Antibody Response to the Influenza Virus Over Time as it Relates to Varying Metabolic States

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April, 2017

APPROVED:

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Table of Contents

5. Figures and Charts

1. Introduction

1.1 Influenza

Influenza has been a historically deadly virus and remains a serious health threat that can affect all populations. In 1918-19, a deadly strain of the influenza virus, known as the Spanish Flu, shook the world, resulting in the greatest pandemic in recorded history, killing approximately 21 million people[8]. Pandemic flu refers to the introduction of a novel virus strain that a large proportion of the population has not previously been exposed to, and therefore does not have pre-existing immunity[9]. These strains are often introduced via zoonotic transfer from animals such as pigs and birds. When this happens, morbidity and mortality from the virus is greatly increased. More recently, during the 2009 swine flu pandemic the A/California/7/2009 (H1N1) strain of the virus was responsible for 151,700 and 575,400 deaths worldwide[8]. A study conducted in California in response to this epidemic found a disproportionate amount of cases, including illnesses and deaths, were in the obese population[7]. The authors of that study concluded that obesity stands alone as an independent risk factor for morbidity and mortality from pandemic influenza. In a study conducted by Sheridan et al. 2011, obesity was found to be associated with an impaired adaptive immune response to influenza vaccination[2]. Body mass index was found to correlate with a greater decline in antibody response 12 months post vaccination as compared to healthy weight individuals.

Beside pandemic flu, seasonal influenza, which refers to circulating annual influenza strains that could have minor changes in their protein structures year-to year, remains a serious health threat. Seasonal influenza can affect virtually all populations because of its relative ease of transmission via droplets from infected persons coughing,

sneezing or talking. The flu season spans from October to May each year in the northern hemisphere. The virus has been known to be especially harmful to very young children, the elderly, pregnant women and persons with specific chronic medical conditions such as immunodeficiency diseases, asthma and chronic heart or lung diseases and more recently obesity.[1] Annual vaccination is still the primary method of defense in terms of decreasing the impact of the influenza infection on a given population.

1.2 Vaccine and Adaptive Immunity

The influenza vaccine is usually administered with an intramuscular injection every year to protect people from what research has projected to be the most common circulating strains that season[10]. Usually the vaccine contains inactivated forms of one influenza A H1N1 strain, one influenza A H3N2 and one or two influenza B strains, making the vaccine either trivalent or quadrivalent. The vaccine is administered to help the body prepare for the potential of actually contracting the virus from the environment, using the bodies adaptive immunity.

The body's immune system is comprised of two distinct segments, the innate and the adaptive immunity. This innate immunity has a broader response and is comprised of lymphocytes like macrophages and natural killer cells. The adaptive immunity is more specific and utilizes B-cells with the help of T-cells to identify antigens and create specific antibodies also known as immunoglobulins. When a vaccine is administered, naïve B-cells are activated by binding to the antigen. T helper cells identify antigen presenting B-cells and secrete cytokines which activate the differentiation and proliferation of antibody producing plasma cells and memory B-cells. If the same pathogen comes back, memory B-cells exhibit a fast and specific response to the antigen

by releasing antigens that bind to the virus, preventing them from binding with other cells in our body and tagging them for destruction. The most common immunoglobulin is IgG1 which responds primarily to soluble protein antigens like viruses[11].

1.3 Obesity

This adaptive immune response was found to be impaired in obese individuals, in response to the influenza virus.[2] This finding has some serious implications because there is an obesity epidemic in the U.S. where more than one third of the adult population is considered obese or extremely obese (BMI of 30+ and BMI of 40+ respectively) and over two thirds of the U.S. adult population is considered to be overweight or obese.[3]

Obesity is associated with systemic inflammation caused by hypertrophy of adipose tissues, especially visceral adipose, that outgrow their matrix. This increased adipocyte growth results in the secretion of inflammatory cytokines like IL-6 and TNFα and chemokines which recruit macrophages to the adipose tissue, further propagating the secretion of inflammatory cytokines.[12] The release of these inflammatory cytokines like IL-6 and TNFα have been found to reduce systemic insulin sensitivity by impairing the insulin signaling pathway.[4] Obesity is also associated with an increase in leptin secretion and is associated with a decrease in adiponectin, which may also play a role in impairing the body's metabolic condition.

