Acute phase proteins and stress markers in the immediate response to a combined vaccination against Newcastle disease and infectious bronchitis viruses in specific pathogen free (SPF) layer chicks

Haider Kaab, Maureen M. Bain and Peter David Eckersall.

Institute of Biodiversity, Animal Health and Comparative Medicine, College of Medicine, Veterinary and Life Sciences, Glasgow University, Glasgow, Scotland, UK.

Corresponding author's email David.Eckersall@glasgow.ac.uk

Phone: +44 (0)1413305732

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ABSTRACT

Vaccination is an important tool in poultry health, but is itself a stressor often resulting in a reduction in feed intake, body weight gain, and nutrient digestibility. In other species vaccination is associated with an immediate acute-phase response. As an important immune parameter, the circulating heterophil/lymphocyte (H/L) ratio is a well-recognized parameter of stress in poultry. In this study, the effects of a routinely used commercial poultry vaccine on the acute phase response (APR) and H/L ratios in specific pathogen free (SPF) layer chicks was examined to determine if post vaccination (PV) stress and an APR occur. A combined Newcastle disease and Infectious bronchitis vaccine (Nobalis Ma5+Clone 30) was administered to SPF chicks by the intraocular route at age 7 days. Acute phase proteins (APP), alpha-1 acid glycoprotein (AGP) and serum amyloid A (SAA) were measured by enzyme-linked immunosorbent assays at 0 (pre-vaccination) and 0.5, 1, 2, 3, 4, 5, 6 and 21 days, PV. Stress was determined in the chicks by measurement of the H/L ratio. The immune response to the vaccine was estimated by measurement of the antibody (IgY) response to the vaccine at day 21.

The antibody titer was significantly ($P < 0.05$) higher in the vaccinated group at 21 days PV confirming stimulation of the immune system. The H/L ratio was also significantly higher in the vaccinated group at 1-2 days ($P<0.01$) and at 3 days ($P<0.05$) PV. The concentration of SAA increased by 2.8-fold, from 63.7 µg/ml in controls to 181 µg/ml in the vaccinated group, ($P<0.05$) at 1 day PV. AGP increased 1.6-fold at 2 days PV, (from 0.75 g/ml in the control group to 1.24 g/ml in the vaccinated group, $P<0.05$).

In conclusion an immediate but mild APR occurred in the chicks following intraocular vaccination, whereas the stress response as measured by H/L ratio seemed to be more specific and sensitive. Measurement of these biomarkers of the host response could be a tool in vaccine development.

INTRODUCTION

Acute phase proteins (APP) are a group of blood proteins involved in restoring homeostatic balance by restricting growth of microorganisms, mediating the inflammatory response and the effects of stress through an antibody-independent response. The APP are also considered to have diagnostic and prognostic potential because of the correlation between their concentration in blood and the response of the host to infection or inflammation (Cray, et al., 2009). There is increasing interest in APPs in chickens as a physiological marker for health and welfare including infection and intriguing vaccine response to both bacterial and viral pathogens (O'Reilly and Eckersall, 2014). Responses of APP in terms of vaccination stimuli have been investigated in other species, for instance, in horses (Andersen, et al., 2012) in sheep (Eckersall, et al., 2008) and in calves (Arthington, et al., 2013). These previous studies reported an acute phase response to a variety of vaccines and therefore suggest the possibility that monitoring the APP may be a means to determine the efficacy of a vaccine in stimulating the innate immune system and as such could be a tool of value in vaccine development.

In chickens, alpha-1 acid glycoprotein (AGP) responds as a moderate positive APP (Chamanza, et al., 1999; O'Reilly and Eckersall, 2014) following experimental infection. Investigation of the plasma AGP response, in White Leghorn SPF chickens at age 3 weeks old, inoculated by an intraocular route with a highly virulent strain of Gumboro disease virus, showed an increase in serum AGP concentration of 4.6-fold at 2-day post immunization which peaked at 6 days at 6.2-fold the pre-treatment level. In contrast, inoculation with an attenuated strain of Gumboro disease virus lead to a peak in AGP of 2.4-fold at day 2 post inoculation (Inoue, et al., 1997).

