 0.187. The mean anti-BSA IgE OD value of the healthy horse samples obtained at 180 days post-vaccination was 0.073. The mean anti-BSA IgE OD value of the reactor horses at this time point was 2.56 times higher when compared to the healthy horses at this time point.

The mean anti-BSA IgE OD value of the 3 reactor horse sample obtained at 270 days post-vaccination was 0.197. The mean anti-BSA IgE OD value of the healthy horse samples obtained at 270 days post-vaccination was 0.073. The mean anti-BSA IgE OD value of the reactor horses at this time point was 2.7 times higher when compared to the healthy horses at this time point.

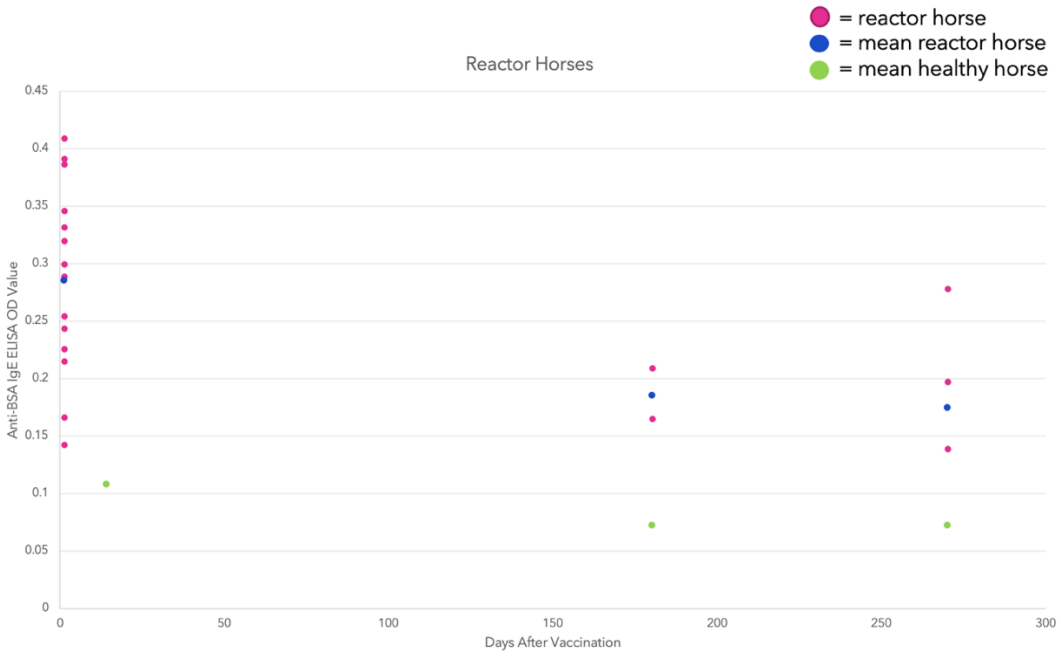


Figure 22: A scatter plot depicting the ELISA OD anti-BSA IgE values for all reactor horses as pink circles. Blue circles depict the mean ELISA OD anti-BSA IgE value for the reactor horses at each time point. The green circles depict the mean ELISA OD anti-BSA IgE value for healthy horses at each time point.

Commercial Plasma Donor Horses

The average anti-BSA IgE OD value on the commercial plasma donor samples (n=10) was 0.122 with samples ranging from 0.057 to 0.172.

Commercial Plasma Samples

The Lake Immunogenics HiGamm plasma which resulted in the fatal plasma reaction had a mean anti-BSA IgE OD value of 0.340. The MG Biologics Immunoglo plasma had a mean anti-BSA IgE OD value of 0.261.

Neonate with Fatal Plasma Reaction

The anti-BSA IgE OD value of the plasma sample obtained at 12 hours of life from the foal that had developed a fatal anaphylactic plasma reaction was 0.067. A sample was not available at 24 hours of life just prior to the administration of the plasma. This value was similar to the non-vaccinated horses (0.062). The commercial plasma that was administered to this foal had an anti-BSA IgE OD value of 0.340. This plasma sample anti-BSA IgE OD value was significantly higher than the mean day 0 anti-BSA IgE OD value in healthy horses (0.108), the mean anti-BSA IgE OD value in commercial plasma donor horses (0.122) (Figure 23).

