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Evaluation of Pro-Inflammatory Biomarkers as Potential Early Indicators of Acute Respiratory Distress Syndrome (ARDS)

Makda Solomon Gebre
Utah State University

EVALUATION OF PRO-INFLAMMATORY BIOMARKERS AS POTENTIAL EARLY INDICATORS OF ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

by

Makda Solomon Gebre

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ABSTRACT

Infections with the 2009 pandemic influenza A(H1N1) virus often leads to acute respiratory distress syndrome (ARDS) in high-risk patients. In these patients, ARDS has a mortality rate of 40-50 % [1]. Patients may die before any intervention is possible since there are few if any early biomarkers that are indicative of ARDS and can be used for diagnosis. In a search for biomarkers that signal early disease progression in mice, multiple acute phase proteins involved with inflammatory responses to infectious stimuli were proposed. Three biomarkers were evaluated for this project. These included C-reactive protein (CRP), Serum Amyloid A (SAA) and Transferrin. To validate these biomarkers as predictors of ARDS, mice were infected with the influenza A California/04/2009 (H1N1)pdm09 virus and serum was collected from infected mice at different time points (0 – 72 hours post infection). Serum samples were tested for amounts of CRP, SAA and Transferrin using an enzyme-linked immunosorbent assay (ELISA). Control mice were mock-infected and tested alongside for comparison. Our results indicate that of the three biomarkers tested, SAA shows strong potential as an early indicator of ARDS with a peak at 72 hours post infection in infected mice serum. Further investigation is in progress to observe the levels of SAA past day 3 (72 hours post infection).

INTRODUCTION

In April of 2009, a novel influenza A(H1N1) virus strain, shown in Figure 1, emerged in Mexico, and quickly spread worldwide. The World Health Organization (WHO) declared this virus outbreak a pandemic in June of 2009 [1]. Eight months later, 59 million estimated illnesses, 265,000 hospitalizations, and 12,000 deaths were reported within the United States [2]. The first pandemic of the 21st century is now over, but the novel H1N1 virus is still circulating worldwide as the major cause of seasonal influenza disease [3].

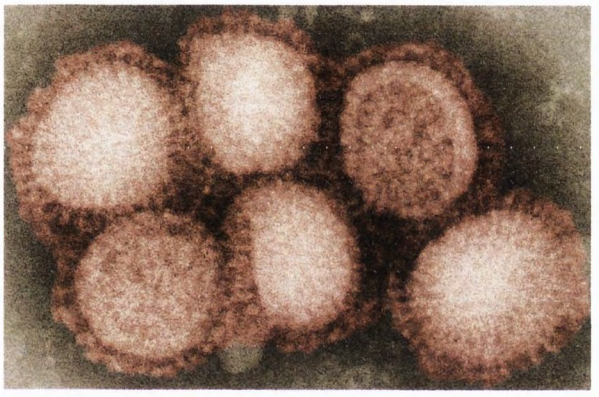

Figure 1: False-color negative stained transmission electron microscope image of Influenza A (H1N1)pdm09 virus particles. (Source of the micrograph, http://en.wikipedia.org/wiki/Influenza_A_virus_subtype_H1N1) [4]

Seasonal influenza is an acute, contagious respiratory infection caused by influenza A and B viruses [5]. Most patients do not need hospitalization and present with mild symptoms to the viruses. However, some high-risk patients (the elderly, the young, pregnant women, and people with immunodeficiency and/or other medical conditions) can develop Acute Respiratory Distress Syndrome (ARDS) and require admittance to the intensive care unit [6]. ARDS is a life-threatening, fast progressing lung condition that can develop 24 to 48 hours after onset of illness and has a mortality rate of 40 – 50% [2].

It causes fluid buildup inside the lungs and thus prevents enough oxygen from getting into the blood and other vital organs [6]. ARDS symptoms include difficulty breathing, low blood pressure, as well as kidney and liver failure. Some surviving patients later suffer from memory loss caused by brain damage. Often, people with ARDS are so sick, they cannot complain about their symptoms. Since patients with ARDS show no clinical evidence of elevated arterial or pulmonary capillary pressure, accurately diagnosing ARDS before it is too late for intervention has proven to be difficult. Thus, the development of biomarkers that could be used to diagnose ARDS more accurately and early in disease progression is vital.

Biomarkers are biological molecules found in blood, and other body fluids, indicating normal or abnormal processes of conditions or disease states [7]. Biomarkers used to show disease progression are usually the result of inflammation or immune system response to an infection. Preliminary unpublished data has indicated that an inflammatory regulatory protein, Interleukin-6 (IL-6), could be a potential early biomarker indicating the severity of influenza virus infection. Elevated levels of IL-6 were associated with severe disease in patients hospitalized with H1N1 virus infection [8]. Serum IL-6 levels associated strongly with the requirement of critical care admission and were predictive of fatal outcome. Preliminary studies performed at the Institute for Antiviral Research (IAR) at Utah State University with influenza virus-infected mice show that IL-6 levels significantly increased at day 2 post infection (Fig 2).

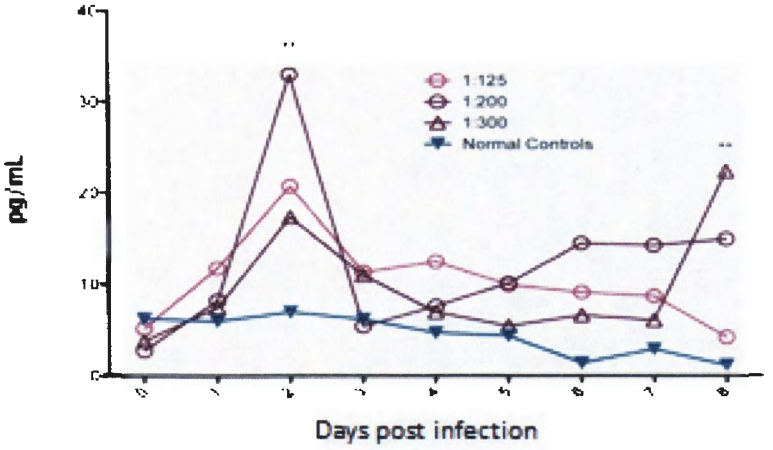

Figure 2: Interleukin (IL-6) levels in serum samples of control mice (blue line) and mice challenged with varying doses of Influenza A (H1N1)pdm09 virus (red lines).

