

The acute phase protein serum amyloid A after routine vaccination in healthy adult horses
by

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Abstract

Background: Serum amyloid A (SAA) is an acute phase protein. Vaccination is intended to induce an antigen-specific inflammatory response. In some instances, this response can be debilitating. Characterizing the acute phase inflammatory response after vaccination in healthy horses will provide a baseline for expected SAA expression.

Hypothesis/Objectives: The objective was to measure the effect of routine vaccination on SAA concentrations in apparently healthy horses. It was hypothesized that SAA would increase following routine vaccination compared to placebo administration.

Animals: Apparently healthy client-owned horses (n=21) and Kansas State University College of Veterinary Medicine teaching horses (n=15) were included.

Methods: In experiment 1 (n=8), a blinded, randomized, prospective, cross-over study was performed. Horses were either vaccinated (rabies, tetanus, West Nile, Eastern and Western equine encephalomyelitis, equine herpesvirus-1/-4, influenza) or administered saline, and SAA was measured at 6, 12, and 24 hours and daily until day 10. In experiment 2 (n=28), a prospective, observational study measured SAA post-vaccination at 12 and 24 hours and daily until day 10.

Results: Over time, vaccination resulted in a significant increase in SAA concentrations compared to placebo without clinical evidence of an adverse reaction. The median SAA post-vaccination peaked on day 2 (median 1872 $\mu\text{g/ml}$, interquartile range:0-2,402.5) and returned to normal (<20 $\mu\text{g/ml}$) by day 9 (median 6 $\mu\text{g/ml}$, interquartile range:0-23.5) post-vaccination. Vaccination did not influence fibrinogen concentration and white blood cell count.

Conclusions and clinical importance: Routine vaccination results in an increased SAA.

This provides evidence for a period of convalescence following vaccination. Measuring SAA following vaccination cannot be used as an indicator of illness.

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Chapter 1 - Background and Introduction

The innate immune system is the early, nonspecific immune response that is stimulated by any local and systemic disturbances to homeostasis, such as trauma (parasites, trauma, surgery, burn injury, neoplasia), infection (bacterial, viral, etc.) and inflammation (ischemic necrosis) [1] [2] [3] [4]. The innate immune system is composed of physical barriers, phagocytes, complement and toll like receptors, that are all aimed at the prevention of infection, elimination of potential pathogens and initiation of the inflammatory process [3] [1]. Stimulation of the innate immune system, thus the acute phase response, occurs through release of inflammatory mediators, such as arachidonic acid metabolites, cytokines, nitric oxide, glucocorticoids and other products of oxidative stress [1][2][3][5][6][7]. These immunomodulators are released when pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) are recognized by cells of the innate immune system through pattern recognition receptors. Components of pathogens (bacterial, viral, fungal, etc.), such as DNA, nucleic acid, lipopolysaccharide (LPS), and other carbohydrate and lipid moieties are examples of PAMPs [8][9]. Molecules released from cell death or connective tissue damage, such as hyaluronic acid, fibrinogen and heat shock proteins are examples of DAMPs [9]. The host cell interactions with PAMP/DAMP molecules results in signal transduction activity that leads to inflammatory mediator expression as part of the acute phase response. The four major classes of factors regulating this response, include: IL-6/gp130 family of cytokines (IL-6, IL-11, ciliary neurotrophic factor, leukemia inhibitory factor), IL-1 type proinflammatory cytokines (IL-1, TNF- α), growth factors (insulin, hepatocyte growth factor, fibroblast growth factor), and corticosteroids [8]. The primary pro-inflammatory cytokines released include IL-1, IL-6 and TNF- α [3][6][10][11]. These cytokines are a measurable marker of inflammation.

However, their nonspecific nature makes interpretation of cytokine immunoassays and bioassays difficult (table 1)[12]. Cytokine concentrations in correlation with disease severity and prognosis have also yet to be well defined [12]. Therefore, cytokines have little clinical utility thus far, with their use restricted primarily to a research setting in the investigation of many disease pathogenesis’.

Parameter	Bioassay	Immunoassay
Measurement	Biologically active molecules	Immunoreactive molecules
Sensitivity	Can be high, often higher than an immunoassay	Can be reasonably high
Specificity	Poor to high (assay dependent); must be confirmed with neutralizing antibodies	Usually high, but dependent on antibodies; unless epitopes determined, the exact form of cytokine detected in unknown, e.g., fragments, bound form, etc.
Accuracy	may be affected by specific and nonspecific inhibitors, cytokine-binding proteins and antibodies present in the sample; may be influenced by blood collection procedure (uptake/release by leukocytes, stability)	May be subject to interference from matrix, e.g., complement, binding proteins, rheumatoid factor, heterophilic antibodies; may be influenced by blood collection procedure (uptake/release by leukocytes, stability)
Precision	Relatively poor (CV, 10-100%)	Usually good (CV, 5-10%)
Ease of Use	Labor intensive (24h-4d)	2-24h
Standardization	Nationally available standards	Nationally available standards

Table 1. Factors to be considered in the interpretation of cytokine assays with biological samples [12]

As immunomodulators cytokines have many functions that are stimulatory and inhibitory, including activation of the adaptive immune response (lymphocyte activation) and release of acute phase proteins [8]. The production of acute phase proteins is described as occurring in two waves. First, the production of type 1 acute phase proteins is followed by the production of type 2 acute phase proteins [6][13]. Interleukin-1 and TNF- α are the primary cytokines that stimulate the production of type 1 acute phase proteins, such as serum amyloid A (SAA) and C-reactive protein. Concentrations of type 1 acute phase protein expression rises rapidly, within hours of an inciting stimulus [6][13][14]. Following the production of type 1 acute phase proteins is the production of the type 2 acute phase proteins, such as haptoglobin,

fibrinogen and α -1 glycoprotein. Production of the type 2 acute phase proteins are stimulated by secondary cytokines and chemokines: IL-6, IL-8 and monocyte chemoattractant protein [6][13][14]. The type 2 acute phase protein concentrations increase later and remain elevated longer, in comparison to type 1 [6]. In reality, these processes are likely occurring simultaneously. The organ primarily responsible for the production of the acute phase proteins is liver, but many acute phase proteins have extrahepatic sites of production [15][6]. Acute phase protein synthesis is influenced by extracellular processes and intracellular control mechanisms. Intracellular production of acute phase proteins is controlled by the golgi complex and endoplasmic reticulum, along with their coinciding mechanisms for protein production and storage [16]. These intracellular processes are complex and are beyond the scope of this review. Extracellular factors that influence the production of acute phase proteins include malnutrition and anorexia (described in humans), massive erythrolysis (ie. lowering haptoglobin levels), and other protein modulators (protein kinase C, 12-O-tetradecanoylphorbol-13-acetate, 1-oleoyl-2-acetyl-sn-glycerol, protein phosphatases, etc.) [7][17][18][19]. Other biological factors that may influence acute phase protein production, such as circadian rhythm, age, sex and breed, are described in some species; however, this has not been fully elucidated for many species [20]. There are also many metabolic effects that result in positive and negative feedback, enhancing or downregulating the acute phase response. As an example, glucocorticoids activate the hypothalamic-pituitary adrenal axis, that acts as a positive feedback mechanism to enhance the expression of some acute phase proteins, but also acts as a negative feedback mechanism to inhibit cytokine gene expression [21][14]. The acute phase response results in many systemic changes. A few examples include suppression of the cytochrome P-450 enzyme system, induction of heat shock proteins, re-distribution of blood flow to brain and muscle instead of

visceral organs, and decreased production of growth hormone [7]. These systemic metabolic changes are physically apparent as fever, anorexia and catabolism of muscle cells [5][7][8].

Along with physical changes of the acute phase response, there are measurable laboratory changes, such as in peripheral leukocyte number, hormonal changes (ie: adrenocorticotrophic hormone), coagulation assays, mineral and electrolyte changes (ie: calcium, zinc, iron, vitamin A, etc.), as well as plasma acute phase proteins [7][8][10]. The magnitude of these laboratory changes is dependent on the inflammatory stimulus, extent of inciting injury and is variable among species. These changes are also distinguishable by duration of ongoing inflammation into acute and chronic. The definition of acute inflammation is a rapid onset of inflammation, with tissue infiltration by neutrophils [9]. Other characteristics of acute inflammation, include a peak of acute phase proteins within 24 to 48 hours of inciting injury, decrease with recovery and resolution (values return within normal limits) within 4 to 7 days [6][9]. In comparison, chronic inflammation is slowly developing or persistent and is characterized by tissue infiltration with macrophages and fibroblasts [9]. Characteristics of acute and chronic inflammation overlap, with individual and species variation in production, peak and duration of the various acute phase proteins.

The Acute Phase Proteins

The major serum proteins include albumin and the four fractions of globulins: α_1 globulins (α_1 antitrypsin, α_1 acid glycoprotein), α_2 globulins (α_2 macroglobulins and haptoglobin), β globulins (transferrin, SAA, fibrinogen and C-reactive protein) and γ globulins (immunoglobulin G) [3]. These proteins can be further categorized by their circulating levels in relation to inflammation. Positive acute phase proteins, such as SAA, ceruloplasmin, complement factor-3 (C3), haptoglobin, fibrinogen, α -globulins and lipopolysaccharide (LPS) binding protein, increase in response to inflammation [1][7][9]. Negative acute phase proteins, such as albumin, transferrin, retinol binding protein (RBP), transthyretin (pre-albumin) and cortisol binding globulin, decrease in response to inflammation [1][7]. The positive acute phase proteins are further divided into minor, moderate and major. Major acute phase proteins are defined by their low level in health, that increase greater than 10, 100 or 1000 times with an inflammatory stimulus [1]. Minor and moderate acute phase proteins increase 1 to 10 times during the acute phase response [1]. Acute phase proteins can be further categorized by inflammatory response time. Acute phase protein magnitude and duration of production is variable among species as shown in Table 2. For example, in the horse, the rapid acute phase protein is SAA; intermediate acute phase proteins are haptoglobin, α -1 acid glycoprotein and C-reactive protein; and delayed acute phase proteins are fibrinogen and ceruloplasmin [1][3].

Species	Major	Moderate
Cat	α -1 acid glycoprotein, serum amyloid A	haptoglobin
Chicken	None	α -1 acid glycoprotein, ceruloplasmin, serum amyloid A, transferrin
Cow	haptoglobin, serum amyloid A	α -1 acid glycoprotein, C-reactive protein, fibrinogen
Dog	C-reactive protein, serum amyloid A	α -1 acid glycoprotein, ceruloplasmin, haptoglobin
Goat	haptoglobin, serum amyloid A	fibrinogen
Horse	serum amyloid A	fibrinogen, haptoglobin
Human	C-reactive protein, serum amyloid A	α -1 acid glycoprotein, fibrinogen, haptoglobin
Mouse	haptoglobin, serum amyloid A, serum amyloid P	C-reactive protein, fibrinogen
Nonhuman primates	C-reactive protein	α -1 macroglobulin, fibrinogen, serum amyloid A
Pig	haptoglobin, serum amyloid A	α -1 acid glycoprotein
Rabbit	haptoglobin, serum amyloid A	α -1 acid glycoprotein, C-reactive protein, fibrinogen
Rat	α -1 acid glycoprotein, α -2 macroglobulin	C-reactive protein, fibrinogen, haptoglobin
Sheep	haptoglobin, serum amyloid A	α -1 acid glycoprotein, C-reactive protein

Table 2. Major and moderate acute phase proteins in different animal species [3]

Serum amyloid A is a family of major acute phase proteins that are evolutionarily conserved among many species including human, hamster, dog, cow, horse, mouse, rabbit, mink, sheep, wallaby, fox, goat and arctic char [2][14][22]. There are several isoforms of SAA described: SAA1 to 4, serum amyloid P and amyloid A [8]. Isoforms SAA1 and 2 are functionally conserved among animal species as inflammatory reactants [21]. The synthesis of SAA1 and 2 in most mammalian species (including horses) occurs in the liver; whereas, SAA3 is synthesized in extrahepatic sites [6][14][23][24]. The regulation of SAA gene expression is complex and involves multiple molecular mechanisms (transcriptionally, post-transcriptionally) [11][23][25][26]. Hepatic SAA production is stimulated from the binding of cytokines (IL-1, IL-6, & TNF- α), through use of nuclear factors to specific DNA sequences upstream from the SAA structural gene [11][26]. Serum amyloid A production is then modulated by other signals,

including endotoxin, lipopolysaccharide, monocyte-conditioned medium, other cytokines (IFN- γ , leukemia inhibitory factor, transforming growth factor- β , etc.), the synthetic glucocorticoid dexamethasone, etc. [23][26]. For isoforms SAA3 and 4, there is species variability in the production and function of isoforms (Table 3) [21]. For example in horses, SAA3 is expressed in mammary gland, joints and colostrum; whereas in humans, SAA3 is a pseudogene and is considered non-functional [1][21][27]. The isoform SAA4, also referred to as constitutive SAA, is the major form carried on normal high-density lipoprotein (HDL) and is not considered a major acute phase reactant [21].