The systemic nature of these metabolic changes suggests that it can also affect immune function and other important bodily functions. Not every individual who is obese, meaning they have a BMI of 30 or greater, exhibits these kinds of inflammatory responses. Obese individuals who are metabolically healthy can also be identified. However, obese individuals who are exhibiting these inflammatory responses to

increased adiposity are at a higher risk of experiencing further complications such as cardiovascular disease, metabolic syndrome and type-2 diabetes.[4]

1.4 Type-2 Diabetes:

A strong association between obesity and type-2 diabetes exists, as excess weight has been established as a risk factor for type-2 diabetes (T2D). The underlying mechanism is again associated with the inflammatory response that is experienced in hypertrophic adipose environments. The release of inflammatory cytokines from adipocytes impairs the insulin signaling pathway, preventing the pathway from signaling the translocation of GLUT-4 to the cell membrane. GLUT-4 is a glucose transporter which allows the passage of glucose from the blood into the cell. This impairment means that more and more insulin is required to allow glucose to enter into the cells and eventually the cells can become resistant to insulin.[12] Insulin resistance in the hallmark of T2D which leads to having a fasting blood glucose of 125mg/dL or higher, where the normal level is between 75-100mg/dL.

Given the close association between obesity and T2D, and that obesity is associated with an impaired immune response to the influenza vaccine, it may be expected that type-2 diabetics would experience a similar response to the vaccine. However, when looking into the effects of diabetes on antibody response to the influenza vaccine, Sheridan et al. found that there was no significant difference between the responses when considering diabetes alone as the risk factor.[5] This poses a new question: What is the difference between obese individuals who are metabolically healthy and obese individuals treated with a diabetic drug in their ability to response to the influenza vaccine?

1.5 Metformin

One thing to consider when differentiating between obese individuals and type-2 diabetics is that, type-2 diabetics often take medications to control their blood glucose. Metformin is the most widely prescribed first medication for patients with T2D, and it was not considered in the Sheridan et al. study. Metformin plays an important role in increasing insulin sensitivity, through its activation of AMPK, which in the liver decreases hepatic glucose production, induces fatty acid oxidation and increases GLUT4 expression on cellular membranes in the skeletal muscle.[6] There is also some evidence to suggest that metformin has a direct effect on the metabolism of adaptive immune cells, potentially increasing their fatty acid oxidation, making their glucose metabolism more efficient.[13]

1.6 Aims and Hypothesis

In previous studies, the antibody response to the trivalent influenza vaccine at 30 days and 12 months post vaccination was analyzed. Subjects were vaccinated between Sept-Oct, and as the peak of the flu season is usually somewhere between December and March[10], a mid-point between 30 days and one year post vaccination would be important to test. It is also important to consider the metabolic profiles of the subjects and whether metformin use can influence the antibody response. Therefore, we initiated an experiment into the investigation of the effect of time and metabolic state (diabetic or non-diabetic) on the humoral and cell mediated immune responses to the A/California/7/2009 (H1N1) pdm09-like virus. We hypothesized that treatment of metformin may normalize the metabolism of the immune cells, therefore their antibody responses may be higher compared with obese people not treated with metformin.

2. Material and Methods:

2.1 Study design and subjects

Study participants were selected from an ongoing prospective observational study carried out by the Beck Lab at the UNC Family Medicine clinic. Samples from year 8 of the study, during which the 2016-17 trivalent vaccine was administered, were called in for three blood draws at consistent time points. The three different time points were classified as "Day 0", which was pre-vaccination, 30 days post vaccination and 6 months post vaccination. Patients were compensated for their participation and were selected to match age, sex and race across three different groups: healthy weight (HW, n=6), obese nondiabetic (OH, n=5) and obese diabetic (OD, n=4). All subjects were white non-hispanic females ages 37 – 58. All obese diabetics were taking metformin and all healthy weight and obese nondiabetics were not. Demographic data displayed in Table 5.1.