As the search for suitable biomarkers continues, three particular biomarkers that could potentially indicate early ARDS progression were tested in the mouse model. These biomarkers included C-reactive protein (CRP), Serum Amyloid A (SAA) and Transferrin. C-reactive protein is a pentameric, acute-phase protein that binds to phosphocholine found on damaged cells to facilitate phagocytosis by macrophages [9]. Levels of CRP have been reported to increase in the serum during inflammation caused by various diseases including viral infections [10]. Serum Amyloid A (SAA) is an acute-phase apolipoprotein, whose function is not yet well understood [11]. It has been shown that concentrations of SAA also increase within the serum during inflammation [11]. Transferrin is a glycoprotein that reversibly binds to free iron within the serum. Transferrin was demonstrated to be a negative acute-phase protein, which means that levels of transferrin were found to decrease with inflammation in humans [12]. In mice however, transferrin is a positive acute-phase protein, much like CRP and SAA.

MATERIAL and METHODS

Animals and Experimental Design

One hundred 8-to-12-week old female mice of the BALB/c strain were used in the experiment to model human influenza virus infections. All mice were comparable in weight (18-20 g). Fifty mice were infected with 90 μL of influenza A/California/04/2009 (H1N1)pdm09 virus - a virus associated with ARDS in both mice and humans. Five mice were put in 10 groups, each group for serum collection at one of ten different time points (0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours post infection (HPI). A 1:150 dilution of the virus, corresponding to a 90% lethal dose, was prepared in PBS (phosphate buffered saline - a carrier solution). The virus was administered intranasally by placing a 90 μL drop of virus solution on the nose after the mice were anesthetized with 100 μL of ketamine/xylazine (50 mg/kg/5 mg/kg). Fifty mice were sham-infected with 90 μL of PBS only, using the same inoculation method.

After infection, blood samples were collected from mice at 0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours post infection (HPI) through cheek bleeding targeting the mandibular blood vessel as shown in Figure 3.

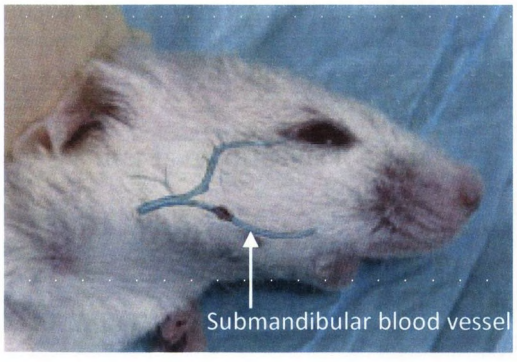

Figure 3: Target submandibular blood vessel used to collect blood samples at 0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours post sham and virus infections. Picture courtesy of Braintree Scientific Inc. [13]

Serum samples were separated from blood and frozen at -80°C. After the last serum collection, infected mice were euthanized and their lungs were collected and homogenized for virus titration, also known as an endpoint dilution assay, to confirm the viral infection of infected mice. To guarantee sufficient sample volume for all proposed tests, serum samples from each animal per time point were later pooled, aliquoted and stored at -80°C until further use.

Virus Titration (Endpoint Dilution Assay)

Virus titration was performed through the endpoint dilution assay by observing virally-induced cytopathic effects in Madin Darby canine kidney (MDCK) cells. Ninety-six-well plates were seeded with approximately 40,000 cells per well and grown overnight to confluency at 37°C and 5% CO_2. The next day, plates were washed twice with 100 µL infection medium (MEM/EBSS supplemented with 10,000 U/ml trypsin, 1 µL/ml EDTA, 50 µL/ml gentamycin). Wells were filled with 100 µL infection medium. Serum samples were diluted ten-fold in infection medium, then 100 µL of each dilution was added into wells as shown in Figure 4. Each sample was titered in quadruplicates. After 6 days incubation at 37°C and 5% CO_2, MDCK cells were microscopically observed for cytopathic effect (CPE) caused by the virus present (Figure 5). The virus dose (or virus titer) that was able to infect 50% of the cell culture ($CCID_{50}$) was calculated by the Reed-Muench method [14]. The virus titer is reported as $\log_{10}$ $CCID_{50}$/0.1 mL.

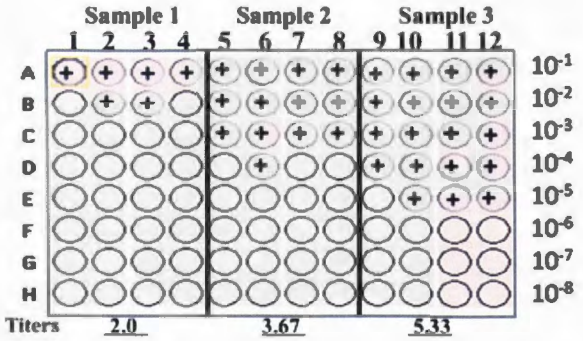

Figure 4: Endpoint dilution assay plate diagram on which samples are serially diluted from 10-1 to 10-8. After 6 days of incubation, each well is microscopically analyzed for virally induced effects and scored as either plus or minus. The numbers of infected wells are used to calculate virus titer values.

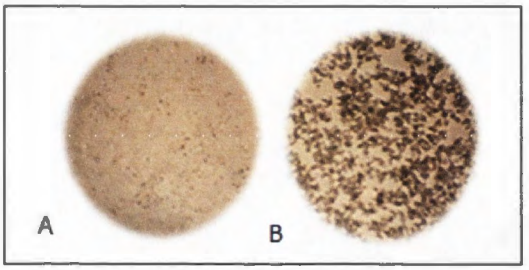

Figure 5: Example of virally-induced cytopathic effects of MDCK cells observed under the light microscope. A) Typical appearance of healthy cells forming a monolayer. B) Rounded off cells indicate cell death caused by the virus. This well would be scored as 'plus'.

Enzyme-Linked Immunosorbant Assay (ELISA)

Serum samples were analyzed for levels of C - reactive protein (CRP), Serum Amyloid A (SAA) and Transferrin using commercially available enzyme-linked immunosorbent assays (ELISA). Assay protocols and deviations are briefly described below.