Species	Extrahepatic sites of serum amyloid A production	Sources
Mouse	kidney, intestine	[14]
Hamster	kidney, stomach muscle, testis, spleen, brain, heart, uterus, lung, intestine, ovary	[14]
Rats	lung, ileum, large intestine	[14]
Mink	ovary, testis, brain, intestine, lung, heart, kidney convoluted tubules, uterine endometrium	[14]
Humans	stomach, small and large intestine, tonsil, breast, prostate, thyroid, lung, pancreas, kidney, skin, epidermis, brain neurons monocyctic/macrophage cell lines, human arteriosclerotic lesions, adipocytes, epithelium of tissues, scattered lymphocytes and plasma cells in stroma, endothelial cell lining blood vessels, breast (lobule and duct epithelium), large intestine, esophagus, kidney, spleen	[28]
Horse	mammary gland, joints	[2][5][14][27][29]
Ovine	mammary gland	[27]
Bovine	mammary gland	[27]

Table 3. Species variability in extrahepatic sites of SAA synthesis

Another protein form belonging to the SAA family of proteins, is amyloid A (AA). This form of amyloid protein is associated with pathologic amyloid deposition, often referred to as “reactive”, “secondary” or “amyloid A” amyloidosis. Chronic inflammatory conditions associated with high levels of circulating apolipoprotein serum amyloid A (apoSAA) and its incomplete catabolism, are thought to function as a precursor to pathologic deposition of β -pleated sheets of amyloid fibrils identifiable by Congo red stain [7][14][21][26][30][31][32]. The

rigid, nonbranching characteristics of AA, as well as its relative resistance to proteolytic digestion in physiological conditions interfere with organ function, which contribute to the pathologic, irreversible and progressive course of amyloidosis [26]. Pathologic deposition of amyloid fibrils can occur in any organ, but is primarily described in the kidney, liver and heart [8]. In humans, amyloidosis is associated with prolonged inflammatory diseases, such as rheumatoid arthritis, some cancers (Hodgkin's disease, carcinoma of the kidney, hair cell leukemia), juvenile chronic arthritis and ankylosing spondylitis, Crohn's disease, etc. [8][14][21][26][33][34][35]. In the human disease, Familial Mediterranean fever, AA is described to accumulate in joint leukocytes and within the serosal surface and is seen with increased SAA [36]. However, only some patients develop systemic amyloidosis [36]. Rheumatoid arthritis is another disease that is commonly described in association with systemic amyloidosis. Migita, et al (1998) have shown that human synovial fibroblasts produce matrix metalloproteinase-2, with its production induced by SAA and is thought to contribute to the pathogenesis of rheumatoid arthritis [37]; although, levels of SAA in rheumatoid arthritis patients with or without amyloidosis are not different [37]. As pathogenic amyloid deposition does not occur in all patients with these chronic inflammatory diseases, other factors are thought to play a role in amyloid formation, such as amyloid enhancing factor, impairment of SAA-degrading proteases, apolipoprotein-E4 and many other factors such as proteoglycans [7]. Polymorphonuclear cells and monocytes are also thought to play a role in converting SAA into amyloid fragment [36]. In horses, amyloidosis has been described as a cause of chronic renal failure and an uncommon nodular dermatosis [38][39]. Cases of amyloidosis are uncommon and more research is required investigating the pathogenesis of amyloidosis across all species.

The evolutionary conservation of SAA among species is a testament to its importance as an immunomodulator with many systemic and local effects. In systemic circulation, SAA binds to high density lipoprotein (HDL) and can be transported to and deposited in inflamed tissues [15]. The local production of SAA is suggested to play a role in localized defense against tissue injury and inflammatory challenges [14][27][29][40]. Serum amyloid A has cytokine like properties that result in a variety of pro- and anti-inflammatory effects [41]. An important pro-inflammatory effect of SAA is its chemoattraction and activation of peripheral blood monocytes and neutrophils, described by Bo su, et al (1999) to be performed through G-protein coupled receptors on phagocytes [42]. Preciado-Patt, et al (1996) described this process further suggesting that the extracellular matrix serves as a temporary anchorage site for SAA and AA, allowing for the regulation of immunocyte recruitment in extravascular inflammatory compartments [43]. Other immune enhancing effects include promoting the synthesis of inflammatory mediators and enhancing cell surface antigen expression, lactoferrin secretion, phagocytosis, polymorphonuclear anti-Candida activity and mast cell migration [1][14][15][44]. The down-regulatory or anti-inflammatory effects of SAA are described at higher serum concentrations [8]. Examples of SAA anti-inflammatory effect include the suppression of T cell and macrophage interactions, decreasing T helper cell function, inhibit leukocyte respiratory burst, phagocytosis and chemotaxis, as well as an inhibitory effect on neutrophil chemotaxis and degranulation [1][14][15][32][45]. Other proposed immune functions of SAA, include stimulating the enzyme production for degradation of extracellular matrix metalloproteinases, inhibiting thromboxane B2 generation and serotonin release by thrombin-activated platelets, inhibiting platelet aggregation and enhancing the formation of inflammatory mediators (thromboxane A2, prostaglandin E2 and prostaglandin F2 α) [1][32][46].

Besides the influence of SAA on the immune system, SAA has other systemic effects. Serum amyloid A influences lipid transport to inflamed tissue. As previously mentioned, the SAA protein family is linked primarily to HDL in circulation, with minor amounts associated with other HDL fractions (low density lipoprotein and very low-density lipoprotein) [26]. There are two hypothesis that exist for how SAA modulates cholesterol transport. The first hypothesis is in cells that have an increased requirement for cholesterol, SAA reverses cholesterol transport to allow delivery of lipid to peripheral cells, thus facilitating tissue regeneration of inflamed cells [14][23][47]. The second hypothesis, is that SAA facilitates large quantities of cholesterol to be liberated from sites of tissue damage during inflammation [14][23][47]. The biological significance of the distribution of SAA among the lipoprotein classes and SAA's effect on cholesterol transport is not completely understood. In humans, SAA's altered lipid metabolism is thought to play an important role in the prevention of atherosclerosis [14][23]. However, further research is required. Serum amyloid A is also proposed to play a role in neoplastic processes, through inhibiting malignant cell attachment to extracellular matrix proteins, induction of enzyme expression to degrade extracellular matrix, as well as the induction of adhesion, migration and tissue infiltration of cells [23]. Mammary gland production of SAA in milk and colostrum is also shown to enhance innate immune system in neonatal intestinal cells in vitro [48]. These are a few examples of SAA's biological functions, with many that are likely yet to be discovered.

Of the acute phase proteins in horses, SAA is a major acute phase protein showing the earliest increases with inflammation, followed by changes in α -1 acid glycoprotein, C-reactive protein, haptoglobin, fibrinogen and ceruloplasmin [13]. In horses, SAA has many properties that make measuring its concentrations useful in clinical monitoring. Serum amyloid A values rapidly

increase to greater than 100-fold in response to tissue injury, infection or inflammation depending on the inciting stimulus [5]. The changes in SAA concentrations can be detectable in a variety of tissue and fluid samples: peripheral blood, peritoneal and synovial fluid [49][50][51]. The SAA values then rapidly decrease with resolution of inflammation. As the acute phase response is highly variable with inflammatory stimuli, there is variation in peak and magnitude of SAA concentrations [20]. In horses, the response of SAA has been investigated in the following inflammatory conditions: endotoxemia [52], neonatal sepsis [53] [54] [55] [56], strangles [57], *Rhodococcus equi* pneumonia [58] [59], pneumonia [60][61][62], noninfectious airway disease [63] [64] [65], diarrhea and enteritis in foals [13], surgical and medical colic [49] [64] [66] [66] [67] [68] [69] [70], reproductive conditions and diseases (induced placentitis, induced endometritis, parturition, abortion, early embryonic death and pyometra) [71] [72] [73] [74] [75] [76] [77], Equine grass sickness [78] [79], septic arthritis and synovitis [50] [80] [81], noninfectious arthritis [82], wound microenvironment [83], penetrating joint wounds [84], in the detection of post-racing trauma [85], habronemiasis [86], inflammatory disease [87] and gastrointestinal parasitism [88] [89] [90]. Serum amyloid A values are used to monitor course of disease, response to treatment and can assist in developing a prognosis. Other factors that do or do not influence SAA concentrations require consideration while interpreting SAA values. Examples of conditions that do not effect SAA concentrations include equine gastric ulcer disease [91], repeat arthrocentesis [51] [92], insemination of mares with frozen-thawed semen [93], anesthesia [57] and ophthalmic disorders [94]. Examples of conditions that do effect SAA concentrations include exercise [85] [95], endurance riding [96] [97], racing [98], vaccination [99] [100] and body condition score [101]. Serum amyloid A values are also used to monitor the expected inflammatory response in equine surgical procedures to allow for early identification of

post-operative complications. This has led to the investigation of SAA concentrations in association with the following surgical procedures: castration [102] [103], laparotomy [57] [104], exploratory celiotomy [105], elective surgical procedures [106], and other surgeries [107]. In general, the expected inflammatory response is characterized by peak SAA values two to three days post-operatively, with values returning to normal pre-operative levels within 7 to 10 days [106] [107]. Alterations to the pattern in SAA values from the expected trend allows for early identification of complications and timely intervention for improved patient care.

Resolution of the acute phase response involves many metabolic changes. At the liver, downregulation of the acute phase response occurs with hepatocyte rapid removal of cytokines, Kupffer cells release of interleukin-10, and interleukin-1 and -4 negative feedback on the acute phase response [7]. Glucocorticosteroids also play a role in downregulation of the acute phase response, by stimulating hepatocyte interleukin-6 and the downregulation of cytokine production by monocytes and macrophages [7]. Among the degradation of other proteins involved in the acute phase response, SAA is also catabolized by the liver independently of other HDL proteins [20] [26] [108] [109]. The liver's capacity to clear apoprotein SAA from circulation is high; however, during an acute phase reaction hepatic catabolism of apoprotein SAA is reported to be decreased by 14% [26]. This accounts for the peak SAA values seen in the acute phase response. The reported apoprotein SAA half-life is short, which accounts for the rapid decrease in measurable SAA values with resolution of the acute phase response [26].

In 1986 equine SAA was first isolated, which was then quantified using the single radial immunodiffusion method in 1993 [13] [110]. Since then other methods have been described for the measurement of SAA in horses including an enzyme linked immunosorbent assay [111], slide reversed passive latex agglutination [112], single radial immunodiffusion [13], latex

agglutination immunoturbidimetric assay [51] [55] and electroimmunoassay [57] [113]. There are also three commercially-available stall-side point-of-care, lateral flow immunoassays for measurement of SAA in horses including the Stablelab SAA (Zoetis Inc, Kalamazoo MI, USA), Equicheck (Accuplex Dx Ltd, Maynooth, Ireland), and SAA test VMRD (Pullman, WA, USA) [99]. The point-of-care assays allow for the assessment of the inflammatory response in real time and timely treatment if necessary. Per manufacturer instructions, SAA can be measured using whole blood, serum or plasma (EDTA or heparin) [114]. There are accepted normal values that differ between adult horses at less than 20 µg/L and foals at less than 50 µg/L [2] [51].

Another acute phase protein in horses is fibrinogen. In horses, fibrinogen is a moderate acute phase protein, with its concentrations increasing 1 to 10 times greater in response to an inciting stimulus over time period of 24 to 72 hours [5]. Hepatocyte synthesis of fibrinogen is stimulated by interleukin-6 and inhibited by interleukin-1, TNF- α and - β [5] [115]. Fibrinogen has functional roles in both the immune system and coagulation cascade. Immune functions of fibrinogen, include the binding of phagocyte integrins (CD11/CD18), initiating intracellular signals to promote the adaptive immune response, as well as providing a matrix for migration of inflammatory related cells [5] [116]. Fibrinogen also plays a role in tissue repair, with its involvement in the formation and organization of the early wound matrix, as a substrate for fibrin formation [5] [116]. The measurement of fibrinogen levels can be performed through several methods, including the heat precipitation method, Ratnoff Menzie assay, and the clot weight method [5]. In horses the normal fibrinogen reference range is 200-400 mg/dL (2-4 g/L). Alterations in fibrinogen can be seen with the following equine inflammatory conditions: systemic inflammation [117] [118], induced noninfectious arthritis [119], inflammatory colic and equine grass sickness [79], *Actinobacillus equuli* peritonitis [120], foal infectious diseases [54],

Rhodococcus equi pneumonia [121] [122], *Lawsonia intracellularis* infection [123], liver disease [124], vaccination [100], colic [68] [125], disseminated intravascular coagulation [125], as well as many other diseases not mentioned. Fibrinogen will also increase in various routine and emergency surgical procedures, post-operatively [104] [105] [106] [126] [127] [128] [129]. As an inflammatory biomarker, fibrinogen's delayed response time to an inciting stimulus and its delay in resolution with inflammation, decreases its clinical utility as a marker of acute inflammation. Fibrinogen concentrations can also be falsely decreased in consumptive coagulopathies or diseases with increased vascular permeability [130]. However, fibrinogen concentrations continued to be measured and utilized clinically due to its inexpensive nature and the ability to be measured in a hospital setting.