2.2 Preparation of Virus and Serum Samples

Virus was inactivated using β-propiolactone and hemagglutination titer (HAU) was measured to be 80HAU/50μL. All serum samples were treated with receptor destroying enzyme (RDE) and diluted in physiological saline.

2.3 ELISA - enzyme-linked immunosorbent assay of IgG1

IgG 1 antibody was quantified using enzyme-linked immunosorbent assay using A/California/7/2009 (H1N1) strain. This strain was chosen because it was found in both the 2015 and 2016 trivalent influenza vaccine. Virus was diluted in a carbonate/bicarbonate buffer and coated onto micro-titration plates. Wells were blocked using a carbonate based milk protein buffer. Plates were washed with PBS-tween (PBSt). Serum serial dilutions were prepared in PBS based milk protein buffer and allowed to

react with virus. Plates were again washed with PBSt. Goat anti-human IgG 1 antibody was diluted in PBS based milk protein buffer and was added to plates and allowed to bind to human antibody. Plates were washed and binding was detected using peroxidase substrate. Color intensity was measured and quantified using a plate reader to measure absorbance at wavelength 450nm. The internal positive control was used on every plate and all three time points for each patient was measured on the same plate.

2.4 HAI - haemagglutination inhibition assay

HAI assays were conducted to determine the level of antibodies in serum as previously described.[5] RDE-treated serum was incubated in duplicate in a 96 well plate for 15 min at room temperature after making serial dilutions of the serum down the plate. After a one hour incubation at 4°C with either 0.5% turkey red blood cells, HAI titer was determined by the reciprocal dilution of the last well. Positive controls were included on each individual plate. An HAI titer of 1:40 is considered to be the threshold for seroprotection against influenza, while a 4-fold increase in HAI titers from pre-vaccination to post-vaccination is considered seroconversion.[5]

2.5 Statistical Analysis

Associations between baseline variables and antibody response at all three time points were assessed using Kruskal–Wallis test for categorical variables (metformin and menopause status). A two-way Anova was conducted on ELIZA relative absorbance values and HAI titers at day 0 (pre-vaccine), 30 days post-vaccine, and 6 months post vaccine. HAI titers fold change was measured between day 0 – 30 day and 30 day – 6-month time points and prevalence of seroprotection and seroconversion were found. All data was analyzed on Prism GraphPad Version 7.

3. Results

3.1 ELISA Data: Antibody response may be influenced by metabolic state

Antibody response at Day 0 across the three groups was similar with no significant differences. As expected all three groups exhibited their lowest antibody response prior to vaccination. Thirty days post vaccination, healthy weight individuals and obese nondiabetics responded similarly to the virus but obese diabetics had a trend towards a higher percent change in their response (p=0.06) (Figure 5.4). While the trend is visibly clear on the graph (Figure 5.3), there was no significant difference in antibody responses between the three groups (p=0.09). All groups showed a decreased response 6 months post-vaccination as compared to 30 days post-vaccination. There was no association between ELISA measured antibody response and menopausal status (p=0.438) or BMI (p=0.627) but there was a significant difference in the antibody response 30 days post-vaccination in subjects taking metformin (p=0.04) (Table 5.2).

3.2 HAI Data: Antibody response not associated with differences among the groups

When measuring for all antibodies that would mount a protective response to the virus, there were no significant differences between the three groups (p=0.32) (Figure 5.5). However, those with the highest HAI titers across the entire sample size were also in the obese diabetic group. When measuring for fold-change in HAI titer across the three groups the results were insignificant, for both fold-increase Day 0-30 Days (Figure 5.6, p=0.89) and fold-decrease 30 Days-6 Months (Figure 5.7, p=0.35).

There was no association between HAI measured antibody response and menopausal status (p=0.96), BMI (p=0.99), or metformin use (p=0.35) (Table 5.2).