The enzyme-linked immunosorbant assay (ELISA) is an immunological test that uses antibodies specific to a target molecule coupled with colorimetric changes that occur after binding of the target molecule with the antibodies to isolate and quantify the target molecule. The principle features of an ELISA are shown in Figure 6.

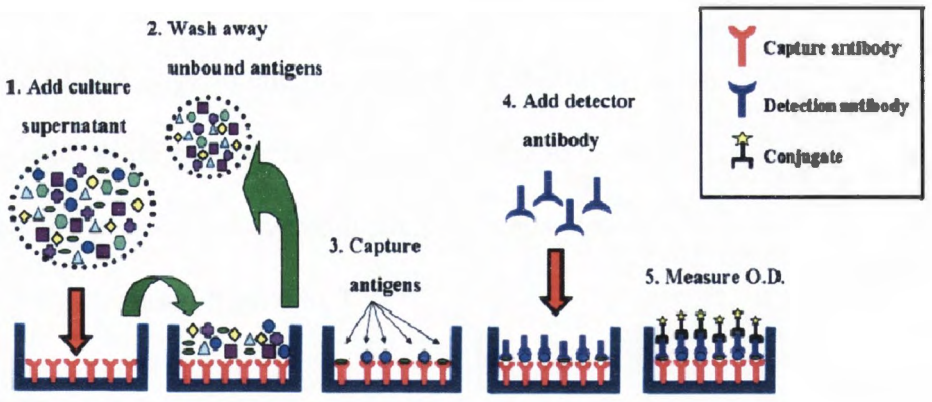

Figure 6: Sandwich ELISA procedure steps that use antibodies to signal the amount of protein present in a sample. Picture courtesy of Sung J. H. et al. [15]

A 96-well microtiter plate is coated with a capture antibody specific to the protein of interest. Samples are added to this plate, and the protein of interest in the sample is given time, usually at room temperature, to bind to the immobilized antibodies. Unbound proteins and other contaminants are washed away in a wash step. A second antibody is added which also binds to the protein/molecule of interest, thereby forming a sandwich and trapping the protein of interest. The second antibody is linked to an enzyme which will produce a colorful substance once it is provided with its specific substrate. The amount of the colorful substance in solution can be measured with a spectrophotometer as the absorbance of that solution and is related to the amount of captured protein/molecule of interest. The exact amount of the protein of interest can be mathematically interpolated from absorbance values obtained from a standard curve.

CRP ELISA

Two CRP ELISA experiments were performed according to manufacturer's suggestions (Life Diagnostics, Inc., Cat. # 2210-1). Initially, three microliters of serum from each samples was used to make 1:100, 1:320, and 1:1000 dilutions of the serum that were

tested on a 96-well ELISA plate. The 1:320 dilution samples were run in duplicates. On the second ELISA experiment, the optimal 1:320 dilution samples were tested in quadruplicate. Wash steps were performed with 250 µL of wash solution instead of the suggested 400 µL to avoid overspill from the wells. The number of wash steps was consequently increased from 6 to 7.

SAA ELISA

Three SAA ELISA experiments were carried out as described by the manufacturer in Mouse Serum Amyloid A (SAA) ELISA test kit (Life Diagnostics, Inc., Cat. # 3400-1). Four microliters of serum from each sample was used and two dilutions, 1:100 and 1:320, were initially tested. For the second ELISA, 3 µL of serum was used to test dilutions 1:100, 1:320 and 1:1000. On third ELISA experiment, 3 µL of serum was used to test SAA levels in the following dilutions: 1:320; 1: 1000; 1: 3,200; 1: 10,000; 1: 32,000; 1:100,000, and 1: 320,000. As with the CRP ELISA, wash steps were performed with 250 µL, and the number of wash steps was consequently increased from 4 to 8.

Transferrin ELISA

Transferrin ELISAs were done the same as outlined in the protocol in Mouse Transferrin ELISA test kit (Life Diagnostics, Inc., Cat. # 3450-1). Two microliters of serum from each sample was used, and 1:100,000; 1:320,000 and 1:1,000,000 dilutions were tested. Samples were run in singles except for the 1:320,000 dilution, which was run in duplicates. Wash steps were increased from 6 to 8.

Data Analysis

The samples were prepared as suggested by the ELISA kit instructions. Absorbance at 450 nm was measured with a spectrophotometer. ELISA data were analyzed by comparing differences in biomarker absorbance between control and infected mice.

RESULTS and DISCUSSION

Virus Titration (Endpoint Dilution Assay)

Serum samples collected at later hours post infection had higher viral titers than those collected earlier (Figure 7). Virus titers of serum collected from mice in groups 1 (0 HPI) and 3 (4 HPI) were below the detection limit of the assay (<0.67 $\log_{10}$ $CCID_{50}$/0.1ml). Almost all serum samples collected at 8 HPI or later showed increased virus titers confirming that the mice were successfully infected with influenza A(H1N1)pdm09 virus. Serum samples collected at 48 HPI showed lower virus titer average because only one mouse out of the five in the group showed a high virus titer result (7.67) while the other four had virus titer below the detection limit (< 0.67). This may be attributed to errors in infecting the mice or performing viral titration. Individual virus titers can be found in Appendix table 1B.

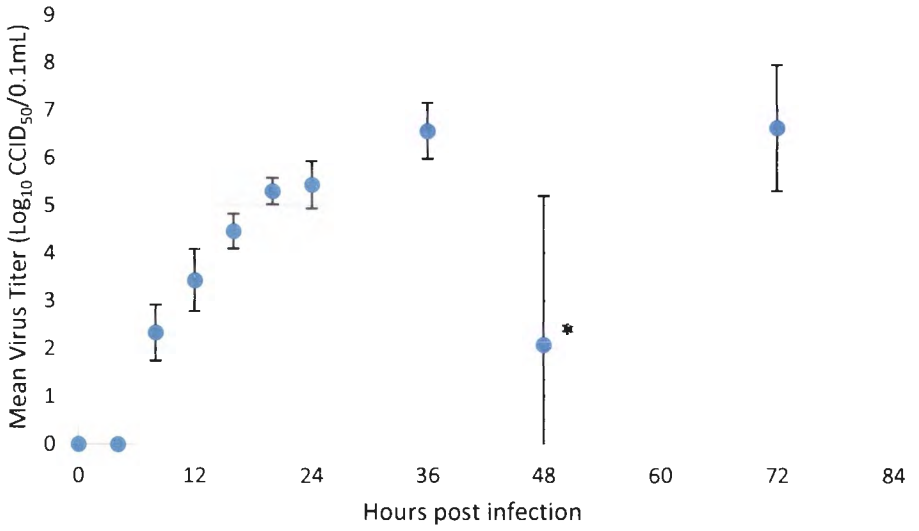

Figure 7: Mean virus titers (reported as $\log_{10}$ $CCID_{50}$/0.1 mL) of serum samples collected from five infected mice per group. Samples were collected at 0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours post infection (HPI). Asterisk at 48 HPI indicates potential technical error during mice infection or virus titration. Refer to Appendix tables 1A and 1B for average and individual titer data tables.