Haptoglobin is another moderate acute phase protein in horses. It is an α -2 globulin that is considered a moderate acute phase protein that increases 1 to 10 times fold [5] [6] [131]. Like other acute phase proteins, haptoglobin is synthesized in the liver in response to interleukin-1, TNF- α , interleukin-6, leukemia inhibitory factor and glucocorticoids [131]. The bacteriostatic effect of haptoglobin is its primary immunologic function by restricting the available iron necessary for bacterial growth [5] [6] [132]. Haptoglobin also complexes with free hemoglobin in blood, preventing the external leak or loss of iron [5]. This function is thought to reduce the oxidative tissue damage caused by free hemoglobin, as well as reduce the renal filtration of free hemoglobin [5] [6] [131]. These haptoglobin- hemoglobin complexes are transported to the liver via the reticuloendothelial system, where the complexes are metabolized by Kupffer cells [6] thus, allowing the reutilization/recycling of iron and amino acids [5]. The functions of haptoglobin that have been described include: binding of CD11b/CD18 integrins, inhibiting neutrophil respiratory burst activity, granulocyte chemotaxis and phagocytosis, cathepsin B22

expression, viral hemagglutination and prostaglandin H synthase expression, suppression of lectin- and lipopolysaccharide-induced proliferation of lymphocytes and B-cell mitogenesis, modulation of macrophage function, angiogenesis, prostaglandin E expression in isolated osteoblasts and synergistic induction of bradykinin and thrombin [5] [6] [7] [131] [132]. In horses haptoglobin concentrations have been investigated in the following conditions: cutaneous habronemiasis [86], colic [133], inflammatory airway disease [63] [65], systemic inflammatory response syndrome [134] [135], surgical procedures [135] [136], induced noninfectious arthritis [119], grass sickness [78] [137], experimentally induced laminitis [138], pregnant mares and parturition [138], etc. It is also important to note that hemolytic conditions result in decreased haptoglobin concentrations [5] [6] [131]. The changes in haptoglobin levels are measurable in many bodily fluids using a variety of techniques: radial immunodiffusion, serum protein electrophoresis, hemoglobin-haptoglobin binding capacity assay, immunoturbidimetry and capillary zone electrophoresis [5] [140]. The normal haptoglobin reference interval in horses ranges between 2 to 10 g/L [5]. In comparison to other inflammatory biomarkers in horses, the need for specialized equipment has restricted the measurement of haptoglobin to a diagnostic laboratory, limited its use in a clinical setting and has confined its use to a research setting.

There are other positive acute phase proteins described in horses that are not discussed in this review, which include α -1 glycoprotein [5] [141] [142] [143], α -2 glycoprotein [78], ceruloplasmin [7] [25] [78] [132] [144] [145] [146] [147] [148] and C-reactive protein [7] [20] [63] [143] [149] [150].

The clinical application of acute phase proteins is important for the diagnosis and prognosis of many equine diseases. Attributes of major acute phase proteins are a testament to their utility including the following: a short lag time from inflammatory onset to measurable

changes in peripheral blood; a short half-life, causing a rapid decrease in plasma concentrations following resolution of inflammation; and low plasma concentrations in healthy animals [130]. These characteristics make the major acute phase protein SAA advantageous over other minor acute phase proteins that remain increased for several days after an anti-inflammatory stimulus [130]. The course of SAA production in equine inflammatory diseases as previously referenced, are clinically useful by providing insight into the magnitude and duration of acute phase response. In a clinical setting, this assists clinicians in disease diagnosis, in determining the length of medical treatment, as well as in providing a prognosis. Serum amyloid A's usefulness extends past the clinical setting to regulatory medicine, such as shown by Oertly, et al (2021) in determining the health status with importation of sport horses [151]. The use of the above described SAA point-of-care assays, provide another advantage of SAA as an inflammatory biomarker over other proteins discussed, such as fibrinogen or haptoglobin.

Chapter 2 - Research

There are many strategies for disease control, including isolation, limit shedding of pathogens, limit contact of infected and susceptible animals, hygienic practices, as well as vaccination. The administration of vaccination has a wide range of goals, from amelioration of infectious disease to prevention. In equine medicine there is limited evidence for the efficacy of vaccination, with administering veterinarians relying on the registration claim of the product and the safety and efficacy requirements for vaccine registration [152] [153]. The difficulty in performing vaccine trials, especially randomized clinical field trials, is due to the necessary sample size, inability to blind researchers or inability to generalize the results to the target population [152]. The evidence provided for equine vaccination relies on experimental challenge studies and is based on field outbreak investigation [152]. Vaccination results in priming of the adaptive immune response [154]. T helper cells, lead to activation of cytotoxic T cells, among other inflammatory cells, as well as antibody expression from B-lymphocytes [56] [152]. The part of the immune system primed by vaccination depends on the adjuvant, type of antigen and route of immunization [152]. There are two broad categories of vaccines: killed vaccines, which are inactivated using heat, chemicals or irradiation and live vaccines, which contain a pathogen with attenuated pathogenicity [152]. The subcategories of killed vaccines are inactivated whole pathogen, protein subunit and recombinant subunit vaccines. The live vaccine category includes modified- live vaccines. The efficacy of killed vaccines depends on the adjuvant, which are chemicals, microbial components or mammalian proteins that enhance the immune response [154].

Vaccination has been used to investigate the acute phase response in many species, including humans [155] [156]. In horses, vaccination has been shown to elicit a strong acute

phase response, with elevations seen in SAA. Andersen, et al (2012) described the acute phase response (SAA, fibrinogen concentrations and white blood cell count) in 10 horses by comparing two vaccines: an inactivated Immune Stimulating CoMplex (ISCOM) vaccine, containing three influenza virus strains and tetanus toxoid and a live recombinant vector vaccine, containing two influenza virus strains and tetanus toxoid at 9, 24, 48, 72 and 96 hours after vaccination [100]. Serum amyloid A concentrations were significantly increased at 24 hours, and remained elevated for the duration of the study. There was variation observed in SAA response between the two vaccines, as well as in individual acute phase response within the two vaccination groups observed [100]. In comparison to other acute phase proteins, white blood cell count was the fastest to respond, with levels changing after 9 hours; and iron, SAA and fibrinogen were significantly changed by 24 hours [100]. The ISCOM vaccine resulted in a higher SAA and fibrinogen concentrations than the vector vaccine. The author's concluded the differences were due to the ISCOM adjuvant, which is highly immunogenic and could account for the more intense acute phase response [100]. The timing of SAA concentrations to normal, pre-vaccination levels did not occur within the 4 day study period [100]. Another study investigating SAA post-vaccination in healthy adult horses was performed by Duran, et al (2020), who described peak SAA concentrations on day 1 after vaccination (1,365.9 +/- 141.4 mg/L for horses) with an inactivated EHV-1/-4 vaccine [157]. However, SAA elevation was not correlated with the intensity of the antibody response [157]. The timing of SAA concentrations to normal, pre-vaccination levels, was not reported by Duran, et al (2020) within the 21 day study period [158]. Another study performed by Skipper and Pusterla (2021), investigated SAA post-vaccination in relation to WNV antibody titer [157]. The vaccine used was Vetera EWT and WNV vaccine containing antigens: eastern and western encephalomyelitis, west Nile virus

(WNV) and tetanus vaccine [158]. Skipper and Pusterla (2021) report elevated SAA concentrations 72 hours post-vaccination (median 554 micgog/ml, IQR 113-784 $\mu\text{g/ml}$) [158]. There was a significant difference in median peak SAA concentration was higher in horses that developed a vaccine reaction compared to those who did not [158]. This is a difference from other studies post-vaccination that are previously referenced that did not report an increased SAA with adverse vaccine site reactions. There was no significant association between peak SAA concentration and fold change in WNV antibody titer [158]. There was no reported timing of when SAA concentrations returned to normal post-vaccination. In summary of studies evaluating SAA response post-vaccination in healthy adult horses, peak SAA concentrations are seen 1 to 3 days post-vaccination, in which vaccine site reactions may influence the level in SAA response post-vaccination. There are no reports of when SAA concentrations return to normal post-vaccination. Serum amyloid A response post-vaccination is not associated with rise in antibody titers.

The American Association of Equine Practitioners (AAEP) Vaccination Guidelines established for healthy equine adults recommend vaccination against core diseases that can cause fatal impact including rabies, tetanus, West Nile, as well as Eastern and Western equine encephalomyelitis. The AAEP vaccination guidelines for healthy equine adults has recommendations for vaccines that are risk based; the most common of which include equine rhinopneumonitis (equine herpesvirus-1 & -4) and influenza.

The objective of the study was to measure the effect of routine vaccination (rabies, tetanus, West Nile (WNV), Eastern and Western equine encephalomyelitis (EEE/WEE), equine herpesvirus-1/-4 (EHV-1/-4), and influenza on SAA concentrations using the Stablelab EQ-1 Handheld Reader in healthy horses. Our hypothesis was that SAA will increase after

administration of routine vaccines in vaccinated horses compared to unvaccinated, control horses.

Materials and Methods

Animals:

Apparently healthy horses from client-owned horses (n=21), as well as the Kansas State University College of Veterinary Medicine teaching horses (n=15), were used for this study. Informed client consent was obtained for client-owned horses. Eight horses were included in experiment 1 (6 Quarter horses, 1 Warmblood and 1 draft), with a mean age of 14 years (age range 2-27 years) and sex distribution of 5 mares, 3 geldings and 0 stallions. Twenty-eight horses were included in experiment 2 (16 Quarter horses, 5 Warmbloods, 1 half-Arabian, 1 Arabian, 2 Paint horses, 1 Thoroughbred and 1 draft), with a mean age of 17 years (age range 4 to 27 years) and sex distribution of 16 mares, 11 geldings and 1 stallion.

Horses were managed following routine management of the teaching herd horses at Kansas State University College of Veterinary Medicine and routine management of client-owned horses. The owner was expected to not transport the horse or perform strenuous exercise with the horse, report administration of any anti-inflammatories or antibiotics, and report any concerns during the 10 day study period.

Ethical Approval:

Approval for this project was granted by the Kansas State University Institutional Animal Care and Use Committee (Protocol number: 4547; Modification number: 4547.1).

Inclusion Criteria:

Horses were included with known vaccine history. Horses were considered healthy based on physical exam and interpretation of baseline bloodwork (complete blood count, chemistry,

fibrinogen, and SAA) performed by the veterinary investigators (CMB, CB, KMD) 0 to 10 days prior to vaccine or control administration. Normal reference range for SAA was considered $<20\mu\text{g/ml}$, based on in-laboratory-derived reference intervals [51]. Comorbidities with no known effect on SAA, as previously stated were included in the study, such as navicular disease, laminitis, or ophthalmic disorders, sinus disorders. There were 8 horses included in experiment 1 and 28 horses included in experiment 2.

Exclusion Criteria:

Horses excluded from the study included if they are determined “unhealthy” determined by bloodwork (complete blood count, chemistry and SAA) and physical exam findings. Foals, pregnant mares, miniature horses, donkeys and mules were excluded as these signalments influence SAA concentrations [113] [157] [159]. Horses were eliminated from the study if they experienced a known cause of increase to SAA as described previously, such as were transported or exercised within the study period or required medical intervention during the study period, such as due to systemic illness or wound (laceration), required the administration of anti-inflammatories or antimicrobials, developed an active episode of equine asthma, or experienced a diet change as these conditions influence SAA values. Due to abnormal SAA values at time point 0, there were 2 horses eliminated from experiment 1 and 3 horses eliminated from experiment 2.

Study Design:

Experiment 1

Experiment 1 (n=8) was a blinded, randomized, prospective, cross-over study. Horses were randomly assigned to vaccinated or unvaccinated, control groups by a computer random number generator. Horses received injection of vaccine or placebo. A whole blood sample (3

mls) was collected via jugular venipuncture using a 20 gauge vacutainer needle, and the horses were evaluated at each time point after injection (0, 6, 12, and 24 hours, and 2, 3, 4, 5, 6, 7, 8, 9, and 10 days). After the initial study period, there was a minimum 2-week interval between studies and then the study was repeated with horses receiving vaccine/placebo. The clinicians evaluating the horses post-injection (vaccine/placebo) were blinded to the treatment each horse received.