4. Discussion

After the 2009 flu pandemic, it was found that obesity is an independent risk factor for morbidity and mortality from influenza[7] and Sheridan et al. reported that obesity impaired the adaptive immune response to the influenza vaccine[2]. Given that obesity and type-2 diabetes are closely associated, in Sheridan et al. published a study examining the immune response to the influenza vaccine of diabetic individuals[5]. Interestingly however, there were no significant differences in the immune response of diabetic and non-diabetic subjects. One important distinction to make between obese non-diabetics and obese diabetics is that many obese type-2 diabetics are taking metformin[6], which is known to improve their metabolic state and could potentially improve their immune cell function[13].

4.1 Metformin could be associated with improved adaptive immune responses

Based on the findings of this study, the ELISA data suggests that obese diabetic subjects taking metformin had a higher antibody response than both healthy weight and obese nondiabetic individuals (Figure 5.3). This indicates that there may be a link between metformin use and antibody response, given that the obese diabetic subjects were the only group taking the drug. However, the HAI antibody response data does not reinforce these findings because the obese diabetic group did not display any significant differences in their response to the virus as compared the other two groups (Figure 5.5). It is interesting to point out, though, that only in the obese diabetic group were 100% of the subjects seroprotected 30 days post vaccination (Table 5.10). These findings suggest the potential of metformin aiding the immune response of obese individuals.

According to the recently published researched, metformin may actually help the immune cells respond to the influenza virus. The mechanism by which this is happening is not entirely clear but there are some suggestions in the literature. One study suggests that metformin blocks a specific signal transduction pathway linked to glucose and anabolic metabolism that ultimately activates NF-κB. In this regard, metformin improves CD8 T-cell memory by modulating fatty acid metabolism.[13]Metformin is currently being used as an immunemodulator for tuberculosis, although it is not clear what cells metformin is targeting.[14] Additionally, metformin is used for anti-tumor effects through inhibiting mitochondrial complex I in the electron transport chain.[14] These are all promising points to suggest that metformin could play an important role in modulating the adaptive immune response, but more research needs to be done to better understand this relationship.

4.2 Peak antibody response does not correlate with peak of flu season

This study found that all three groups had a lower response to the influenza virus 6 months post vaccination as compared to 30 days post vaccination (Figure 5.3). Considering that many people get their vaccination in early September, when the flu vaccine is first made available, they may not be as protected by the time the peak of the flu season hits. In North Carolina, during the 2016-17 flu season, circulation of the virus peaked in early March and again at the beginning of April[16], about 6 – 7 months after the flu vaccine was released to the public. This could mean that those who were vaccinated early in the season may not be as well protected as they would have been had they been vaccinated closer to the peak of the season. However, during the 2014-15 flu season, the influenza illnesses peaked at the beginning of January.

4.3 Strengths and Limitations

This study used two proven methods of measuring antibody response and the same positive control was used on every plate. The antibody response was also found over varying time points to help create a timeline. This helped us determine that the peak antibody response for our subjects did not correlate with the peak of the flu activity. We also used medical records and current serum glucose levels to identify diabetics and metabolically healthy obese individuals.

There were several weaknesses of this study. The duration of diabetes was not considered in study subjects. It is possible that the length of time the subjects had diabetes had an effect on their immune response. The sample size was small (n=15), making the data underpowered. The study only represented white non-Hispanic women ages 37-58. The subjects' serum was tested for response to one virus, but the vaccine that they were given was trivalent. It could have been that subjects responded differently to different vaccine strains, but that was not captured in the scope of this study.

4.4 Future Research

Because of the abundance of evidence in the literature to suggest that metformin has beneficial effects on the immune cells, it is important to look further into how metformin specifically affects the adaptive immune cell function. This can be done collecting more in-depth metabolic data, including T-cell and B-cell metabolism, respiration rates and the fuel sources they are using to generate energy. It is also important to investigate the effects of metformin on antibody response in a more diverse group of people. To ensure a larger sample size, mouse models could also be used to investigate metformin in a more controlled environment.