AGP levels have also been shown to increase significantly ($P<0.05$), at 12-48 h post IV injection with lipopolysaccharide (LPS) from *Escherichia coli* (E. coli) in male broiler chickens at 3 weeks old (Takahashi, et al., 1998)

Inoculation of commercial layer chickens with *Mycoplasma gallisepticum* vaccine caused a significant increase in serum AGP concentration, which started at 1 day PV(Peebles, et al., 2014). In this study the vaccine was administered via the intraocular route in one group and subcutaneous injection in another group. The concentration of AGP remained significantly higher, in both administration routes, than in the control group for up to 21d post inoculation.

Serum amyloid A (SAA) is another major positive APP in chickens (Alasonyalilar, et al., 2006) and is immunomodulatory, inhibiting pyrexia and down regulating pro-inflammatory events during an APR (Shainkinkestenbaum, et al., 1991; Uhlar and Whitehead, 1999). However due to the lack of commercial assay systems there have been few reports on this APP in chickens and none in relation to post vaccination stimulation. In this study we were able to monitor SAA in chicken serum using a newly available species specific ELISA system and therefore determine if this major APP is also stimulated by vaccination.

Changes in white blood cell count, especially in Heterophils/Lymphocyte (H/L) ratio have been used as a measure of stress in chickens (Gross and Siegel, 1983; Shini, et al., 2008) (Crowther, 2009; Ohara, et al., 2015). The H/L ratio of birds can be affected by health disturbance or stress (Crowther, 2009), including transport stress (Huff, et al., 2005; Matur, et al., 2016) possibly due to the transition of leukocytes from the marginal pool to peripheral circulation (Duncan JR, 1987).

In this study we measured the AGP, SAA and the H/L ratio in specific pathogen free (SPF) chickens following routine vaccination via the intra-ocular route to determine whether the immediate post-vaccination innate immune or stress response was most sensitive to the mild or severe vaccination stress. Our specific aim was to determine if an immediate APP response occurs following intra-ocular vaccination as well as via the intra muscular route (Peebles, et al., 2014) which would allow subsequent investigations on the pathophysiology of avian APPs and their interaction with the protection of the host provided by such vaccination. The vaccine chosen for the study, a combined Newcastle disease and Infectious Bronchitis (N/B) Live, freeze-dried virus vaccine, is routinely used in chicken production and this dual vaccine was selected to stimulate an APP response under conditions similar to those on commercial farms. Vaccination routes recommended for this vaccine are by spray, in drinking water or by eye drop. Intraocular vaccination (eye drop) was selected to ensure every individual bird received the same dose, which could not be guaranteed with the other routes. To evaluate the success of the vaccine, the amount of specific antibody (IgY) raised against the immunogen proteins of the vaccine after 21 days, was also determined.

MATERIAL AND METHODS

SPF Chicks and Housing

One hundred and eighty 1-Day old SPF White leghorn layer chicks were hatched out at the experimental farm (Cochno Research Farm, University of Glasgow) and divided into two batches each containing 90 chicks. Each batch of chicks was then placed in a separate controlled environmental room (R1 an R2) in one of two pens (n=45 per pen). The two pens in

each room had a litter of wood shavings and were fitted with a brooding ring. The stocking density was 12chicks/m^2.

The chicks were fed *ad libitum* with a commercially available chick crumb formulated to meet or exceed National Research Council (NRC, 1994) guidelines and the birds had access to fresh water throughout the study period. The light, temperature and ventilation within each room were automatically controlled and adjusted according to management guide recommendations. The chicks were allowed to adjust to their environment for the first 7 days before the experiment commenced. Strict biosecurity was applied to prevent cross contamination between the two rooms and each pen, not least the controls (R2) were always visited first and then the vaccinated room (R1).