Data recorded at each time point included: physical exams findings (mentation, temperature, heart rate, respiratory rate, mucous membranes, capillary refill time, gastrointestinal sounds, digital pulses), vaccine site reactions (abscess, swelling, pain to palpation, stiff movement or any other abnormal activity/behaviors. Signalment (age, breed, sex) was also recorded. Rectal temperature was collected using a digital thermometer.

Experiment 2

In Experiment 2 (n=28), a prospective, observational study was performed, in which horses were routinely vaccinated. A whole blood sample (3 mls) was collected via jugular venipuncture using a 20 gauge vacutainer needle, and the horses were evaluated at each time point (0, 12, and 24 hours, and 2, 3, 4, 5, 6, 7, 8, 9, and 10 days). Data recorded at each time point was the same as experiment 1.

Vaccination/Placebo:

Horses in the study received a single dose (1 ml intramuscular, left neck) of Vetera 2XP, an equine rhinopneumonitis-influenza vaccine containing influenza (Clade 1 and Clade 2), Herpes (EHV-1 and EHV-4) killed virus and the CARBIMMUNE adjuvant (Boehringer Ingelheim Vetmedica Inc., St. Joseph MO 64506, USA); and a single dose (1 ml intramuscular, right neck) of CORE EQ INNOVATOR vaccine containing rabies, West Nile, Eastern and

Western equine encephalomyelitis killed virus, tetanus toxoid and the MetaStim adjuvant (Zoetis, Kalamazoo, MI 49007, USA). These vaccinations were administered per AAEP guidelines for core and common risk-based vaccines as a component of horse's routine preventative care management and according to veterinarian recommendations. As part of experiment 1, horses received 0.9% sodium chloride injection (1ml intramuscular, right and left neck) as a placebo.

Laboratory analysis:

Both experiments involved collection of a whole blood sample (3 mls) at time 0, 6*, 12, and 24 hours, and then 2, 3, 4, 5, 6, 7, 8, 9, and 10 days. Serum amyloid A was analyzed using the point-of-care lateral flow membrane-based immunoassay, Stablelab EQ-1 Handheld Reader (EPONA biotech Limited, Sligo, Ireland; Zoetis) by trained personnel per manufacturer instructions and within 12 hours of sample collection. This assay has been previously validated [160]. For each horse, the sample of EDTA-anti-coagulated blood underwent a complete blood count via an automated analyzer performed by Kansas State Veterinary Diagnostic Laboratory (KSVDL) and a slide exam, performed by the clinical pathology laboratory at KSVDL, on days 0, 4, and 7 post-injection. Fibrinogen was measured using the heat precipitation method. Blood was analyzed within 24 to 48 hours of collection and refrigerated as needed.

*After completion of experiment 1, no increase in SAA was observed 6 hours post-vaccination and this collection time was omitted from experiment 2.

Statistical Analysis

The changes in SAA, white blood cell count, fibrinogen and temperature were described in relation to time post-vaccination (experiment 1 and experiment 2). All statistical analyses were conducted in RStudio (RStudio, Version 4.0.3, Boston, MA). A linear mixed effect model with

repeated measures over time blocked by horse was used to test the effect of treatment on SAA post-vaccination (experiment 1). A repeated measures correlation was used to test the correlation between SAA and body temperature post-vaccination (experiment 2).

Results

Serum amyloid A:

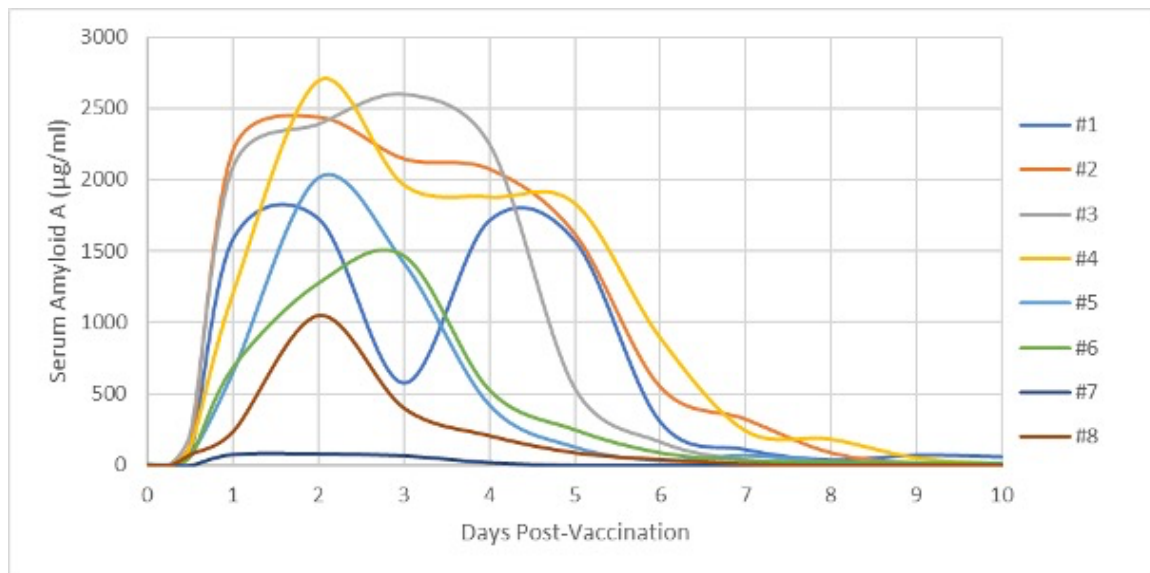


Figure 1. Experiment 1 SAA (µg/ml) post-vaccination (treatment: n=8)

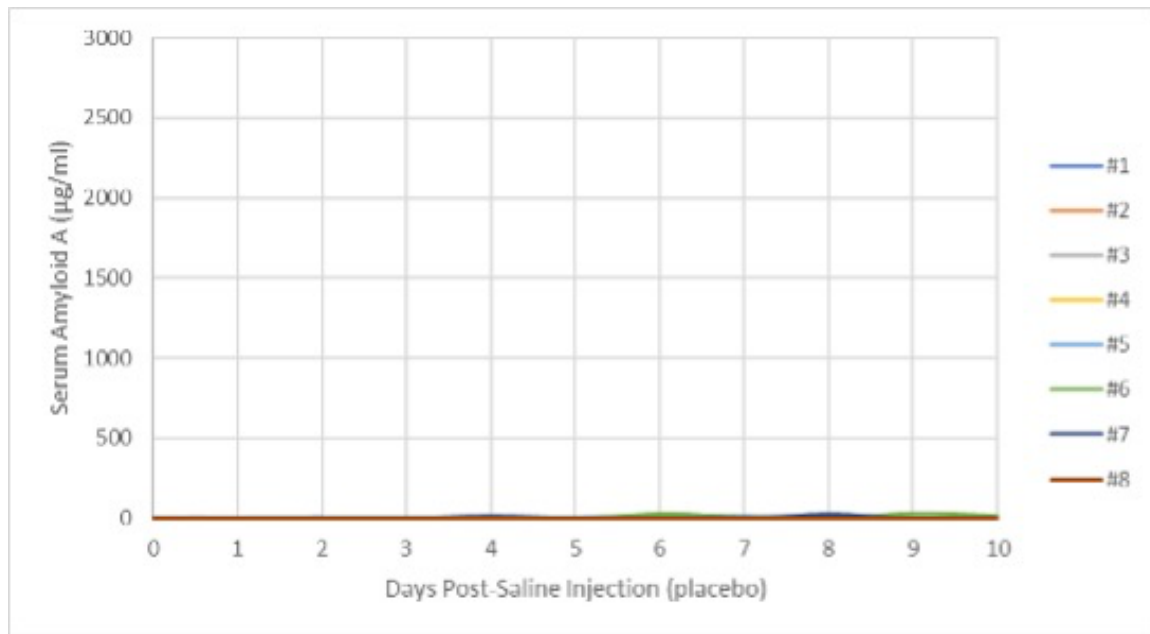


Figure 2. Experiment 1 SAA (µg/ml) post-saline injection (placebo- control: n=8)

Over time, vaccinated horses had increased SAA (Figure 1) compared to unvaccinated horses (Figure 2). Initial elevation in SAA post-vaccination was observed by 12 hours (0.5 day) in 87.5% of horses (7/8) (Figure 1). Post-vaccination, SAA peaked between day 2 to 3 (SAA: 1726 to 2698 µg/ml) (Figure 1). Timing of return to normal SAA to normal post-vaccination (within reference range, <20 ug/ml) was variable among horses (day 4 to day 10). One out of 8 horses 1/8 horses, SAA returned to normal on day 4 post-vaccination, 1/8 horses, SAA returned to normal on day 7 post-vaccination, 2/8 returned to normal on day 8 post-vaccination, 2/8 horses returned to normal on day 9 post-vaccination, 1/8 horses returned to normal on day 10 post-vaccination and 1/8 horses did not return to normal within the 10 day sampling period (Figure 1). In 87.5% (7/8) horses, SAA returned to normal (within reference range, <20 µg/ml) by day 10 (Figure 1). There was variation observed between horses: monophasic and biphasic trends in SAA response post vaccination, as well as, degree of SAA elevation and return to normal post-vaccination. Over time, horses administered placebo (post-saline injection) had no

change in SAA associated with placebo (post-saline injection) (Figure 2). Increases in SAA were observed sporadically in 37.5% of horses (3/8) administered placebo (Figure 2, Table 15).

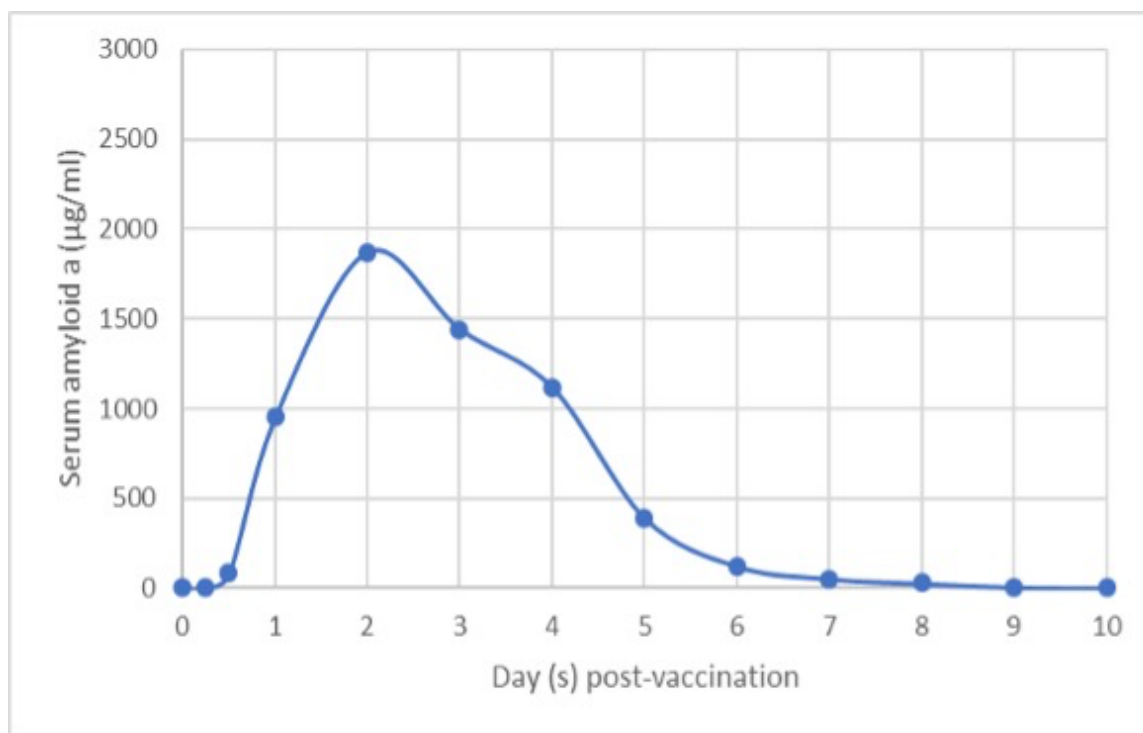


Figure 3. Experiment 1, model-adjusted median SAA (µg/ml) post-vaccination (treatment: n=8)