5. Figure and Charts

Table 1. Demographic data of study subjects

Variable	HW	OH	OD
Sex - no. (%)			
Female	6 (100.0)	5 (100.0)	4 (100.0)
Age - yr			
Mean ± Std	48 ± 7	51 ± 6	49 ± 11
Range	40 - 58	44 - 57	37 - 58
Race - no. (%)			
White	6 (100.0)	5 (100.0)	4 (100.0)
Ethnicity - no. (%)			
Non-Hispanic	6 (100.0)	5 (100.0)	4 (100.0)

Table 2. ELISA and HAI antibody related to menopausal status, BMI and metformin use

		Menopausal	BMI	Metformin	Antibody
HAI Titer Day 0	95% CI	(-0.634, 0.3944)	(-0.6057, 0.4324)	(-0.1577, 0.7641)	(-0.4416, 0.5985)
	p-value	0.605	0.673	0.143	0.7027
30 Day		(-0.5004, 0.5478)	(-0.5272, 0.5218)	(-0.2772, 0.7066)	(-0.5787, 0.4657)
		0.961	0.992	0.352	0.7823
6 month		(-0.3684, 0.6519)	(-0.5111, 0.5376)	(-0.3657, 0.6536)	(-0.3189, 0.6829)
		0.479	0.950	0.519	0.3721
Antibody Day 0	95% CI	(-0.4773, 0.5687)	(-0.5271, 0.5219)	(-0.1355, 0.7734)	-
	p-value	0.864	0.995	0.138	-
30 Day		(-0.6679, 0.3436)	(-0.4185, 0.6164)	(0.04851, 0.8378)	-
		0.438	0.627	**0.037**	-
6 month		(-0.5687, 0.4773)	(-0.448, 0.5934)	(-0.1355, 0.7734)	-
		0.864	0.724	0.138	-