Experimental Design

The experiment commenced when the chicks were 7 days old. There were 9 sampling time points; pre (0) and post vaccination (PV) at 12h, 24h, and then 2, 3, 4, 5, 6, and 21 day thereafter. At each time point 12 chicks were weighed and culled and samples collected, 6 per treatment and 3 per replicate pen. Full ethics approval was granted in advance by the University of Glasgow MVLS College Ethics Committee.

Vaccine and Vaccination

A commercially available combined Newcastle disease and Infectious Bronchitis (N/B) Live, freeze-dried virus vaccine (Nobalis Ma5+Clone 30, MSD Animal Health) was used in this experiment. After re-constituting the vaccine in sterile saline solution, each dose contained at least $10^{3.5}$ EID_{50} of the IB strain Ma5 and 10^6 EID_{50} Newcastle disease virus strain Clone 30.

The chicks in pens 1 and 2 in R1 were weighed and the vaccine was administered by the intra-ocular route with one drop applied to one eye. A sterile saline solution was administered by the same route to control animals in pens 1 and 2 which were housed in R2.

Blood Sampling and Assessment

At each sampling time point three chicks were chosen from each replicate pen, weighed then humanely culled by dislocation of the neck followed by decapitation. Following decapitation, approximately 1.5 ml of blood was collected from the major vessels in the neck using heparinized tubes. Fresh blood was used to make blood smears to determine the H/L ratio; the remainder was centrifuged (3000 × g) for 15 min at 4 ˚C and the plasma immediately frozen at -20˚C . SAA and AGP levels were measured in all samples collected using commercially available ELISA kits described below. Plasma from samples on day 0 and 21 days were used to estimate the antibody titers as detailed below.

Heterophil / Lymphocyte Ratios:

Differential WBC counts were carried out on the blood smears stained with the May-Grunwald-Giemsa stain. Two hundred leucocytes were counted and classified per slide. The H/L ratio was calculated by dividing the total number of Heterophils by the total number of Lymphocytes (Gross and Siegel, 1983; Ohara, Oyakawa, Yoshihara, Ninomiya and Sato, 2015). Samples used for this technique were at 0, 1, 2, 3, 4, 5, 6 day post vaccination and we dropped 12h post vaccination for technical issues.

Antibody Titer Raised Against the Vaccine:

A modified antibody (Ab) titration assay (Crowther, 2009; Snyder, et al., 1983) was used to determine the amount of IgY Ab raised against the vaccine in this study. A 96 well plate (Costar Assay Plate, CORNING) was coated with the Newcastle and Infectious Bronchitis vaccine (Nobalis Ma5+Clone 30) diluted to a protein concentration of 20µg/ml in 0.2M carbonate bicarbonate buffer at pH 9.5 and incubated at 4^{o}C overnight. The protein content of the vaccine had been determined by a Bradford protein assay (Sigma Chem Co. Poole, UK) with bovine serum albumin as the standard. Each well was then aspirated and washed 4 times using Tris-buffer saline (TBS) 50 mM Tris-Cl, pH 7.5 containing 0.05% tween-20. Wells were blocked with 200µl of 5% (w/v) Marvel milk protein diluted in TBS-T (0.05%) over an hour at room temperature on a rocking plate. The plate was then washed as above.

Antibody standards were made by serially diluting pooled samples collected from vaccinated birds sampled at 21day post vaccination and by diluting 1:20 in TBS-T with 0.5% Marvel milk. This was standard 1 (S1) and given a value of 100 arbitrary units (AU) of antibody. It was then diluted in a 6-fold serial dilution to S6 (3.13 AU) using TBS-T with 0.5% Marvel milk. The chicken serum samples also were diluted 1:80 in TBS-T 0.5% Marvel milk. To duplicate wells a 100 µl aliquot of diluted standard or sample was added. After 1 hour incubation with constant shaking at room temperature, the plate was washed as above. The second antibody, anti-Chicken IgY VHH Single Domain Antibody conjugated to horse radish peroxidase (HRP) (Abcam, Cambridge, UK), was diluted to a concentration of 1:5000 with TBS-T 0.5% Marvel milk and added before incubating for another 1 hour at room temperature by constant shaking then washed as above. 100µl of tetra-methylbenzidine (TMB, KPL laboratories, *Inc*., Maryland, USA) was then added to each well for 20 minutes at room temperature whilst rocking until a blue color developed, then 100µl of stop solution (2M H_2SO_4) was added. This caused the

color to change to yellow. The absorbance of the resulting solution was measured at 450nm using an OPTIMA absorbance microplate reader (BMG Labtech Ltd, Bucks, UK). A standard curve using 4-parametre fit curve was used to determine the antibody response.