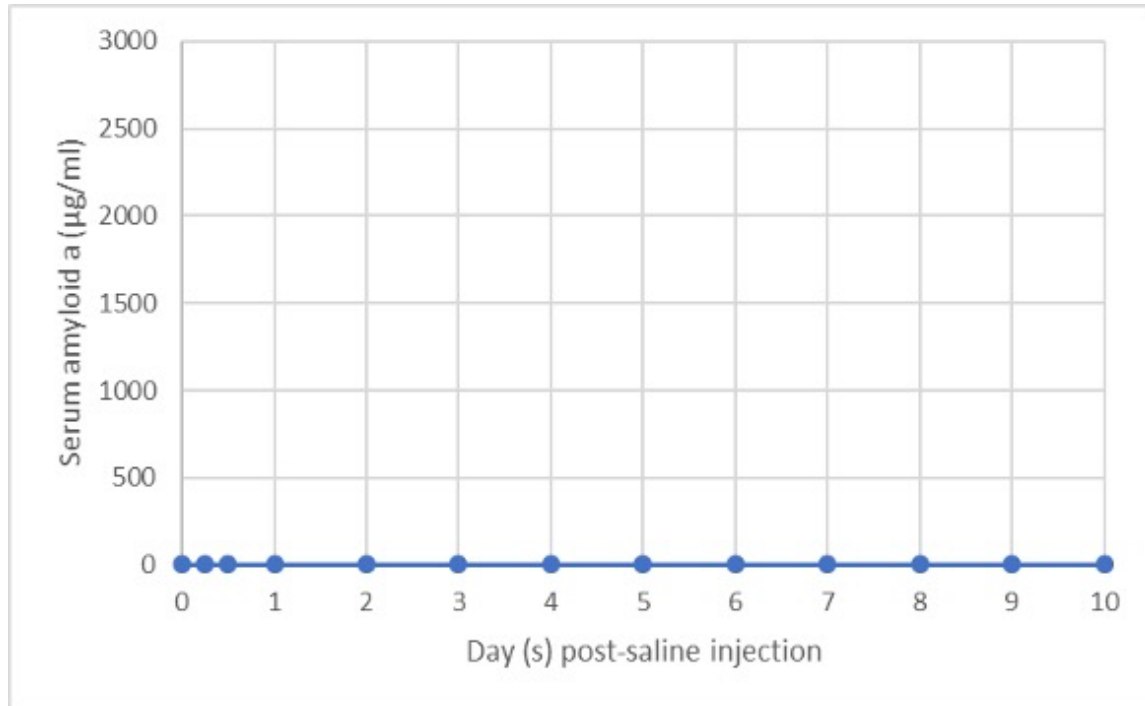


Figure 4. Experiment 1, model-adjusted median SAA (µg/ml) post-saline injection (placebo-control: n=8)

Over time, vaccinated horses had increased model-adjusted SAA (Figure 1) compared to unvaccinated horses (Figure 3). Median SAA post-vaccination peaked on day 2 (median: 1872 µg/ml, IQR: 0- 2402.5 µg/ml). Median SAA post-vaccination normalized by day 9 (median 6 µg/ml, IQR: 0-23.5 µg/ml) (Figure 3). There was no change in median SAA after placebo administration (Figure 4).

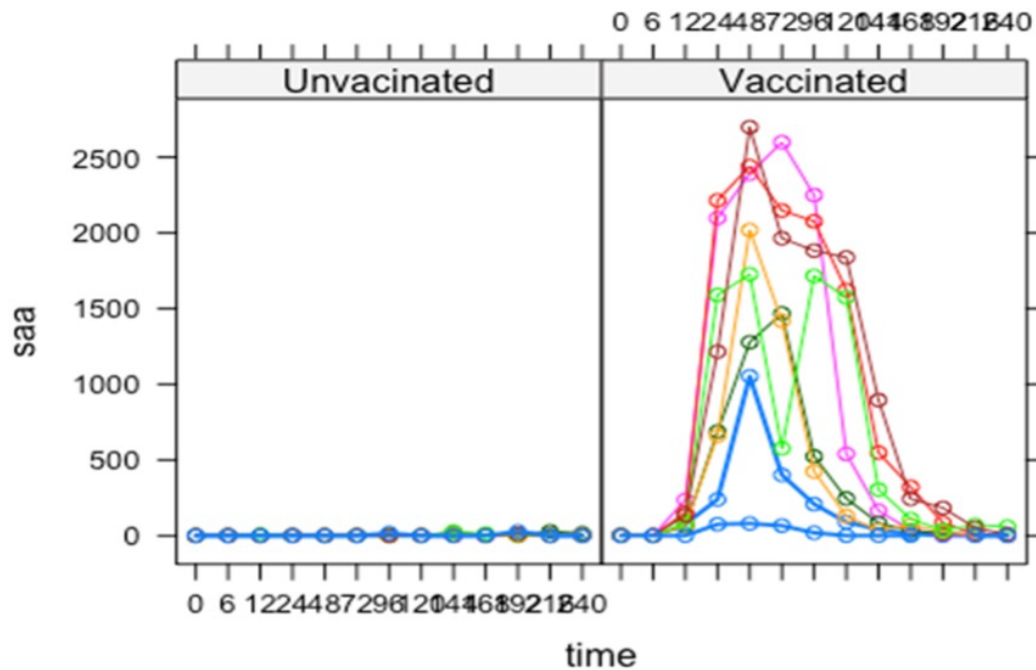


Figure 5. Experiment 1, Linear mixed effect model showing comparison of SAA (µg/ml) in vaccinated and unvaccinated horses (n=8)

The linear mixed effect model using repeated measured over time blocked by horse is shown in Figure 5. Over time, vaccinated horses had increased SAA compared to unvaccinated horses.

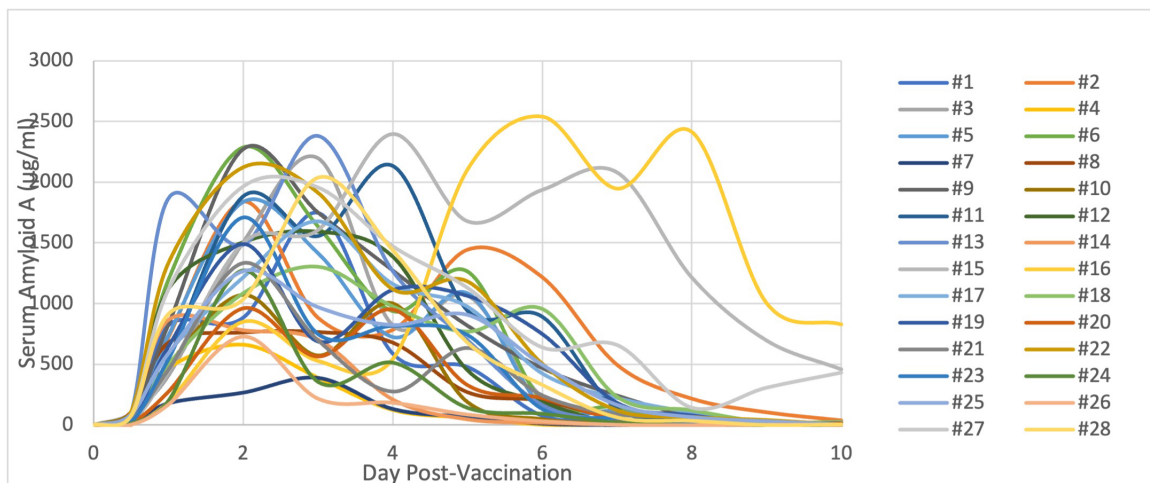


Figure 6. Experiment 2, SAA (µg/ml) post-vaccination (n=28)

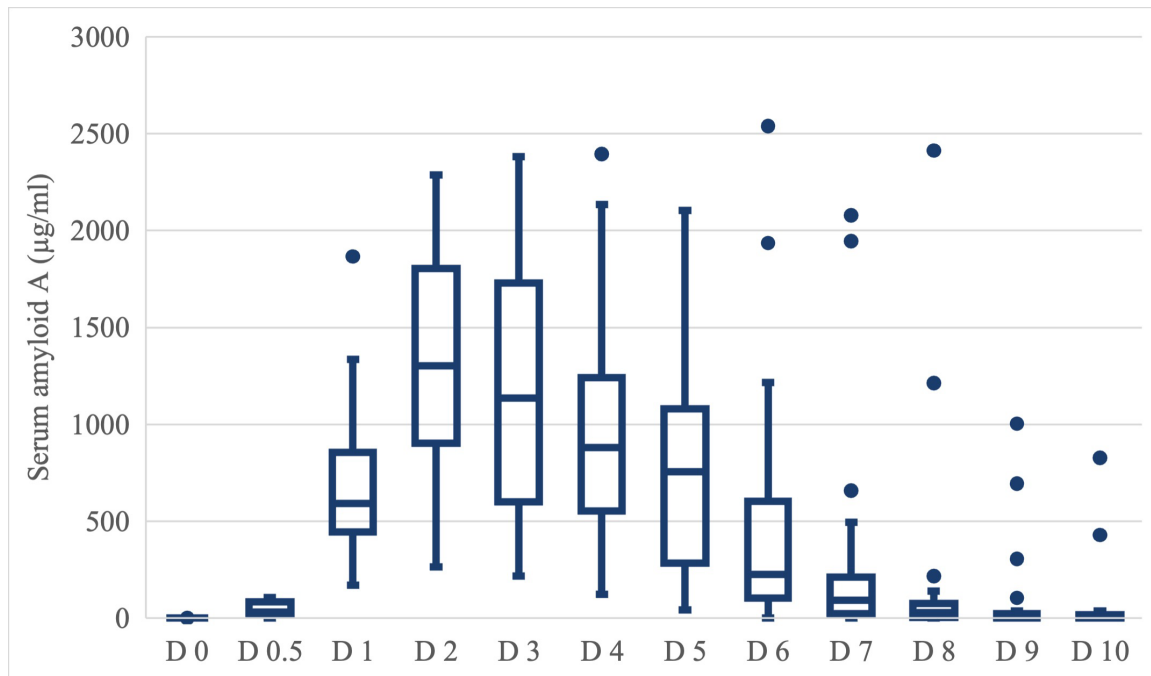


Figure 7. Experiment 2, box and whisker plot of SAA (µg/ml) concentrations post-vaccination (n=28)

Over time, vaccinated horses had increased SAA (Figure 6). Variation observed between horses: mono-phasic elevation in SAA occurred in 57.1% (16/28) of horses and bi-phasic elevation in SAA occurred in 42.9% (12/28) of horses (Figure 6). Initial elevation (>20 µg/ml) in SAA post vaccination was observed by 12 hours (0.5 day) in 71.4% (20/28) of horses and by 24 hours (1 day) in 28.6% (8/28) of horses (Figure 6). Serum amyloid A peaked between day 2 and 6, with a variable degree in peak SAA elevation (SAA: 387-2539 µg/ml): 3.6% (1/28) horses had peak SAA day 1 post-vaccination, 50% (14/28) horses had peak SAA day 2 post-vaccination, 32.1% (9/28) horses had peak SAA day 3 post-vaccination, 3/28 horses had peak SAA day 4 post-vaccination, 3.6% (1/28) horses had peak SAA day 6 post-vaccination (Table 4. The day post-vaccination when SAA returned to normal (within reference range, <20 µ/ml) was variable between horses (between day 6 to day 10): 3/28 horses returned to normal by day 6 post vaccination, 3/28 horses returned to normal by day 7 post-vaccination, 4/28 horses returned to normal by day 8 post-vaccination, 11/28 horses returned to normal by day 9 post-vaccination,

2/28 horses returned to normal by day 10 post-vaccination (Table 4). Serum amyloid A returned to normal (within reference range, $<20\text{ }\mu\text{g/ml}$) by day 10 in 78.5% (22/28) of horses (Table 4). Serum amyloid A did not return to normal (within reference range, $<20\text{ }\mu\text{g/ml}$) within the 10 day sampling period in 17.9% (5/28) of horses (Table 4).