Figure 5.3

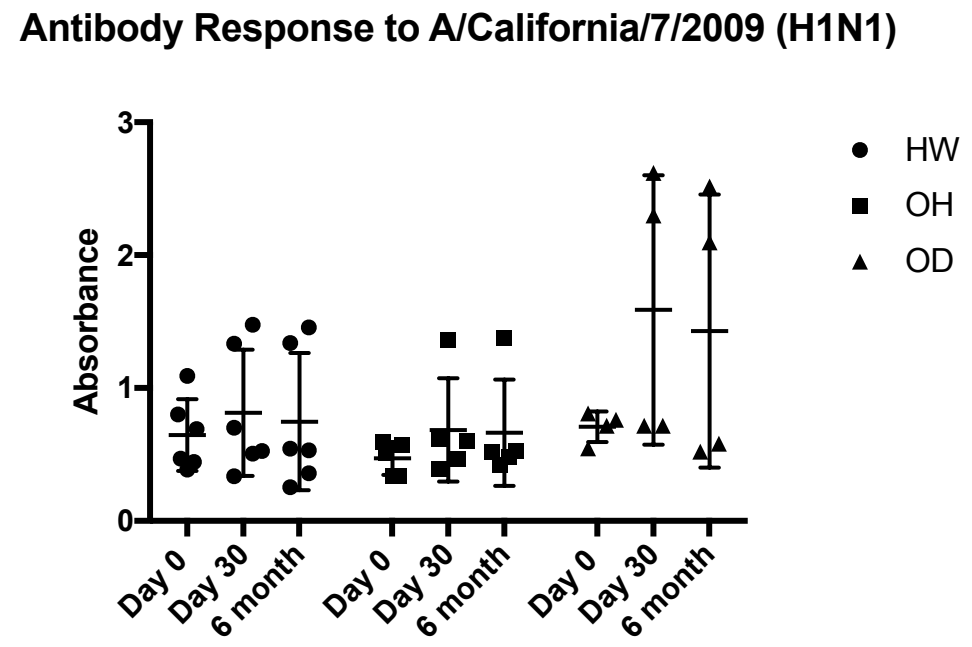

Figure 5.4

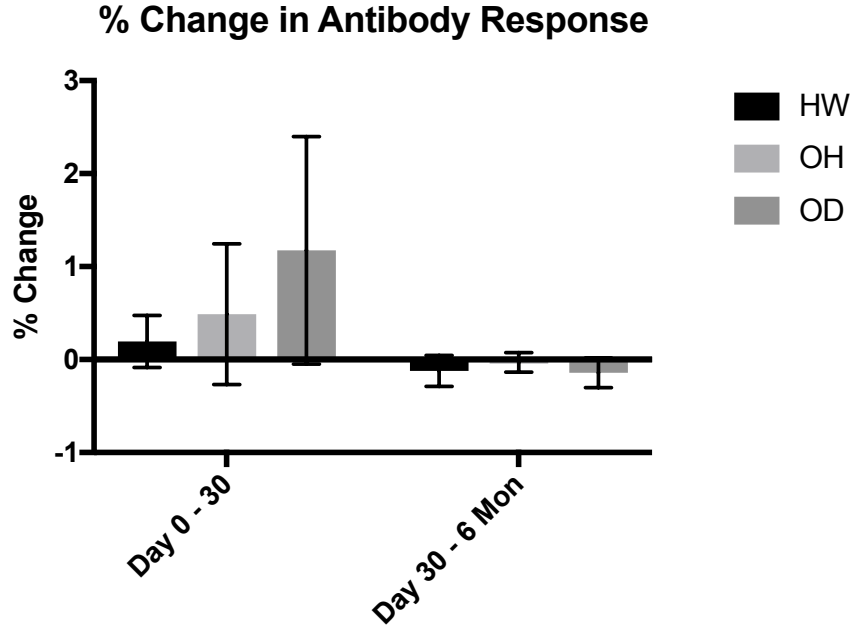

Figure 5.5

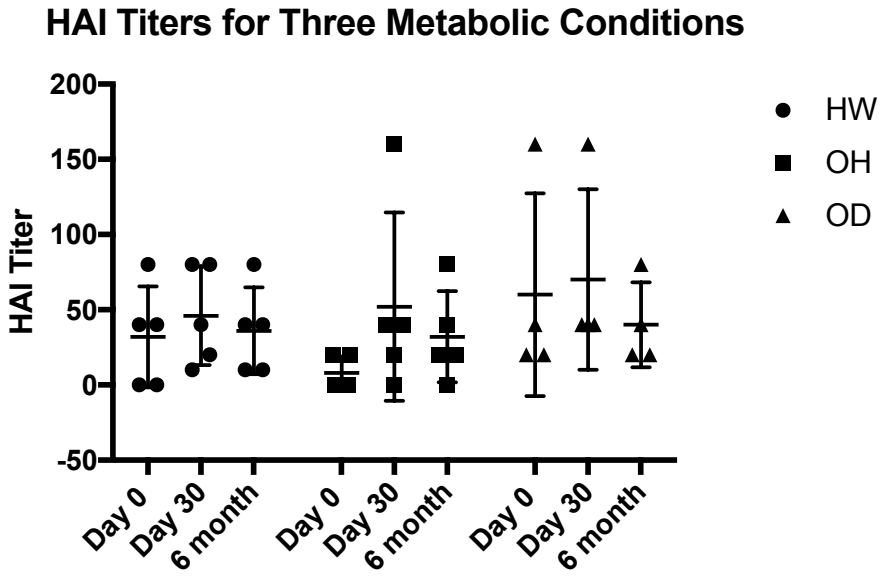