Enzyme-Linked Immunosorbant Assay (ELISA)

CRP ELISA

Control samples showed an infection-like curve with a peak between 12 and 36 hours in the serum samples that were diluted 1:100 (Figure 8a). However, at 72 HPI, infected serum samples had greater levels of CRP than did the control mice. More optimal absorbance results were obtained from samples diluted 1:320, where control mice sera have minimal levels of CRP while infected mice sera showed a drastic increase in CRP levels at time points 48 and 72 HPI (Figure 8b). The third dilution evaluated (1:1000) was too diluted and absorbance levels were too low to draw any conclusions (Figure 8c). CRP concentrations in serum samples could not be quantified due to inconsistencies in the absorbance values for the standard.

Interestingly, we could not replicate the results in a second ELISA experiment using the same samples (Figure 9). Both infected and control samples show an increase in CRP levels simultaneously starting from 48 HPI. However, this trend only continues for infected samples. At 72 HPI, CRP levels were higher for infected samples compared to control samples. Overall absorbance levels also decreased as compared to the first ELISA experiment. This could be due to potential degradation of CRP during extended storage at -80°C, which in this case was about a month.

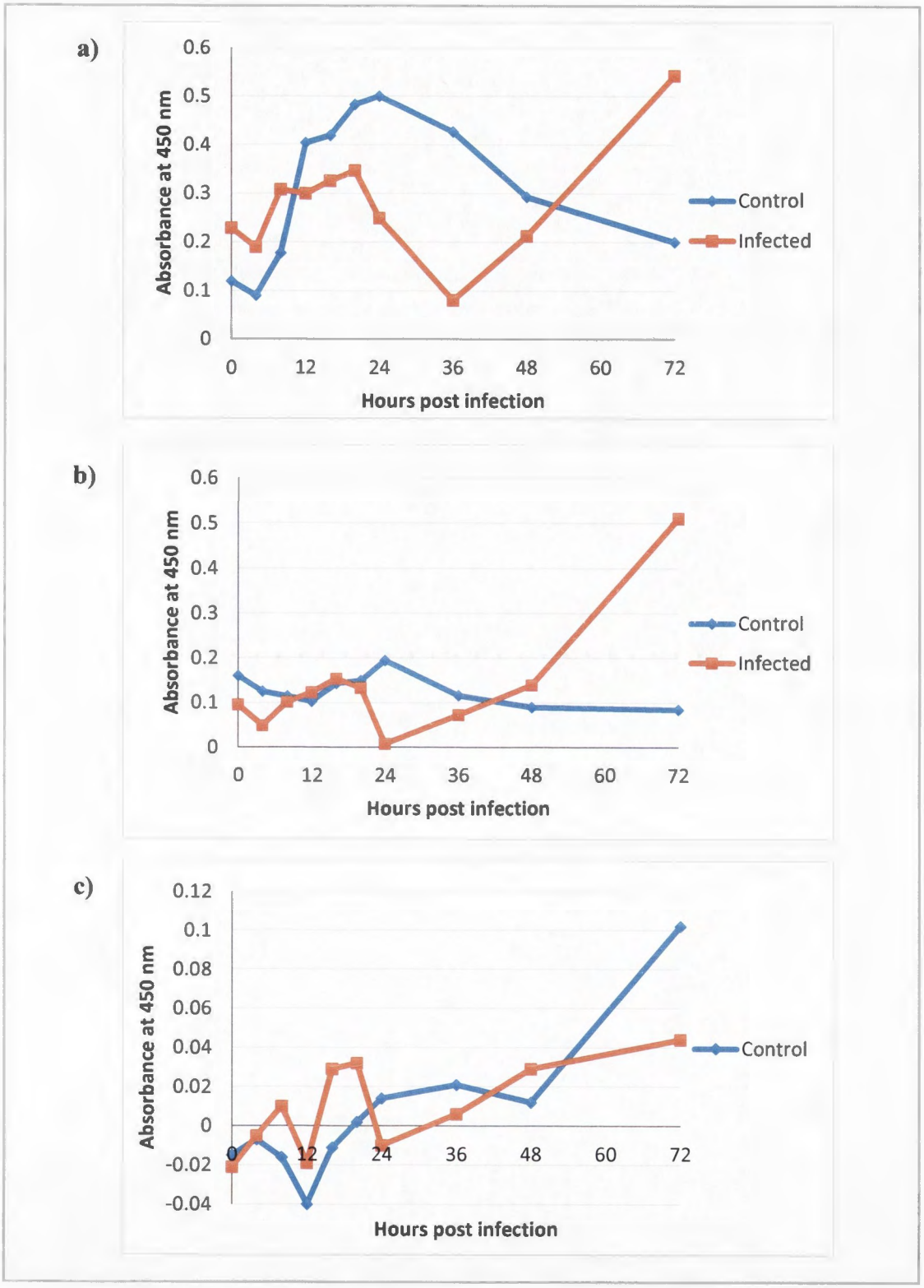

Figure 8: Absorbance readings from the first ELISA experiment for mouse CRP in serum samples from influenza virus-challenged (red) and control mice (blue) as measured 450 nm. **(a)** Samples were diluted 1:100 and were run as singles. **(b)** Samples were diluted 1:320. Samples were run in duplicates and mean values are plotted. **(c)** Samples were diluted 1:1000 and run as singles.