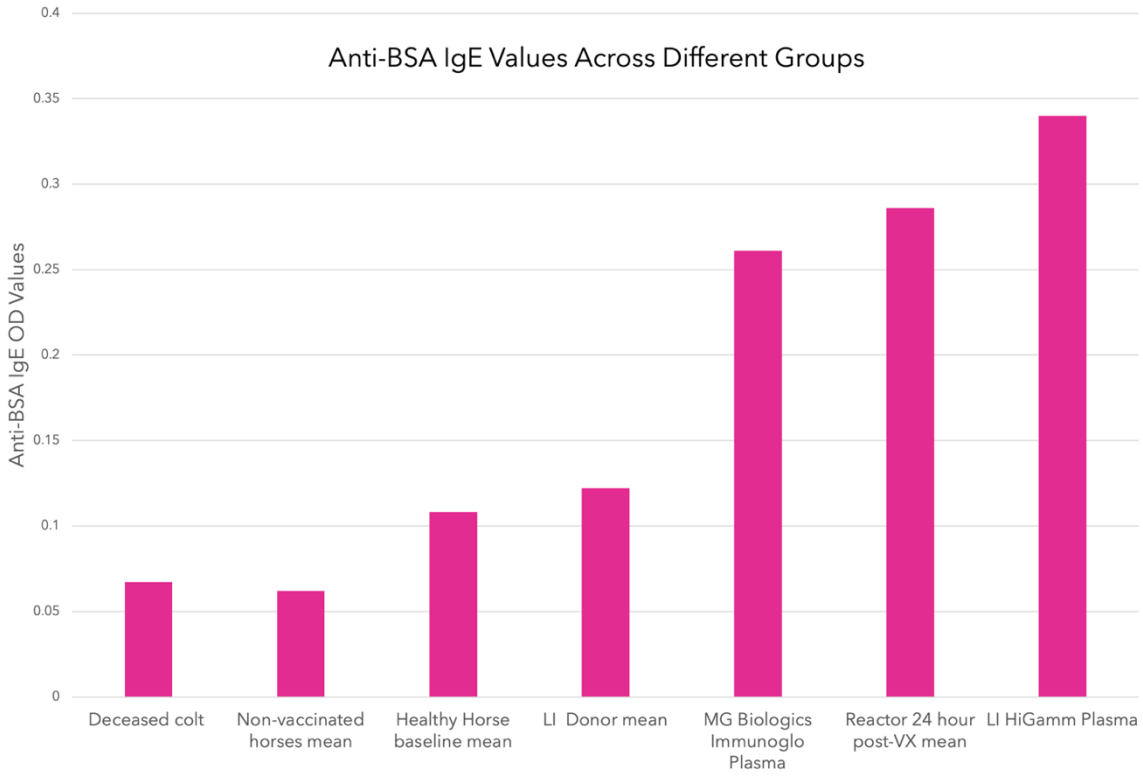


Figure 23: ELISA OD Anti-BSA IgE values of the 12 hour of life serum sample from the deceased colt, non-vaccinated horses mean, healthy horses baseline mean, Lake Immunogenics donor horse mean, MG Biologics Immunoglo plasma sample, reactor horses 24 hours post-vaccination mean, and the Lake Immunogenics HiGamm plasma causing the fatal reaction represented by a bar graph.

Discussion

The results of this study demonstrate that horses produce anti-BSA IgE in response to vaccination. There appears to be an association with vaccination and expression of anti-BSA IgE. These findings are consistent with post-vaccination studies in both humans[5] and horses[8].

As expected, horses that had never been vaccinated did not demonstrate detectable levels of anti-BSA IgE using ELISA. A larger subset of horses would be needed to further investigate whether non-vaccinated horses produce anti-BSA IgE. Foals receiving commercial powdered colostrum supplementation should also be investigated to determine if they are producing anti-BSA IgE.

Horses that had either historically or currently experienced an immediate vaccine reaction all had detectable levels of anti-BSA IgE on ELISA. The majority of these horses had blood samples collected within 24 hours post-vaccination. Our study did not measure healthy horse anti-BSA IgE at 24 hours following vaccination. Ten out of the 12 reactor horse samples at 24 hours post-vaccination had an anti-BSA IgE OD value greater than 0.200 (83.3%). Whereas only 9 out of the 65 healthy horses had an anti-BSA IgE OD value greater than 0.200 at 14 days post-vaccination (13.8%). Twenty-four-hour post-vaccination blood samples from horses not experiencing a vaccine reaction could be investigated by ELISA to determine a reference range for anti-BSA IgE in healthy horses. A very small subset of these adverse reaction horses had blood samples from 180 and 270 days post-vaccination. While their anti-BSA IgE levels appeared to be higher than the healthy horses in this study, these values were not statistically different from healthy horses. A larger sample of horses is needed in order to further investigate whether adverse reaction horses have a significantly increased anti-BSA IgE level at different time points post-vaccination. With this information, horses developing a vaccine reaction could be tested in order to determine if they have elevated anti-BSA IgE levels. With reference ranges available, it could be possible to screen horses prior to vaccination to determine if they are at a higher risk for developing a vaccine reaction specifically against BSA.