Enzyme Linked Immunosorbent Assays (ELISA)

The ELISA assays for chicken APPs were obtained from Life Diagnostics Inc, (West Chester, USA). They were performed according to the manufacturer's instructions with a dilution factor for the serum samples of 1:10000 for AGP and 1:20 for SAA. Each individual sample was run in duplicate.

ELISA Assay for AGP: Diluted samples and standards were mixed thoroughly and 100µl of each sample or standard was dispensed into duplicate wells of 96-well microtiter plate. This was then incubated on an orbital microplate shaker at 150 revolutions per minute (rpm) at room temperature (RT) for 45 min. Contents of the wells were then discarded and the wells were washed five times each using 1x wash buffer. After ensuring all residual droplets in the wells were removed by striking plates onto absorbent paper, 100µl of the secondary antibody-HRP conjugate was then dispensed into each well and incubated on the shaker at RT for 45 min. The wash step was repeated and 100µl of TMB reagent (HRP substrate) was dispensed into wells and a blue color development was allowed to proceed for 20 min on the shaker at RT. The reaction was stopped by adding 100µl of stop solution per well into the wells. Absorbance was read using a FLUOstar Optima plate reader at 450 nm within 15 min of stopping the reaction. A four-parameter logistic curve (4PL) was used as described above. Intra assay coefficients of variance (CVs) were 4.7% at 76.2g/L ± 3.6 (mean ± SD) and 3.7%, at 32.6g/L ± 1.48 (mean ±

SD) (n= 40). Inter assay CVs were 2.7%, at 43.6g/L ± 1.17 (mean ± SD) and 5.8%, at 70.8g/L ± 6.4 (mean ± SD) (n= 5) and the limit of detection was 1.7g/L (3 SD from zero sample)

ELISA Assay for SAA: Serum samples (50µl) were first incubated in a heat block at 60ºC for one hour to dissociate SAA from lipoproteins. Following heat treatment, the diluted samples and standard (100µl) were incubated in the antibody-coated microtiter wells in duplicate together with HRP conjugate (100µl) for one hour. As a result, SAA molecules become sandwiched between the immobilization and detection antibodies. Contents of the wells were then discarded and wells washed five times each using 1x wash buffer. After ensuring all residual droplets in the wells were removed by striking plates onto absorbent paper, 100µl of TMB Reagent was added and incubated for 20 minutes. Color development was stopped by the addition of 100µl Stop Solution, changing the color from blue to yellow and the optical density was measured at 450 nm. A four-parameter logistic curve (4PL) was used as described above. Intra assay CVs were 5.7 % at 57.5µg/L 4.8 (mean ± SD) and 3.07 % at 25.9µg/L ± 1.8 (mean ± SD) (n= 40), inter assay CVs were 7.6% at 10.5µg/L ± 0.6 (mean ± SD) and 5.3 % at 103.7µg/L ± 6.2 (mean ± SD) (n= 5) and the limit of detection was 0.21 µg/L (3 SD from zero sample)

Data Handling and Statistical Analysis

The antibody response to the vaccine, H/L ratios, and acute phase proteins (SAA, AGP) of the vaccinated and control groups, were compared at each sampling time point using a Mann-Whitney Test for non-parametric distribution (Minitab 17.1.0). In all analyses, $P < 0.05$ was used to represent statistical significance.

RESULTS

Chick Weights:

The chicks in each group were within the same range, 69.85g (56.5-84.2) median (range) for the control group and for the vaccinated group 76.4g (60.8-81.2) median (range) at 7 day old. There were no significant differences in body weight between the groups in response to the vaccination (Table 1).