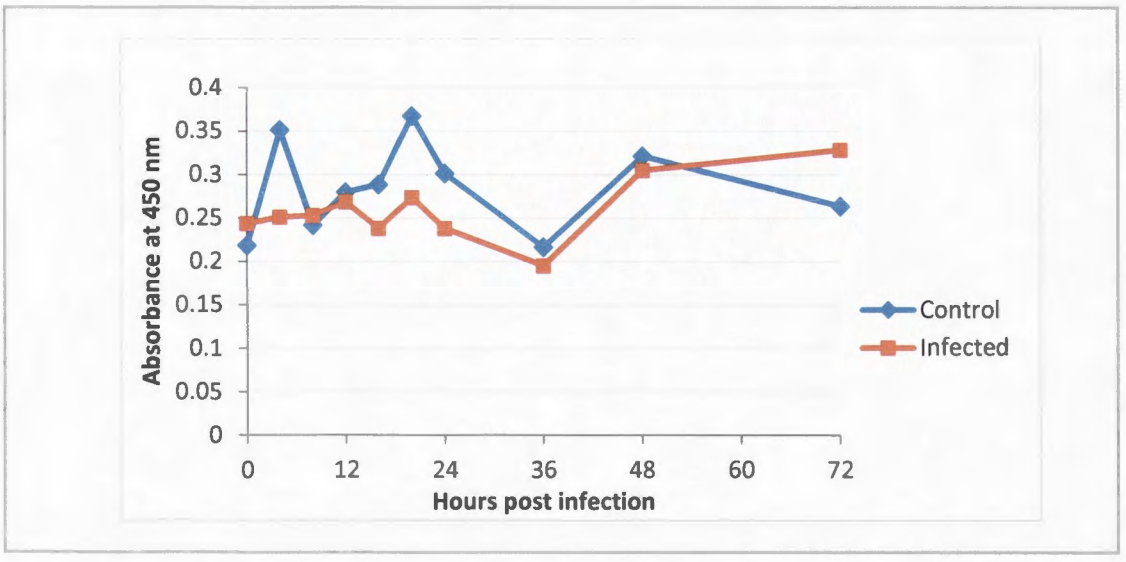

Figure 9: Absorbance readings from the second ELISA experiment for mouse CRP in serum samples from influenza virus-challenged and control mice as measured at 450 nm. Samples were initially diluted 1:320 in assay diluent. Samples were run in quadruples and mean values are plotted.

SAA ELISA

SAA appears to be a strong biomarker candidate. In the first experiment, serum samples collected from control animals showed only a baseline absorbance reading (Figures 10a and 10b) with minor fluctuations in the first 36 hours post sham-infection. Serum samples collected from virus-infected animals, however, indicated a clear increase in SAA starting at 48 HPI with a peak at 72 HPI.

A second experiment with samples diluted with dilutions 1:100, 1:320, and 1:1,000 was performed to validate that SAA was a good potential biomarker (Figures 11a, 11 and 11c). As expected from the first experiment, infected samples showed higher concentrations of SAA at 72 HPI in all dilutions, although minimal peaks could also be observed at 20 HPI in control samples.

In both experiments, absorbance values did not decrease with increased dilutions of samples at 72 HPI, indicating that SAA concentrations were too high for the spectrophotometer to detect the difference (Figures 10a, 10b and 11a, 11b and 11c). To further observe the effect of dilution on absorbance reading and to obtain a viable dilution factor that can be used in further investigations, a third ELISA test was performed. As shown in Figure 12, sera diluted higher than 1:1000 show greater differences in absorbance for the time point of interest, 72 HPI. If sera were diluted any further than 1:32,000 SAA levels were too diluted for detection. To avoid high and low ends of absorbance levels that cannot be detected by the spectrophotometer, the dilution 1:10,000 was selected as an optimal dilution factor for future ELISAs. The estimated concentration of SAA at 72 HPI was 2,428 μg/mL (1:10,000 dilution) with the 4-parameter regression model. The standard curve 4-parameter regression had an R^2 value of 1.