	Day (s) Post-Vaccination											
Horse	0	0.5	1	2	3	4	5	6	7	8	9	10
#1	0	31	820	884	1748	587	477	75	31	2	0	17
#2	0	0	722	1839	883	783	1443	1217	496	217	105	38
#3	0	92	407	1505	2198	820	1067	229	99	40	7	1
#4	0	11	467	660	388	122	57	1	0	0	0	0
#5	0	24	738	1836	1414	724	1084	156	115	22	15	0
#6	0	82	1198	2287	1636	938	1265	204	149	24	13	18
#7	0	10	179	266	387	132	67	12	0	0	0	0
#8	0	107	681	764	767	680	271	202	53	4	4	0
#9	0	80	828	2271	1749	1272	817	468	242	73	11	0
#10	0	91	501	1071	571	1000	187	46	7	3	0	1
#11	0	32	543	1883	1555	2134	940	890	131	66	17	3
#12	0	89	1120	1503	1590	1385	450	187	11	4	0	0
#13	2	101	1867	1484	2381	1267	698	164	38	25	0	5
#14	0	80	866	780	702	208	44	17	0	0	5	0
#15	0	25	442	1500	1605	2396	1677	1936	2080	1214	695	457
#16	0	2	191	850	527	542	2103	2539	1946	2413	1004	828
#17	0	13	593	1217	1678	1162	975	425	224	104	18	16
#18	0	29	503	1084	1302	976	769	955	233	122	25	27
#19	0	24	646	1489	686	1111	1062	748	181	78	13	14
#20	0	19	274	960	563	950	327	221	48	22	6	11
#21	0	46	455	1333	697	275	634	243	88	67	8	0
#22	0	107	1335	2124	1914	1118	1182	503	119	62	38	0
#23	0	24	542	1710	748	815	741	130	53	5	19	0
#24	0	14	201	1273	356	514	142	94	21	0	0	0
#25	0	86	591	1268	971	824	908	496	165	64	30	4
#26	3	4	170	729	218	184	87	33	3	0	3	2
#27	0	77	1118	1965	1957	1472	1129	638	658	141	306	430
#28	0	76	915	1035	2037	1440	678	331	63	35	0	6

Table 4. Experiment 2, SAA post-vaccination (n=28). Orange= initial increase in SAA (>20 µg/ml), red= peak SAA concentrations, green= return to normal (<20 µg/ml), blue = SAA concentrations did not normalize within sampling period

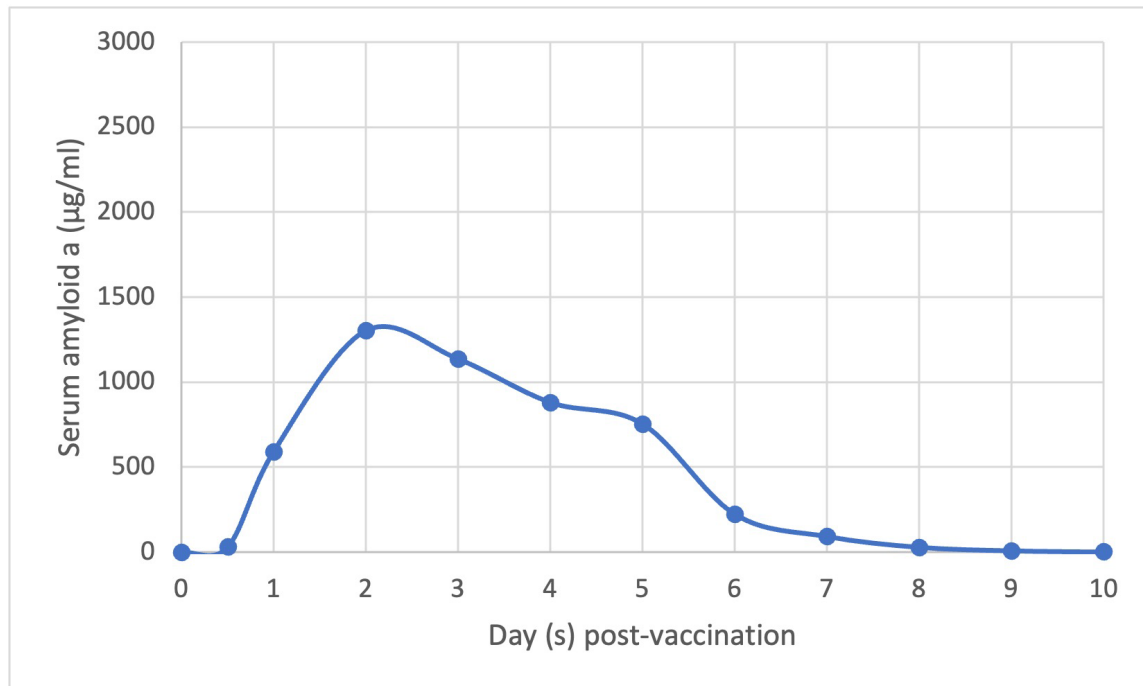


Figure 8. Experiment 2, model-adjusted median SAA (µg/ml) post-vaccination (n=28)

Over time, vaccinated horses had increased model-adjusted SAA Figure 8Median SAA post-vaccination peaked on day 2 (median: 1303 µg/ml, IQR: 941- 1741.5 µg/ml) (Figure 8). Median SAA post-vaccination normalized by day 9 (median 9.5 µg/ml, IQR: 0-20.5 µg/ml) (Figure 8Table 5).

Day post vaccination	Minimum	Quartile 1	Median SAA	Quartile 3	Maximum	Interquartile Range
0	0	0	0	0	3	0
0.5	0	17.75	31.5	83	107	65.25
1	170	451.75	592	837.5	1867	385.75
2	266	941	1303	1741.5	2287	800.5
3	218	657.25	1136.5	1695.5	2381	1038.25
4	122	575.75	881	1188.3	2396	612.5
5	44	313	755	1071.3	2103	758.25
6	1	121	225	536.75	2539	415.75
7	0	28.5	93.5	191.75	2080	163.25
8	0	3.75	30	74.25	2413	70.5
9	0	0	9.5	20.5	1004	20.5
10	0	0	2.5	16.25	828	16.25

Table 5. Experiment 2, calculated statistics reported for SAA ($\mu\text{g/ml}$) post-vaccination: minimum, interquartile 1, median interquartile 3, and maximum

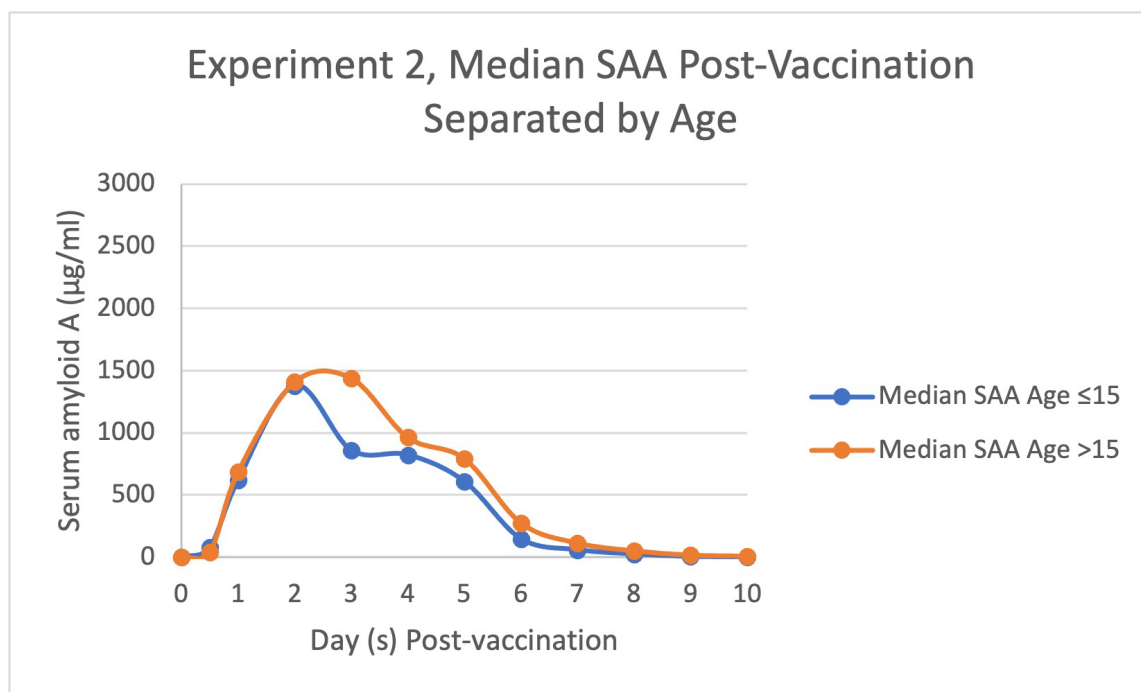


Figure 9. Experiment 2, model-adjusted median SAA ($\mu\text{g/ml}$) post-vaccination (n=28)

There was no difference in trend in model-adjusted median SAA post-vaccination between horses that were distinguished by age less than or equal to 15 years old and greater than 15 years old (Table 6Figure 9. Experiment 2, model-adjusted median SAA ($\mu\text{g/ml}$) post-vaccination (n=28)).

Day Post-Vaccination	Median SAA Age ≤15 years old	Median SAA Age >15 years old
0	0	0
0.5	78	39
1	618.5	684
2	1378.5	1408.5
3	859.5	1440.5
4	822	963
5	609	793
6	147	273.5
7	58	112
8	24	49.5
9	4	14.5
10	0.5	7.5

Table 6. Experiment 2, median SAA (µg/ml) post-vaccination separated by horses age ≤ 15 years old and >15 years old (n=28)

White blood cell count:

Day (s) Post-placebo	Minimum	Quartile 1	Median	Quartile 3	Maximum	Interquartile Range
1	6	6.525	7.4	8.575	9.6	2.05
4	4.7	5.65	6.2	8.4	9.3	2.75
7	5.2	5.9	6.1	7.45	10	1.55

Table 7. Experiment 1, calculated statistics reported for white blood cell count (K/uL), collected days 1, 4 and 7 post-vaccination: minimum, interquartile 1, median, interquartile 3, and maximum

Day (s) Post-placebo	Minimum	Quartile 1	Median	Quartile 3	Maximum	Interquartile Range
1	6	6.525	7.4	8.575	9.6	2.05
4	4.7	6.65	6.2	8.4	9.3	2.75
7	5.2	5.9	6.1	7.45	10	1.55

Table 8. Experiment 1, calculated statistics reported for white blood cell count (K/uL) collected days 1, 4 and 7 post-placebo (saline) administration: minimum, interquartile 1, median, interquartile 3, and maximum

In experiment 1, white blood cell count showed no trend post vaccination Table 7 or post-placebo (saline) administration (Table 8).

Day (s) Post-Vaccination	Minimum	Quartile 1	Median	Quartile 3	Maximum	Interquartile Range
1	4.2	6.15	6.65	7.55	11.6	1.4
4	4	5.85	6.9	7.55	11.1	1.7
7	4.4	6.35	7.3	8.625	10.8	2.275

Table 9. Experiment 2, calculated statistics reported for white blood cell count (K/uL) collected days 1, 4 and 7 post-vaccination: minimum, interquartile 1, median, interquartile 3, and maximum

In experiment 2, white blood cell count showed no trend post vaccination (Table 9).

Physical Exam Parameters

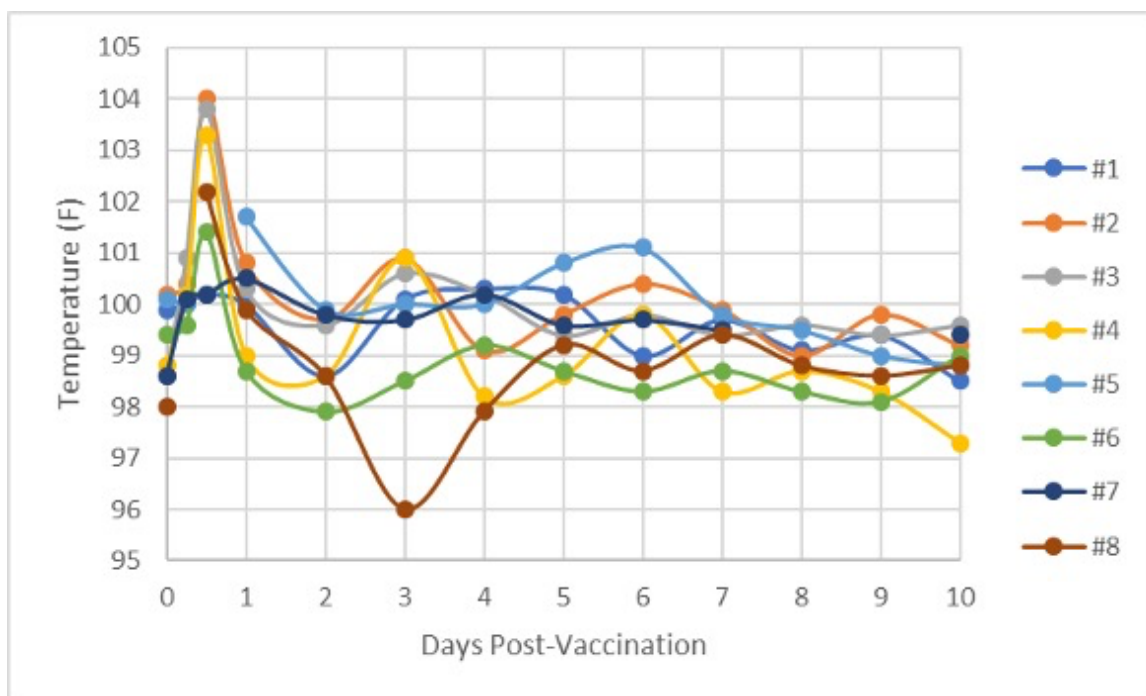


Figure 10. Experiment 1, Temperature (F) post-vaccination (n=8)