Antibody Titer Raised Against the Vaccine:

The antibody titer was significantly elevated by 3.1-fold ($P<0.01$) after 21 days to be 488AU (346-781) median (range) in the vaccinated group compared to the control group 154 AU (130-186) median (range) (Figure 1).

The Heterophil/Lymphocyte Ratio:

The H/L ratio increased significantly ($P<0.01$) to reach a peak of 0.58 (0.39-0.65) median (range) in the vaccinated group (V) compared to the control group (C) 0.20 (0.08-0.32) median (range) by on day-1 post treatment (Figure 2). This increase remained significant on day 2 ($P<0.01$) and day 3 ($P<0.05$) post treatment. From day-4 there was no significant difference in the H/L ratio between the control and vaccinated groups.

Acute Phase Proteins

Alpha-1 Acid Glycoprotein (AGP) in the vaccinated group was statistically different from the AGP levels in the control group on day-2 post treatment. AGP increased 2-fold from 0.47g/L (0.22-0.49) median (range) in the control group to1.007g/L (0.45-1.39) median (range) in the vaccinated group, $P< 0.05$ (Figure 3). At day-3 post treatment the AGP levels were not statistically different from the control animals. At day -6 post treatment, another significant increase ($P< 0.05$) in the levels of AGP in the vaccinated group over controls was observed with a 1.9-fold increase from 0.54g/L (0.37-0.68) median (range) in the control group to 0.99g/L (0.53-1.29) median (range) in the vaccinated group.

Serum Amyloid- A (SAA) levels were significantly higher in the vaccinated group on day-1 post treatment ($P< 0.05$) with a 2.5-fold increase from 39.5µg/L (20.6-63.7) median (range), in the control group to 103.5µg/L (61-180.9) median (range), in the vaccinated group. Thereafter there was no significant difference except on day-6 post vaccination when the SAA concentration was significantly higher in the vaccination group with a 2.8-fold ($P<0.01$) increase from 42.5µg/L (22.7-63.3) median (range) in the control group to 105.5µg/L (63.8-238.1) median (range) in the vaccinated group (Figure 4).

DISCUSSION

This investigation showed that an intra-ocular vaccination of SPF layer chicks with a dual vaccine against Newcastle disease virus and infectious bronchitis causes an immediate but mild APP response with small increases in AGP and SAA on day 2 and day 1 post vaccination respectively. We also demonstrated that the vaccinated birds underwent a mild vaccination stress response with H/L ratios remaining higher in the vaccinated birds for up to 3 days post

vaccination. The efficacy of the vaccine to produce antibody to the immunogens contained, was confirmed by demonstration of the presence of specific antibody on day 21 post vaccination.

The SAA concentration in many species is related to the severity or virulence of the pathogen, and it can be used as marker for detection of inflammation (Ceron, et al., 2005; Eckersall, 1995) serving as an indicator of the immediate innate immune response to stimulations such as by vaccination. SAA usually increases within several hours (5-6 hours in humans) of an inflammatory event and decreases after 48 hours and typically increases 10 to100-fold during a response (Gruys, et al., 2005; Kushner and Rzewnicki, 1994). In the current study, the intraocular administration of N/B vaccine stimulated only a mild increase in the SAA concentration. Though, the serum SAA increased significantly at 24h post treatment, with a 2.4-fold elevation, same as seen with vaccination in other species. For instance, SAA levels increased sharply from 5 h to hit the peak at 24 h to be 3.6-fold higher than the previous level before inoculation following intratracheal inoculation of beef calves with inactivated *Pasteurella multocida,* (Dowling, et al., 2004). In lambs, subcutaneous vaccination with Heptavac P (companied Clostridia and Pastural vaccine) also caused a significant increase ($P<0.01$) in both SAA and haptoglobin (Hp). The level of SAA peaked at 24 h reaching a 400-fold increase and did not return to normal concentration until 4 day (Eckersall, et al., 2008).