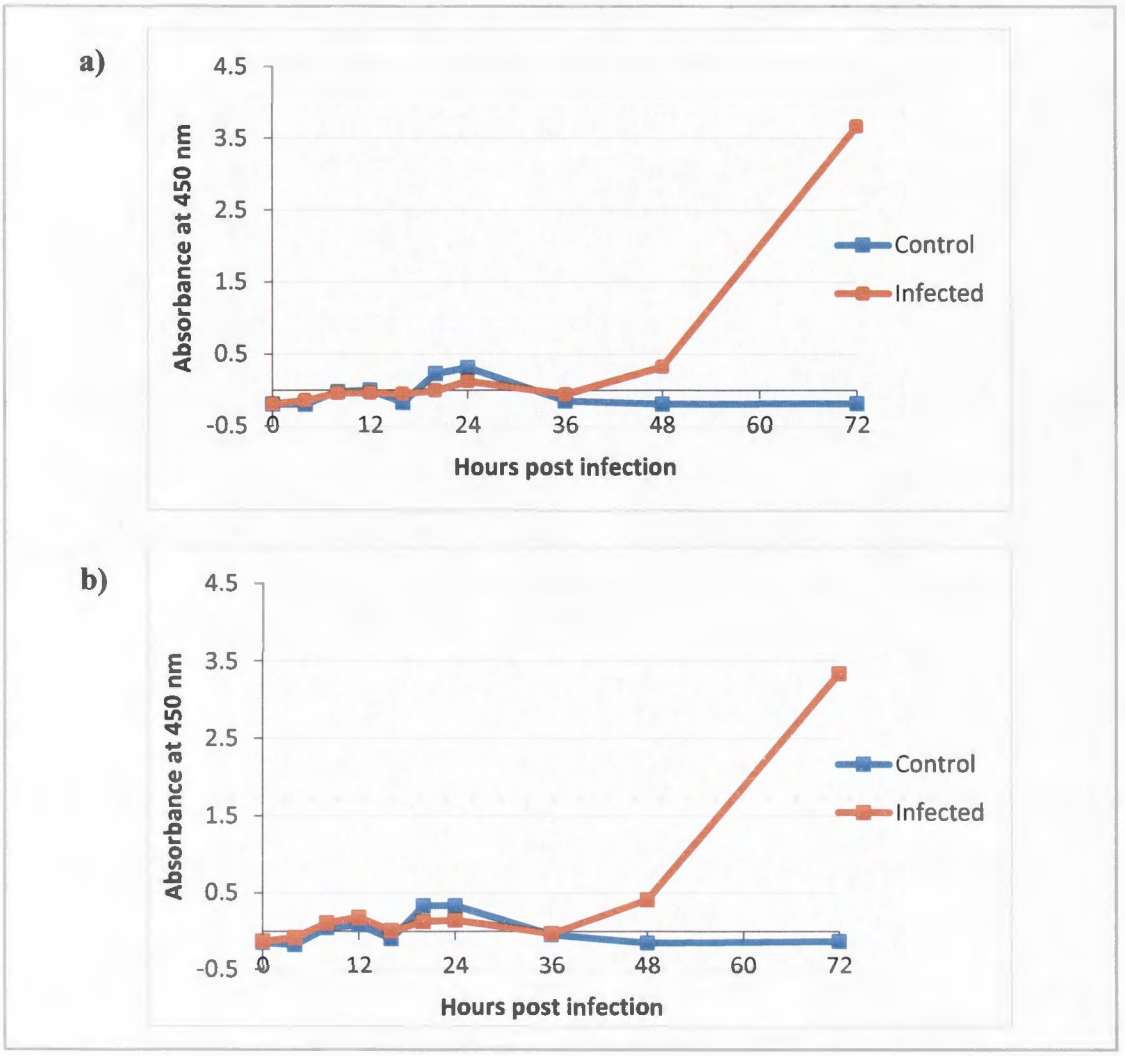

Figure 10: Absorbance readings from the first ELISA experiment for mouse serum amyloid A (SAA) samples from infected (red) and uninfected mice (blue). Values represent the mean of duplicates. (a) Serum samples were diluted 1:100 in assay diluent. (b) Serum samples were diluted 1:320 in assay diluent.

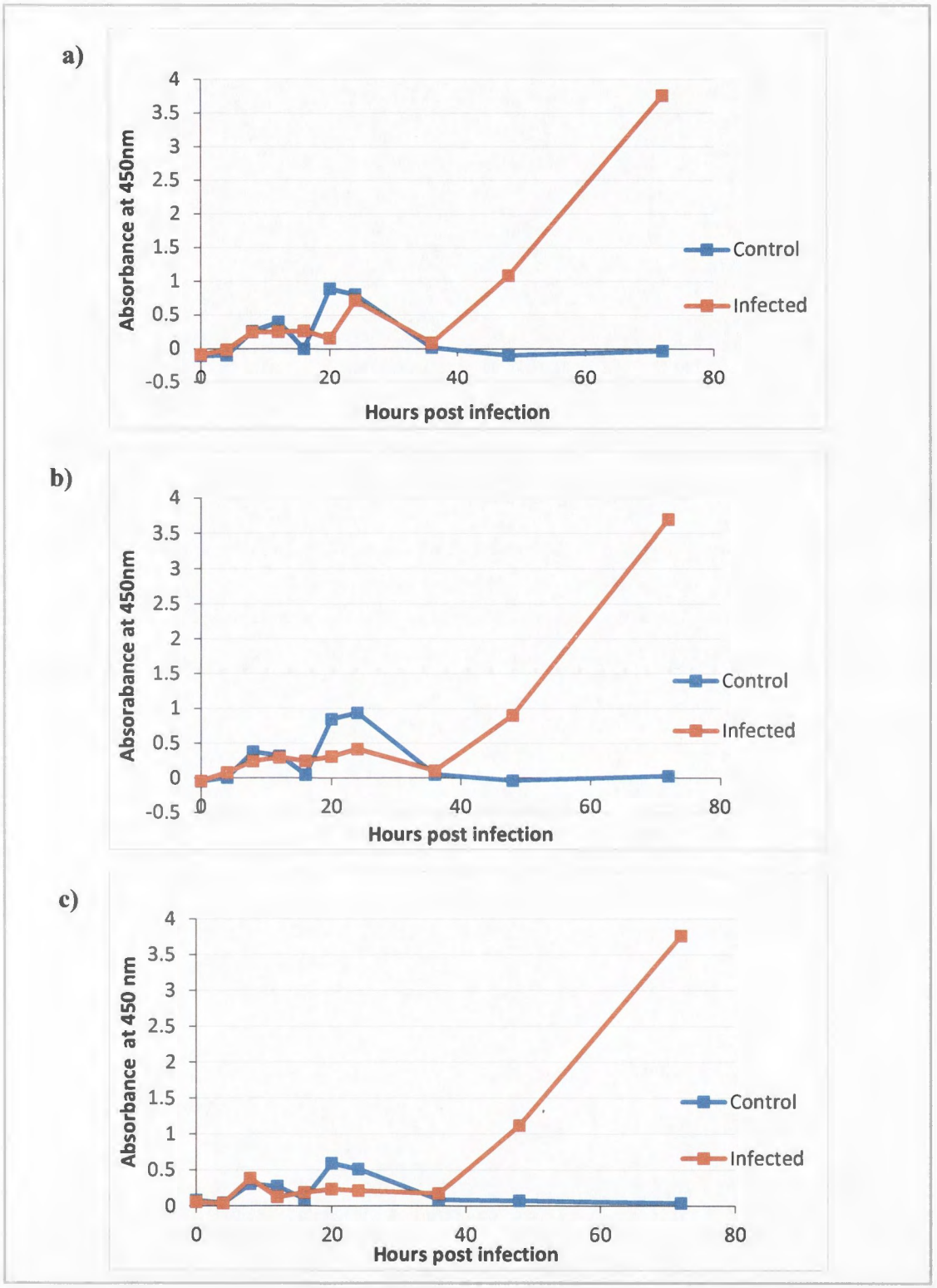

Figure 11: Absorbance readings from the second ELISA experiment for mouse serum amyloid A (SAA) samples from infected (red) and uninfected mice (blue). **(a)** Serum samples were diluted 1:100 in assay diluent and were run in singles. **(b)** Serum samples were diluted 1:320 in assay diluent and were run in duplicates. Values represent the mean of duplicates. **(c)** Serum samples were diluted 1:1000 in assay diluent and were run as singles.