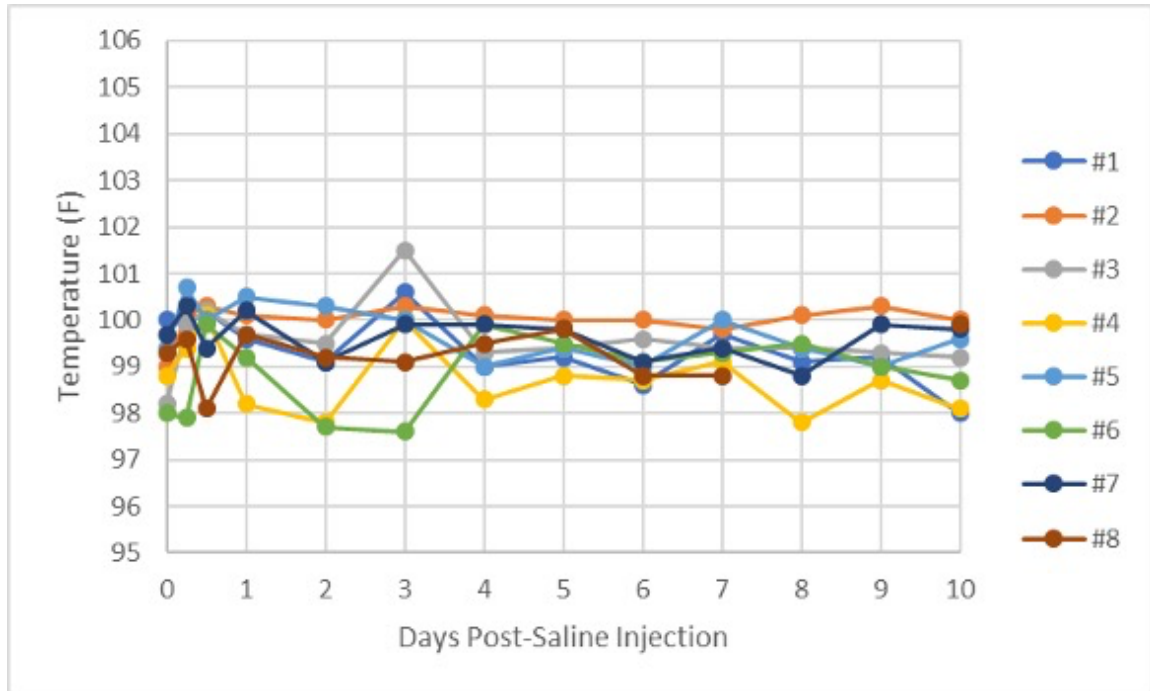


Figure 11. Experiment 1, Temperature (F) post-saline injection (n=8)

In experiment 1, temperature peaked (101.4-104F) at 12 hours (0.5 day) in 50% (4/8) of horse's post-vaccination (Figure 10. Experiment 1, Temperature (F) post-vaccination (n=8)). Temperature peaked (101.7F) at 24 hours (1 day) in 12.5% (1/8) of horse's post-vaccination (Figure 10. Experiment 1, Temperature (F) post-vaccination (n=8)). Temperature remained within normal limits (98.1F-100.9F) in 25% (2/8) of horse's post-vaccination (Figure 10. Experiment 1, Temperature (F) post-vaccination (n=8)). Temperature returned to within normal limits in all horse's, without intervention 12 to 24 hours post-elevation (Figure 10. Experiment 1, Temperature (F) post-vaccination (n=8)). Temperature remained within normal limits (rr: 98.1F-100.9F) post-saline administration (Figure 11. Experiment 1, Temperature (F) post-saline injection (n=8)).

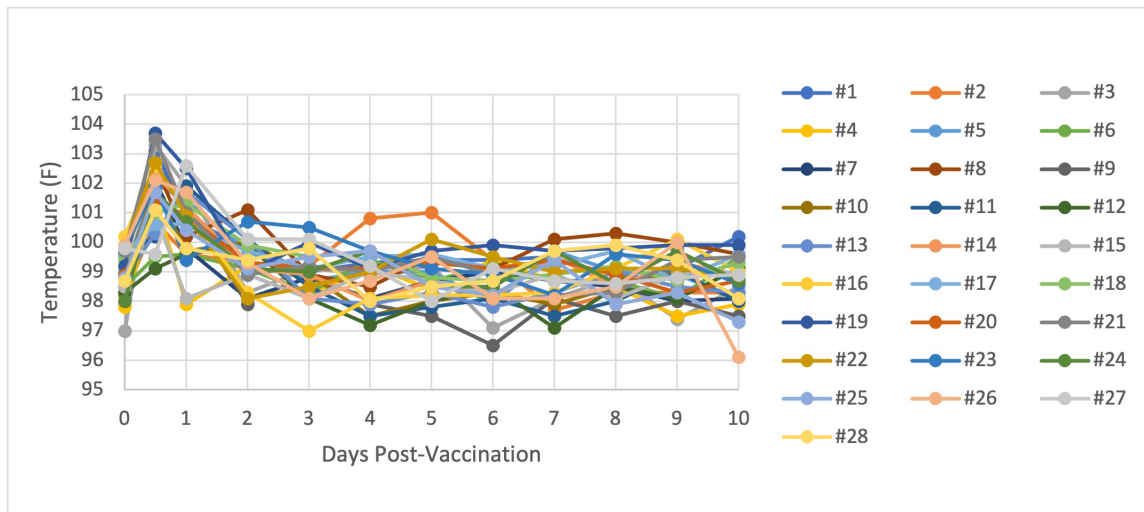


Figure 12. Experiment 2, Temperature (F) post-vaccination (n=28)

In experiment 2, based on repeated measured correlation body temperature and SAA concentrations were not correlated ($R^2=0.0009$; $P=0.78$). A fever (temperature greater than 101.2F) was observed in 78.5% (22/28) of horse's post-vaccination (Figure 12. Experiment 2, Temperature (F) post-vaccination (n=28), Table 16). Temperature peaked (101.1F- 103.7F) at 12 hours (0.5 day) in 71.4% (20/28) of horse's post-vaccination (Figure 12. Experiment 2, Temperature (F) post-vaccination (n=28), Table 16). Temperature peaked (101.1F- 102.6F) at 24 hours (1 day) in 14.3% (4/28) horse's post-vaccination (Figure 12. Experiment 2, Temperature (F) post-vaccination (n=28), Table 16). Temperature remained within normal limits in 14.3% (4/28) of horse's post-vaccination (Figure 12. Experiment 2, Temperature (F) post-vaccination (n=28), Table 16). Temperature returned to normal by day 1 in 42.9% (12/28) of horse's and by day 2 in 42.9% (12/28) of horse's post-vaccination (Figure 12. Experiment 2, Temperature (F) post-vaccination (n=28), Table 16).

No changes were appreciated in other physical exam parameters (mentation, heart rate, respiratory rate, mucous membranes, capillary refill time, gastrointestinal sounds, digital pulses).

Fibrinogen

Horse	Day 1	Day 4	Day 7
#1	600	300	400
#2	200	300	500
#3	500	400	500
#4	300	400	700
#5	300	300	400
#6	500	400	300
#7	200	100	400
#8	*	*	400
Minimum	200	100	300
Quartile 1	250	300	400
Median	300	300	400
Quartile 3	500	400	500
Maximum	500	700	700
Interquartile range	300	100	100

Table 10. Experiment 1, Fibrinogen (mg/dl) values collected days 1, 4 and 7 post-vaccination (n=8). Calculated statistics reported: minimum, quartile 1 (Q1), median, quartile 3 (Q3), maximum, and interquartile range 9IQR). *indicating areas of missing data

In experiment 1, fibrinogen showed no trend post vaccination (Table 10). Fibrinogen remained within normal limits in 37.5% (3/8) of horse's post-vaccination. Fibrinogen was elevated in 25% (2/8) of horse's on day 1, then normalized on day 7 in 12.5% (1/8) of horse's or remained the same on day 7 in 12.5% of horse's (1/8) post-vaccination (Table 10). Fibrinogen had no change from Day 1 to Day 7 12.5% (1/8) of horse's post-vaccination (Table 10). Insufficient data recorded for 1 horse (Table 10).

Horse	Day 1	Day 4	Day 7
#1	200	400	400
#2	300	300	300
#3	200	300	100
#4	300	200	200
#5	300	500	*
#6	400	200	200
#7	200	100	300
#8	200	300	300
Minimum	200	100	100
Q 1	200	200	200
Median	250	300	300
Q 3	300	325	300
Maximum	400	500	400
IQR	100	125	100

Table 11. Experiment 1, Fibrinogen (mg/dL) values collected days 1, 4 and 7 post-saline (placebo-control) injection n=8). Calculated statistics reported: minimum, quartile (Q1), median, quartile 3 (Q3), maximum, and interquartile range (IQR). *indicating areas of missing data

In experiment 1, fibrinogen showed no trend after placebo (post-saline injection) administration (Table 11). Fibrinogen remained within normal limits (0-400 mg/dL) in 87.5% (7/8) of horse's on days 1, 4 and 7 (Table 11). Fibrinogen increased from day 1 to day 7 in 37.5% (3/8) of horse's post-saline injection (Table 11). Fibrinogen decreased from day 1 to day 7 in 37.5% (3/8) of horse's post-saline injection (Table 11). Fibrinogen did not change from day 1 to day 7 12.5% (1/8) of horse's post-saline injection (Table 11). Insufficient data in 1/8 horse's (Table 11).

Horse	Day (s) Post-Vaccination		
	Day 1	Day 4	Day 7
#1	200	400	500
#2	200	400	300
#3	300	400	300
#4	200	300	200
#5	400	400	100
#6	200	200	200
#7	200	400	400
#8	100	400	200
#9	300	200	400
#10	200	400	400
#11	300	400	400
#12	300	400	300
#13	200	300	300
#14	300	*	200
#15	500	600	300
#16	400	500	400
#17	400	300	400
#18	200	400	400
#19	300	400	300
#20	400	400	300
#21	200	400	400
#22	400	200	300
#23	200	300	300
#24	200	300	400
#25	200	400	400
#26	300	400	200
#27	300	400	500
#28	200	400	300

Table 12. Experiment 2, Fibrinogen (mg/dL) values collected days 1, 4 and 7 post-vaccination (n=28). *indicating areas of missing data

In experiment 2, fibrinogen showed no trend post-vaccination within this population of horses (Table 12). Fibrinogen remained within normal limits (0-400 mg/dL) in 92.9% (26/28) of horse's post-vaccination (Table 12). Fibrinogen increased from day 1 to day 7 in 53.6% (15/28) of horse's post-vaccination (Table 12). Fibrinogen decreased from day 1 to day 7 in 21.4%

(6/28) of horse's post-vaccination (Table 12). Fibrinogen did not change from day 1 to day 7 in 25% (7/28) of horse's post-vaccination (Table 12)

Day post-vaccination	Minimum	Q1	Median	Q3	Maximum	IQR
Day 1	100	200	250	300	500	100
Day 4	200	300	400	400	600	100
Day 7	100	300	300	400	500	100

Table 13. Experiment 2, calculated statistics reported for fibrinogen (mg/dL) values collected days 1, 4 and 7 post-vaccination: minimum, interquartile 1, median, interquartile 3, and maximum

Discussion

Routine vaccination for core and common risk-based diseases induced a prominent inflammatory response leading to increases in SAA concentrations in healthy adult horses compared to horses receiving placebo. These results are consistent with other studies performed monitoring the acute phase response post-vaccination [100] [157] [158]. The trend observed in SAA concentrations post-vaccination were observed with an initial increase at 24 hours after vaccination, with peak concentrations observed at 48 hours to 72 hours post-vaccination [158] [100]. In other studies, not all horses SAA concentrations returned to normal within the study's sampling period (4 days) [100] or was not reported [157] [158]. This study is the first in horses to follow SAA concentrations for 10 days, in which 78.5% of horses SAA returned to normal (within reference range, $<20 \mu\text{g/ml}$) by day 10 (table 4).