Horses used as commercial plasma donors had anti-BSA IgE levels similar to healthy, non-reactor horses. However, the history of these horses was unknown. Important information would include the age of these horses, how frequently they were vaccinated, when their last vaccine was administered and how long they had been in the program for plasma collection.

The foal in the case presented here had plasma collected at 12 hours of life which was analyzed for anti-BSA IgE and the value was similar to a non-vaccinated horse. The plasma that

was administered to this foal was analyzed for anti-BSA IgE which was higher than healthy non-reactor, reactor horses and those horses that were utilized as commercial HiGamm plasma donors. Based on this finding, it appears that at least an individual commercial plasma sample had elevated anti-BSA IgE levels and this could occur in other donor horses, but it may go undetected. In the described case, the interaction of the high circulating IgE with the presence of BSA presumably bound on mast cells leads to an immediate hypersensitivity reaction in the described foal.

Lake Immunogenics HiGamm commercial plasma is USDA approved for administration in equine neonates for the treatment of failure of passive transfer. This product is guaranteed to have an IgG concentration of at least 2800 mg/dL. If plasma is high in anti-BSA IgE and it is administered to a foal that has been exposed to BSA in commercial powdered colostrum this could lead to a fatal anaphylactic reaction. Among available commercial plasma products, some manufacturers explicitly state that the product should not be administered to neonatal foals that have received a commercial powdered colostral product for this specific reason. Further investigation is indicated to determine the frequency of elevated anti-BSA IgE among commercial plasma donor animals. Depending on the frequency of these donors, horses could be removed as plasma donors for the use in foals.

Forty-six of the 65 healthy horses demonstrated an increase in anti-BSA IgE during at least one time point post-vaccination compared to baseline. Sixteen of the 65 healthy horses showed a greater than 1.5-fold increase in anti-BSA IgE at 14 days post-vaccination compared to baseline. Six of these 16 horses had a greater than 4-fold increase in anti-BSA IgE at 14 days post-vaccination. All of these 6 horses were between 3 and 11 years of age. Interestingly, none of these 65 horses developed clinical signs associated with an adverse vaccine reaction. This is

suggestive that the presence of anti-BSA IgE or the rise in anti-BSA IgE does not directly correlate with the development of an adverse vaccine reaction. The question remains as to what, if any, fold increase is required in order to develop an adverse reaction or what other factors must be present for a hypersensitivity reaction to occur.

The youngest age group consisting of horses ages 3-9 years of age had significantly more horses displaying a greater than 1.5-fold increase in anti-BSA IgE at 2 weeks post-vaccination compared to baseline. This suggests that younger horses develop a greater immune response to BSA than older horses.

A future direction of this study is the development of an anti-BSA IgGT ($IgG_{3/5}$) ELISA assay. IgGT is the IgG isotype associated with immediate skin reactions and is known to play a role in mast cell activation.[13] Currently we are working on purifying IgGT in order to develop this ELISA.

This study supports the theory that horses can mount an immune response to BSA in vaccines by producing anti-BSA IgE. Further investigation needs to be performed to evaluate the levels of anti-BSA IgE in commercial plasma labeled for use in horses and especially foals. At this time, veterinarians should practice extreme caution upon administration of commercial plasma to a neonate that has consumed powdered colostrum supplementation. One commercial plasma company (MG Biologics) has taken the steps to apply a warning label for this situation on their plasma products. It would be recommended at this time for other plasma companies to apply this warning label as well.

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Appendix A - Study Questionnaire for Reactors

Evaluation of antibodies in a horse post-vaccination reaction

Owner Name:

Veterinarian Name:

Phone Number:

Primary Contact:

E-Mail Address:

Survey Questions:

1. **How long have you owned this horse?**
2. **Does this horse receive the same vaccines regularly? Please elaborate as best as possible.**
3. **Has this horse experienced an adverse reaction post-vaccination in the past? If so, please explain the symptoms observed and type of vaccine administered to the best of your ability.**
4. **Please describe the symptoms observed during this current vaccine reaction:**
5. **Which vaccines were administered on the day of this current adverse reaction?**
6. **Were any medications administered prior to the blood draw? If so, please list:**
7. **Time of vaccination _____**
   Time of observed symptoms _____ Time of blood draw_____

Additional comments:

****Please attach this survey with the signed client consent form and send with blood sample to KSU-VDL attn: Dr. Elizabeth Perry**