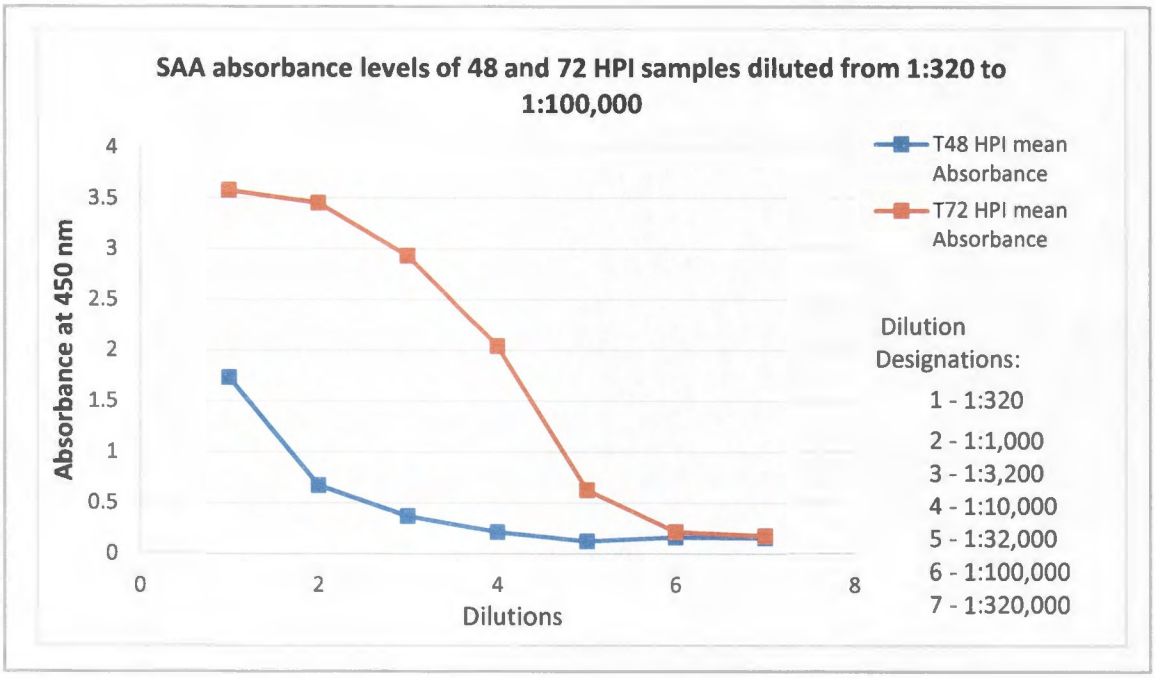

Figure 12: Absorbance values from the third ELISA experiment of serum samples collected at 48 and 72 HPI diluted in half logs from 1:320 to 1:320,000 to show the effect of dilution and fine tune the best dilution factor.

Transferrin ELISA

In the sera of infected mice, transferrin concentrations showed a sharp peak at 16 HPI followed by a return to base levels. The peak was about twice as high as in serum samples taken from control animals at the same time point. This trend could be observed from all three tested dilutions (1:100,000; 1:320,000; 1:1,000,000). Some absorbance readings at 1:1,000,000 dilution of sample were out of range (Figure 13c). Except for the time points 16 and 20 HPI, the transferrin concentrations in serum samples from control animals were actually higher than those levels in sera taken from infected animals. Thus transferrin may not be a strong early biomarker candidate.