The SAA response differed markedly between individual horses. The individual variation of SAA response was consistent with other studies evaluating SAA post-vaccination [100]. This seems to be consistent across species and has been shown in cattle [161], mice [162] and humans [163]. There is some speculation that high interindividual variation in immune response may be

genetic, as some individual's immune response can be categorized as hyper- or hypo-responsive [163] [164]. Other factors that may have an effect on SAA response, include age, breed and gender. Skipper and Pusterla (2021) reported that age had a significant effect on SAA concentrations, with a lower SAA concentration following vaccination in older horses [158]. As discussed this may be an effect of immunosenescence, decreased immune response with increasing age. Studies performed in small animals have not reported a significant age-related difference in serum acute phase proteins in healthy animals; however, response to some inflammatory conditions have shown alterations in acute phase proteins in adult animals [132]. Our study population was overrepresented by an older population (>15 years old) of horses. Our study population was also predominately Quarter horses. Breed related differences have not been reported in horses; however, other studies investigating SAA response post-vaccination were of more heterogenous populations that reported similar trends in SAA following vaccination [157] [158]. Likely, one can conclude breed does not affect SAA concentrations in horses post-vaccination. It is also possible there would be reduced variance with a larger population of horses.

The results of this study also show that measuring SAA following vaccination cannot be used as an indicator of illness within at least 10 days post-vaccination in 78.5% of horses (table 4) and even longer in some horses. Further investigation into a larger population of horses following SAA concentrations for a longer sampling period is required to determine the length of time required for some horses SAA values to return to normal. In this study, a fever post-vaccination was observed in 85.7% of horses that were otherwise clinically normal with no adverse vaccine reactions. Clinically normal was defined as eating, drinking and other physical exam parameters within normal limits. Peak temperatures were seen at 12 to 24 hours post-

vaccination. These changes in SAA and temperature post-vaccination are significant when evaluating horses for illness. Within the described time period post-vaccination, SAA for 10 days post-vaccination and temperature 12 to 24 hours post-vaccination cannot be used as indicators of illness. This is especially important in regulatory medicine, when it is not uncommon to vaccinate horses prior to transport for sporting events or importation purposes. This is important to clinicians when evaluating a patient's clinical status to not rely on one clinical parameter.

There was no significant change observed in fibrinogen values post-vaccination. This was different from Andersen, et al (2012), who found a significant increase in plasma fibrinogen concentrations post-vaccination [100]. Andersen, et al (2012) followed the positive acute phase proteins SAA, fibrinogen and white blood cell count following vaccination with an inactive Immune Stimulating COMplex vaccine (ISCOM) containing three influenza strains and a tetanus toxoid and a live recombinant vector vaccine containing two influenza strains and tetanus toxoid with adjuvant. The difference was likely because fibrinogen concentrations were measured more frequently in the study performed by Andersen, et al (2012) (9, 24, 48, 72 and 96 hours post-vaccination), in comparison to this study protocol that measured fibrinogen on day 1, 4 and 7 post vaccination. Similarly, there were no significant changes observed in total white blood cell count or leukocyte count post-vaccination in this study. This was different from Andersen, et al (2012), who found a significant increase in white blood cell count following vaccination with the ISCOM vaccine, in comparison to the live recombinant vector vaccine [100]. The difference could be attributed to more frequent sampling in the study performed by Andersen et al [100]. However, the variation in response in SAA, fibrinogen and white blood cell count observed by Andersen et al between the ISCOM vaccine and live recombinant vector vaccine could be

attributed to the variation in type of vaccine. A limitation of this study was infrequent sampling of other inflammatory biomarkers (white blood cell count and fibrinogen).

The Stablelab point-of-care (POC) immunoassay has been previously validated for use in horses [160] [165]. Our study measured SAA concentration using only the Stablelab POC assay, without comparison to a turbidimetric immunoassay (TIA). Previous studies comparing the Stablelab reader to the previously-validated human and veterinary TIA found acceptable accuracy and precision with SAA concentrations [160] [165]. However, sampling error due to machine error could have been accounted for by implementing the use of two Stablelab readers for each sample at each time point into the study design, thus increasing the accuracy of SAA value at each time point post-vaccination. Outlier SAA values or individuals that did not follow the monophasic trend in SAA values could have been attributed to machine error. Schwartz, et al (2018) discussed the linearity and precision of the Stablelab POC reader with acceptable accuracy and precision in equine plasma with SAA concentrations up to at least 1000 µg/ml, with low inter-batch precision at high concentrations [160]. To increase precision and accuracy of the SAA values, repeat testing could have been performed on each sample at each time point.

Caution is recommended to not overinterpret SAA concentrations post-vaccination. Studies in horses investigating SAA post-vaccination have found no correlation between the intensity of SAA concentrations and antibody response [157] [158]. The antigens used in the studies include West Nile Virus [158] and EHV-1 and -4 [157]. The type of vaccine, mono- or multivalent, or adjuvant utilized may play a role in intensity of SAA response and differing antibody response post-vaccination. Further studies investigating SAA response in correlation to antibody response post-vaccination are required.

There were several limitations to our study. Although health status was determined by no abnormalities on physical exam and bloodwork, diseases such as equine asthma, equine pituitary pars intermedia dysfunction (PPID) and equine metabolic syndrome were not fully ruled out. However clinical signs of an active equine asthma or heaves episode were not detected on physical exam (increased respiratory rate, effort or nasal discharge). There is conflicting evidence to show that equine asthma may influence SAA concentrations [63] [65] [149]. However, it is important to consider that a developing episode of equine asthma may have influenced SAA concentrations in horses that had elevated SAA concentrations extended past the 10 day sampling period. The effect of PPID on SAA concentrations has not been evaluated. However, it is important to consider that horses with equine PPID are susceptible to secondary infections and immunosuppression is suspected to play a role. This may have had an effect on peak SAA concentrations and elevated SAA concentrations past the 10 day sampling period. The effect of equine metabolic syndrome on SAA concentrations has not been investigated; however, obesity in mice and humans has been associated with an increase in acute phase proteins [101]. Body condition scores were not included in our inclusion or exclusion criteria. However, this may have influenced SAA concentrations. Another limitation of the study was variation in season in which horses were vaccinated. Horses were vaccinated in accordance with AAEP guidelines for core and common risk-based “spring” vaccines. Horses were vaccinated between February to July 2021-2022. There are no published reports on the effect of season or insect bites on SAA concentrations. Horses vaccinated in the spring and summer months were exposed to higher levels of insects. However, these are normal environmental conditions horses are exposed to.

In conclusion, routine vaccination results in an increased SAA. This may provide evidence for a period of convalescence following vaccination when advising owners considering travel or competition. Further studies investigating the effect of travel or competition on antibody response are required. Measuring SAA following vaccination cannot reliably be used as an indicator of illness. Future studies could be aimed at investigating the effect of monovalent versus multivalent vaccine protocols, as well as the investigating the effect of other diseases on SAA concentrations (equine PPID, equine metabolic syndrome, etc).

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Appendix A - Supplemental Tables and Figures

	Day Post Vaccination												
Horse	0	0.25	0.5	1	2	3	4	5	6	7	8	9	10
#1	0	0	87	1589	1726	576	1715	1573	304	109	37	70	59
#2	0	0	153	2214	2440	2147	2076	1622	548	321	85	10	0
#3	0	0	236	2097	2390	2598	2249	540	164	36	10	1	0
#4	0	0	134	1215	2698	1964	1882	1837	895	243	183	52	9
#5	0	0	73	660	2018	1418	424	129	35	70	27	2	0
#6	4	0	62	687	1277	1467	524	245	83	32	17	14	10
#7	0	0	0	75	80	66	19	0	0	0	*	0	0
#8	0	*	77	239	1052	401	208	89	41	11	0	0	0

Table 14. Experiment 1, SAA (µg/ml) post-vaccination (n=8) *indicating areas of missing data

Day(s) Post-vaccination	Quartile 1	Median SAA	Quartile 3
0	0	0	0
0.25	0	0	0
0.5	0	82	138.75
1	0	951	1716
2	0	1872	2402.5
3	0	1442.5	2009.8
4	0	1119.5	1930.5
5	0	392.5	1585.3
6	0	123.5	365
7	0	53	142.5
8	0	27	61
9	0	6	23.5
10	0	0	9.25

Table 15. Experiment 1, median SAA (µg/ml) post-vaccination

Horse	Days Post-Vaccination											
	0	0.5	1	2	3	4	5	6	7	8	9	10
#1	97	103.1	100.9	99.7	99	99.3	99.4	99.4	99.5	98.6	99.1	100.2
#2	98.2	100.7	99.6	99.3	99.2	100.8	101	99.4	97.7	98.3	98.3	98.3
#3	97	103.3	101.9	98.3	99.1	98.9	99.2	97.1	98.1	98.8	97.4	98.5
#4	97.8	101.5	97.9	99.2	98.2	99.2	98.4	99.4	98.7	98.5	97.5	97.9
#5	99	102	101.4	99.7	98.7	99	99	97.9	99.7	99	98.9	98.1
#6	98.5	99.5	99.6	99.1	98.3	99.4	98.7	99.2	98.7	98.7	98.1	99.5
#7	98	102.1	99.8	98.1	98.8	98.1	98.7	99	98.7	98.5	98	98.1
#8	99.8	102.6	100.2	101.1	98.9	98.5	99.5	99.1	100.1	100.3	100	99.6
#9	99.5	102.6	101.2	97.9	*	97.9	97.5	96.5	98.1	97.5	98	97.5
#10	98.9	101.8	99.7	100	98.9	97.5	98	98.3	97.9	98.5	99	98.9
#11	99.6	100.2	101.9	100.1	98.5	97.5	97.8	98.1	97.5	98	99	98
#12	98.3	99.1	99.6	99.9	98.1	97.2	98	98.5	97.1	98.5	98.1	99.1
#13	98.8	100.3	101.7	100.1	98.1	97.9	98.3	97.8	98.3	99	98.5	98.3
#14	99.5	102.3	101.2	99.1	98.9	98	98.7	98.1	99.5	98.5	99.3	98.9
#15	98.5	101	98.1	98.9	98.1	99	98.7	98.3	98.9	98.1	98.9	99
#16	100.2	102.6	101.1	98.3	97	98.1	98.2	98.2	98.3	99.1	100.1	99
#17	99.5	100.6	100.6	99.7	99.1	99.5	99.6	99.1	99.2	99.7	98.7	99.6
#18	99.4	101	101.3	99.9	99.5	99.7	98.8	98.7	98.9	99.2	98.7	99.3
#19	99.2	103.7	102.5	99	100	99.1	99.7	99.9	99.7	99.8	99.9	99.9
#20	98.9	101.4	100.9	99.3	99.1	99.1	99.3	99.1	99.4	98.9	98.3	98.7
#21	99.7	103.5	101	98.9	99.2	99	99.5	98.9	98.2	98.3	99.4	99.5
#22	99.8	102.7	100.9	98.1	98.5	99	100.1	99.5	99	99.1	99.1	98.8
#23	99.8	101.9	99.4	100.7	100.5	99.7	99.1	98.9	98.2	99.6	99.4	98.6
#24	98	101.7	100.7	99.1	99	99.7	98.5	98.5	99.7	98.6	99.7	98.7
#25	98.8	101.7	100.4	99.1	99.5	99.7	98.5	98.2	99.4	97.9	98.3	97.3
#26	99.9	102.1	101.7	99.3	98.1	98.7	99.5	98.1	98.1	98.5	100	96.1
#27	99.8	99.6	102.6	100.1	100.1	99.2	98	99.1	98.7	98.6	98.8	98.9
#28	98.7	101.1	99.8	99.4	99.8	98	98.5	98.7	99.7	99.9	99.4	98.1

Table 16. Experiment 2, Temperature (F) post-vaccination (n=28). Orange= low-grade fever (101.2-102.5F), Red= fever (temperature >102.5F) *indicating areas of missing data

	Days Post-Vaccination												
Horse #	0	0.25	0.5	1	2	3	4	5	6	7	8	9	10
#1	99.9	100	100.2	100	98.6	100.1	100.3	100.2	99	99.7	99.1	99.4	98.5
#2	100.2	100.4	104	100.8	99.7	100.9	99.1	99.8	100.4	99.9	99	99.8	99.2
#3	98.6	100.9	103.8	100.3	99.6	100.6	100.2	99.4	99.8	99.4	99.6	99.4	99.6
#4	98.8	100.2	103.3	99	98.6	100.9	98.2	98.6	99.8	98.3	98.7	98.3	97.3
#5	100.1	100	*	101.7	99.9	100	100	100.8	101.1	99.8	99.5	99	98.8
#6	99.4	99.6	101.4	98.7	97.9	98.5	99.2	98.7	98.3	98.7	98.3	98.1	99
#7	98.6	100.1	100.2	100.5	99.8	99.7	100.2	99.6	99.7	99.5	*	*	99.4
#8	98	*	102.2	99.9	98.6	96	97.9	99.2	98.7	99.4	98.8	98.6	98.8

Table 17. Experiment 1, Temperature (F) post-vaccination (n=8). Orange= low-grade fever (101.2-102.5F), Red= fever (temperature >102.5F) *indicating areas of missing data