Figure 5.6

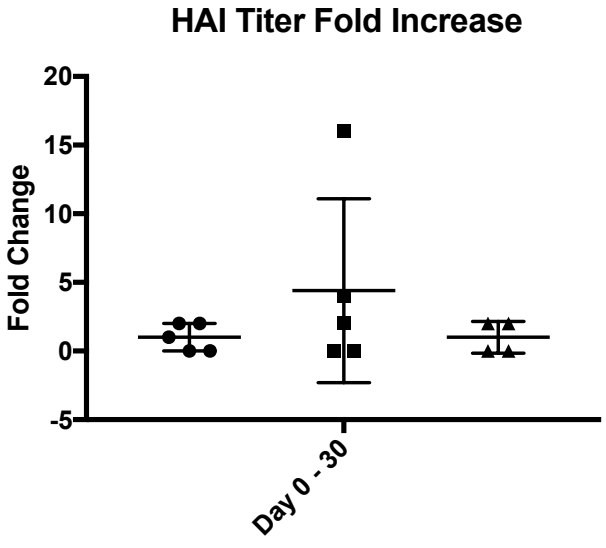

Figure 5.7

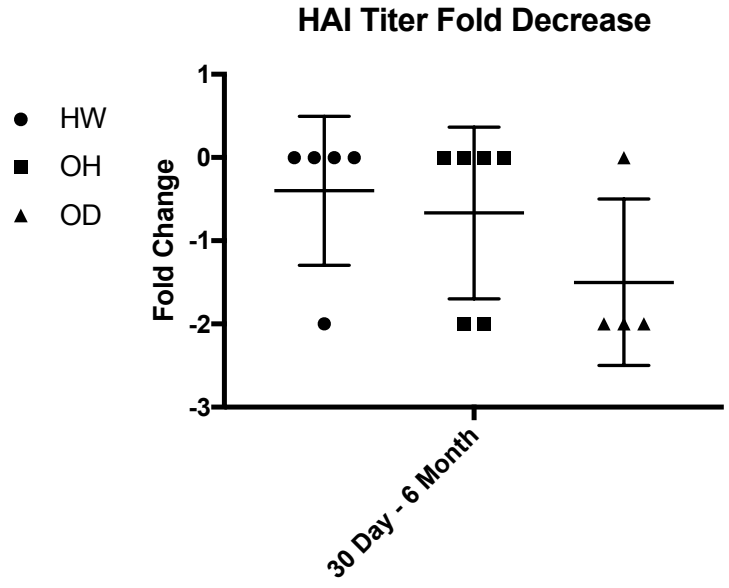

Figure 5.8[16]