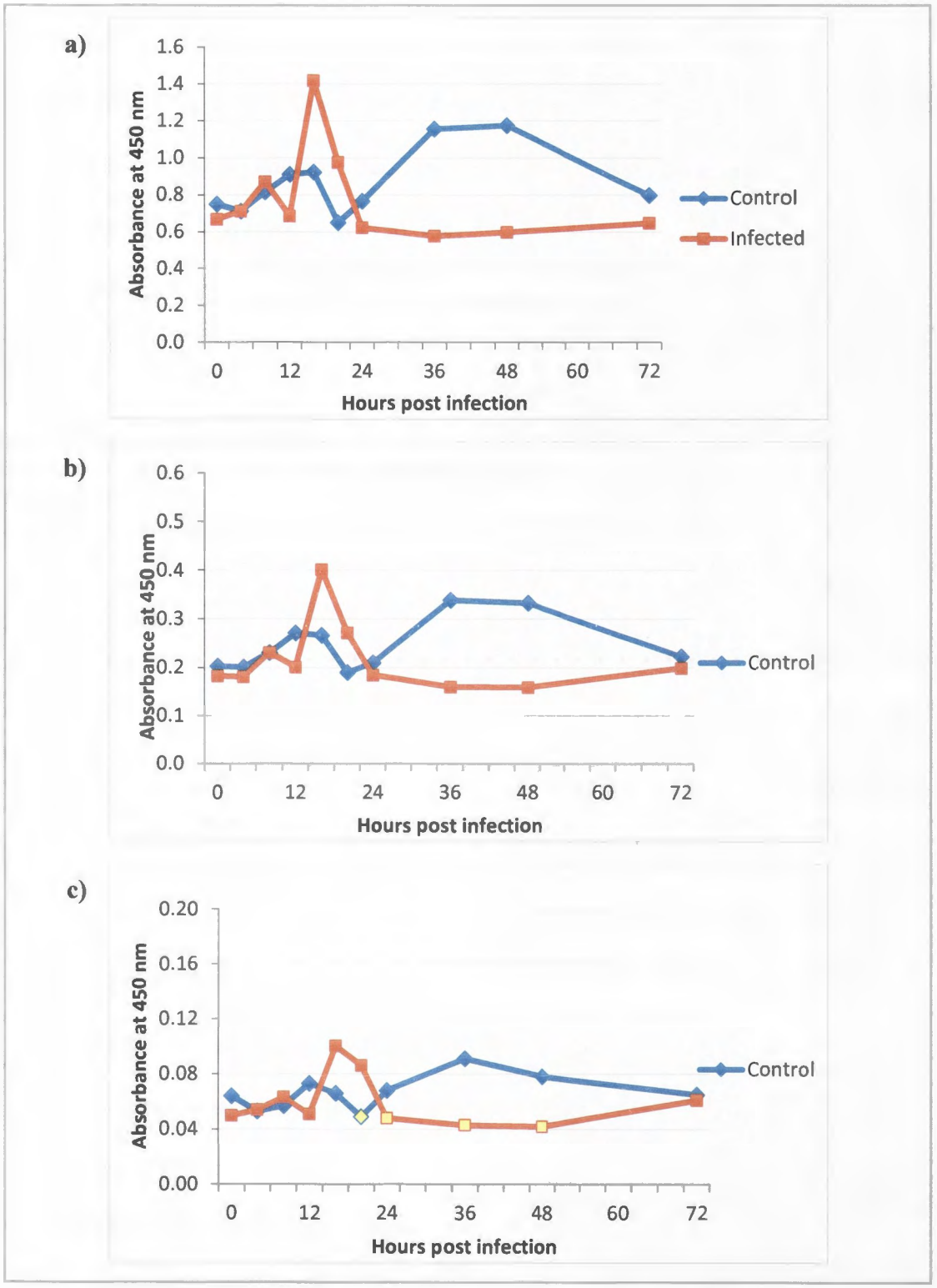

Figure 13: Absorbance readings for Transferrin of mouse serum samples from infected (red) and uninfected mice (blue). (a) Serum samples were diluted 1:100,000 in assay diluent and run as singles. (b) Samples were diluted 1:300,000 and run in duplicates. The mean of duplicate readings is shown. (c) Samples were diluted 1:1,000,000 in assay and run as singles. Yellow-filled markers indicate out-of-range readings.

CONCLUSIONS

The most promising biomarkers (CRP, SAA, and Transferrin) are presented and discussed in this thesis in detail. Their assessment, along with four other biomarkers (Complement Factor 3, Hydrogen Peroxide, Alpha 2, 6 – sialyltransferase and Prostaglandin E_2) tested in the project as potential early indicators of ARDS, are listed in Table 1.

Table 1: Summary of tested biomarkers and their potential as early indicators of ARDS. Seven biomarkers (CRP, SAA, Transferrin, Complement factor 3, H_2O_2, alpha 2,6-sialyltransferase, and prostaglandin E_2) were tested for their potential use as early indicators of ARDS. Biomarkers shaded in grey are discussed in this thesis.

Biomarker tested	Potential early indicator of ARDS
C-Reactive Protein (CRP)	No
Serum Amyloid A (SAA)	Yes
Transferrin	No
Complement Factor 3 (CF3)	No
Hydrogen Peroxide (H_2O_2)	No
Alpha 2,6-sialyltransferase	No
Prostaglandin E_2	No

The 72-HPI peak observed in the first CRP ELISA experiment could not be replicated in the second CRP ELISA experiment where samples were diluted with the ideal dilution of 1:320 and tested in quadruples. Thus, CRP does not appear to be a good potential early indicator of ARDS.

SAA proved to be a strong biomarker candidate for early detection of ARDS on day 3 (72 HPI) of infection. Serum collected from control mice show a baseline of SAA levels while those of infected mice show a steep increase of SAA after 48 HPI. This makes SAA an ideal biomarker. Further experiments are in progress to evaluate the levels

of SAA after 72 HPI. Changes in SAA levels in mice that have been treated with effective antiviral drugs should also be evaluated to further correlate SAA levels with ARDS.

At this point, it is not certain whether transferrin is a good biomarker candidate. Although infected mouse serum showed a much earlier peak in transferrin at 16 HPI, the validity of the peak seems questionable since control samples appear to have higher transferrin concentrations than infected samples at later time points.

In summary, SAA has been found to be the most promising biomarker that could be used for the early detection of ARDS in mice. Further experimentation is underway to measure SAA levels after 72 HPI. To further correlate SAA levels with ARDS, the influence of antiviral drugs on SAA serum levels will also be monitored in a separate experiment.

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APPENDIX

Table A1: Average virus titers (log_{10} $CCID_{50}$/0.1 mL) of serum samples collected from five infected mice in ten different groups. Each group was assigned to one time point at which serum was collected (0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours post infection, HPI).

Mouse group ID	Serum collection (HPI)	Average virus titer	Standard deviation
1	0	< 0.67	0.00
3	4	< 0.67	0.00
5	8	2.33	0.59
7	12	3.43	0.65
9	16	4.47	0.36
11	20	5.30	0.27
13	24	5.43	0.49
15	36	6.57	0.59
17	48	2.07*	3.13
19	72	6.63	1.33

*Low virus titer may be attributed to technical errors in mice infection or viral titration.

Table A2: Individual virus titers (log_{10} $CCID_{50}$/0.1 mL) of serum samples collected from five infected mice in ten different groups from which serum was collected at 0, 4, 8, 12, 16, 20, 24, 36, 48, and 72 hours post infection (HPI).

Group ID	Serum collection (HPI)	Mouse ID	Virus titer per mouse	Average group virus titer	Standard deviation
1	0	1_1	< 0.67	< 0.67	0.00
		1_2	< 0.67		
		1_3	< 0.67		
		1_4	< 0.67		
		1_5	< 0.67		
3	4	3_1	< 0.67	< 0.67	0.00
		3_2	< 0.67		
		3_3	< 0.67		
		3_4	< 0.67		
		3_5	< 0.67		
5	8	5_1	2.67	2.33	0.59
		5_2	2.00		
		5_3	3.00		
		5_4	2.50		
		5_5	1.50		

Group ID	Serum collection (HPI)	Mouse ID	Virus titer per mouse	Average group virus titer	Standard deviation
7	12	7_1	3.50	3.43	0.65
		7_2	4.33		
		7_3	2.50		
		7_4	3.50		
		7_5	3.33		
9	16	9_1	4.50	4.47	0.36
		9_2	5.00		
		9_3	4.50		
		9_4	4.00		
		9_5	4.33		
11	20	11_1	5.50	5.30	0.27
		11_2	5.00		
		11_3	5.00		
		11_4	5.50		
		11_5	5.50		
13	24	13_1	5.67	5.43	0.49
		13_2	4.67		
		13_3	5.50		
		13_4	5.33		
		13_5	6.00		
15	36	15_1	6.67	6.57	0.59
		15_2	5.67		
		15_3	6.67		
		15_4	7.33		
		15_5	6.50		
17	48	17_1	< 0.67	2.07	3.13
		17_2	< 0.67		
		17_3	< 0.67		
		17_4	< 0.67		
		17_5	7.67		
19	72	19_1	7.33	6.63	1.33
		19_2	7.50		
		19_3	7.33		
		19_4	4.33		
		19_5	6.67		