In the current study, we observed a second elevation of SAA in the vaccinated group compared to the control group on day 6 post treatment, which also coincided with a second peak in AGP concentration. A limitation of our study was that we were not able to repeat

sample the same individual chicks and so it is not possible to determine if this second peak in APP was present because some individuals responded more slowly to the vaccination or if some other factor was coming into play e.g social stress. The most likely explanation is that the second APP peak was due to individual differences in the APP response (Elsasser, et al., 2005; Verschuur, et al., 2004).

In the current study AGP in the vaccinated group was significantly higher by a 2-fold increase at 2-day post treatment. This result is similar to that reported by Sylte and Suarez (2012) who reported an AGP peak serum concentration of 2.6 -fold at 48h post-experimental infection with influenza virus in 4wk old White Leghorn chickens. However, we did not see a prolonged increase in AGP unlike the 96h increase reported by these authors. Notably the intraocular inoculation with a vaccine strain of Gumboro disease virus lead to a 2.4-fold increase in plasma AGP at day 4 post treatment in 3wk old white leghorn SPF chickens (Inoue, et al., 1997) whereas intraocular inoculation with a highly virulent strain of Gumboro disease virus lead to an increase of plasma AGP at day 2 which peaked at day 6 post treatment (6.2-fold increase).

Measuring the H/L ratio has been established as means of evaluating stress in chickens (Maxwell, et al., 1992; McFarlane and Curtis, 1989; Post, et al., 2003; Puvadolpirod and Thaxton, 2000). In the current study, we observed an increase in Heterophils and a decrease in Lymphocytes in the vaccinated group, whereas the control animals did not show any significant change in their H/L ratios. The changes we observed in the H/L ratios was therefore likely to be related to the vaccine action and its effects on the innate immune response. These inflammatory mediators also initiate and modulate the APR, which by diffusing into the extracellular fluid and circulating in the blood, leads to the activation of the hypothalamic-

pituitary-adrenal axis, decrease production of growth hormone and physiological changes as fever, lack of apatite and catabolism of muscle cells (Gruys et al., 2005). As a result, inflammation may have a combined effect on the H/L ratio by increasing Heterophil concentrations due to increased granulocytopoiesis and decreasing lymphocyte concentration due to cytokine-mediated increases in corticosterone concentration (Clark, 2015). Change of the H/L ratio has been reported in birds to be between 0.19 - 64.67 in different diseases (Clark, 2015) so for our vaccinated birds a change in H/L from 0.11 to 0.54 is suggestive of a mild stress response.

Taken together, the vaccination procedure used in this study caused only a mild APR with only small increases in AGP and SAA, and a mild change in H/L ratios. This contrasts with results of post vaccination acute phase reactions in other species and with other types of vaccine. In previous reports, the use of an adjuvant may have aided and enhanced the APR e.g. in rabbit (Destexhe, et al., 2013) and sheep (Eckersall, et al., 2008). Thus, the absence of an adjuvant in the current N/B vaccine and administration by the intra-ocular route may have led to a moderate stimulation of the acute phase and stress responses.

In conclusion vaccination of chicks with N/B vaccine by the intra-ocular route produced a mild acute phase response and vaccination stress response in 7 day old SPF layer chicks. Of the two methods, the H/L ratio was more sensitive and consistent in terms of measuring the mild vaccination stress response under the conditions employed in this experiment. Whilst the SAA and AGP levels increased within 1 and 2 days post vaccination respectfully a limitation of the design of this experiment was that individual differences in the APR response could not be taken into account. For future work, it will be of interest to monitor the APR in the same

individuals using different vaccines and routes of administration and to assess whether the post vaccine APPs response is correlated to both the H/L ratio and the subsequent antibody Titer. In addition, characterising post vaccination responses in chickens could be a way of assessing the dynamic APR and evaluating its potential as a biomarker for disease resistance.