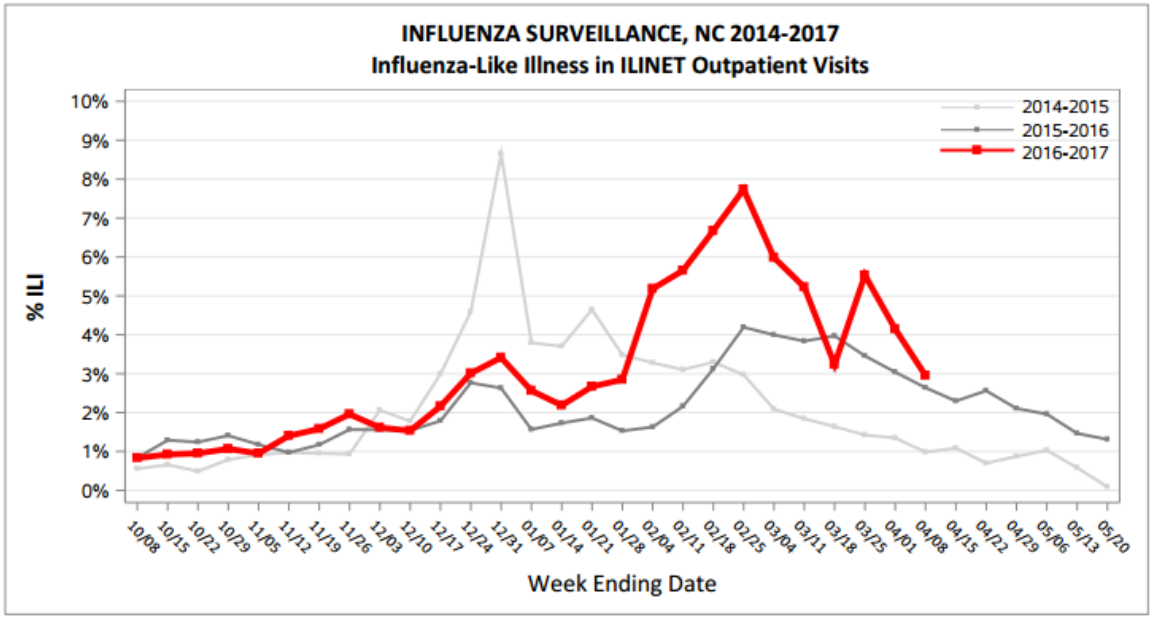

Figure 5.9[16]

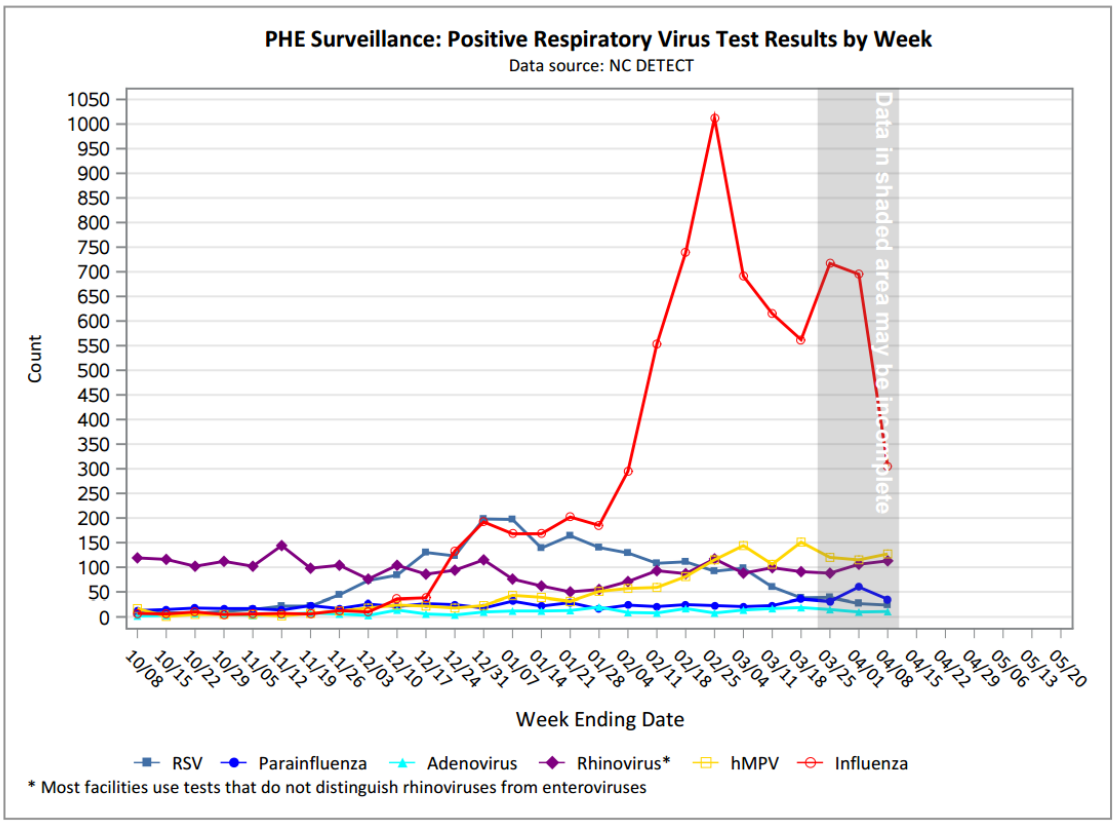

Table 5.10 HAI: Seroprotection and Seroconversion of the Three Groups Post Vaccination

Seroprotected	HW	OH	OD
30 Days	60%	80%	100%
6 Months	60%	60%	50%
Serconverted	HW	OH	OD
30 Days	0%	40%	0%

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