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Table 1

Age/ day	Sampling time	Control Median (range)	Vaccinated Median (range)	P value
7	0	69.85 (56.5-84.2)	76.4 (60.8-81.2)	0.5752
8	1	77.55 (68.2-87.6)	78.6 (66.6-99.1)	1.0000
9	2	85.1 (77.8-97.1)	85.2 (79.4-94.3)	0.7488
10	3	92.7 (60.5-102.5)	95.45 (80.7-100)	0.0927
11	4	105.1 (90.4-122.3)	96.3 (58.9-109.6)	0.0929
12	5	105.35 (93.2-124)	103.5 (90.8-132.1)	0.4712
13	6	105.05 (95.6-138.9)	122.7(91.1-142.7)	0.2980
28	21	332.95 (294-390.7)	297.1 (271.5-387.9)	0.0957

Table-1; shows median ±SD of body weight for control and vaccinated groups at each sampling time. There were no significant differences by Mann-Whitney test, at any sampling time between the groups, N= 6 chicks per sampling time.

462 **Legend to Figures**

463 Figure-1, Antibodies levels pre-treatment 0 and 21 day post treatment in the control (C) (n=6
464 per time point) and vaccinated (V) groups (n=6 per time point). The difference was significant
465 P≤ 0.01 at day 21 post treatment. (Horizontal bars with star indicate statistical differences
466 between groups (** = P≤0.01). Data presented as box and whisker plots with median in the
467 box, with 25-75 percentile range as the box and the whisker as 10-90 percentiles

468

469 Figure-2: The Heterophil/Lymphocyte ratio was significantly higher in the vaccinated group
470 (V) (n=6 per time point) at 1, 2 and 3 days post treatment compared to the control group (C)
471 (n=6 per time point). (Horizontal bars indicate statistical differences between groups (** =
472 P≤0.01, * = P≤0.05). Data of 7 sampling time points are presented as box and whisker plots
473 with median in the box, with 25-75 percentile range as the box and the whisker as 10-90
474 percentiles.

475

476 Figure-3: Comparison of AGP concentrations in vaccinated (V) (n=6 per time point) and
477 control groups (C) (n=6 per time point) over the time course of this study. Significant
478 differences (Horizontal bars) were detected at day 2 and day 6 post treatment (P< 0.05). Data
479 of 8 sampling time points are presented in median with 25-75 percentile range as the box and
480 the whisker as 10-90 percentiles.

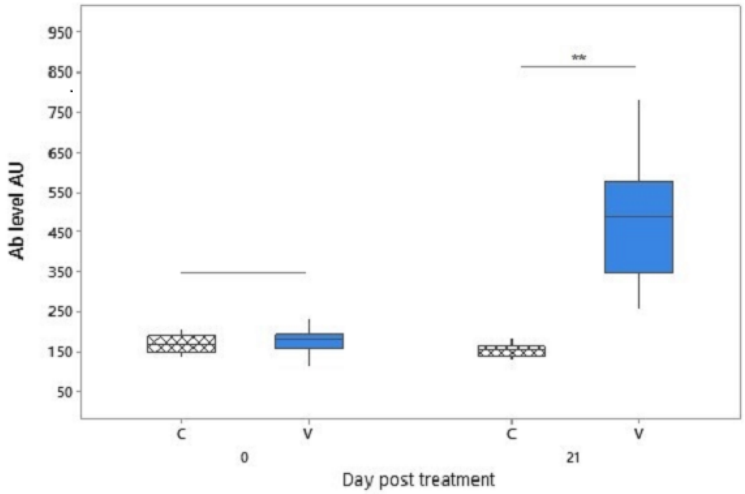
481

482 Figure-4: Comparison of SAA concentration in control (C) (n=6 per time point) and vaccinated
483 groups (V) (n=6 per time point) over the time course of this study. Significant differences were
484 detected (Horizontal bars) at day 1 and day 6 post treatment, ** = P≤0.01, * = P≤0.05. Data of

485 8 sampling time points are presented as box and whisker plots with median in the box, with
486 25-75 percentile range as the box and the whisker as 10-90 percentiles.

487

Figure 1


Ab level AU
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250
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**
C
V
C
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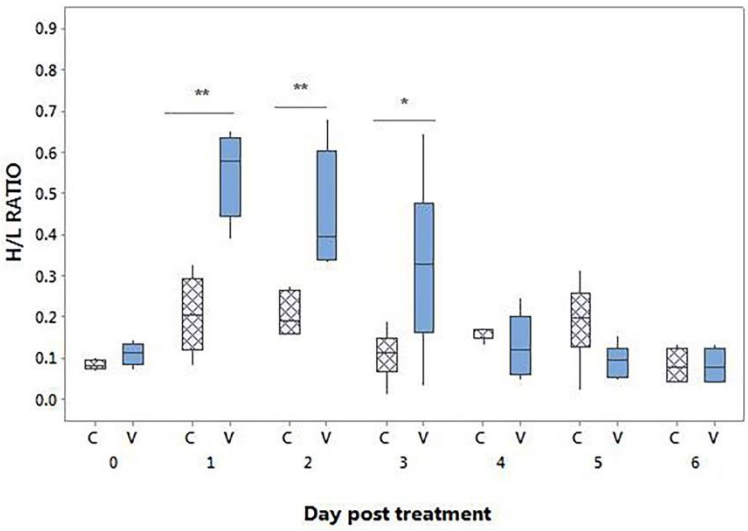
0
21
Day post treatment

Figure 2


H/L RATIO
0.9
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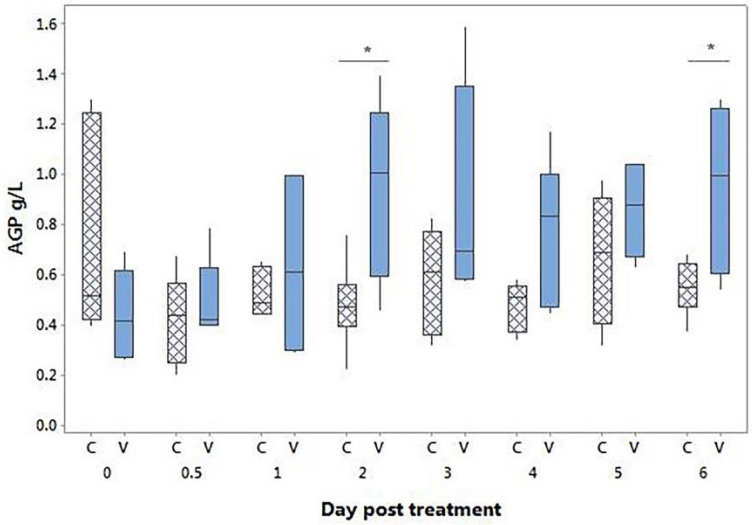
C V C V C V C V C V C V C V
0 1 2 3 4 5 6
Day post treatment

498 Figure 3



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502 Figure 4

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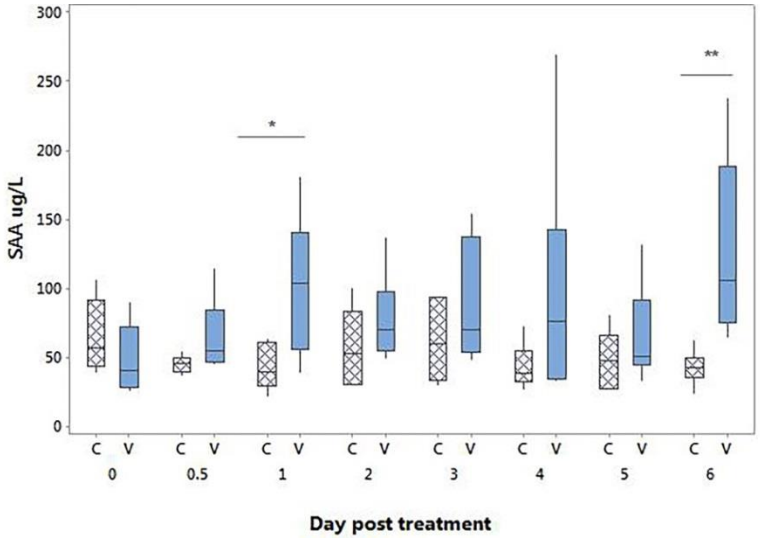

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