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APPLICATION OF FLOW CYTOMETRY TO DETECT *STAPHYLOCOCCUS AUREUS* IN MILK SAMPLES

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ABSTRACT

Mastitis is inflammation of the udder typically caused by bacteria, fungi and yeast. Of the various pathogenic organisms that cause mastitis, *Staphylococcus aureus* is one of the most commonly isolated pathogens from mastitic milk. In this study, flow cytometry was explored as a new diagnostic tool for detection of *S. aureus* in milk samples. The study was conducted in two phases. In the first phase, *S. aureus*, *Escherichia coli*, *Staphylococcus warneri* and *Staphylococcus epidermidis* were inoculated in Brain-Heart Infusion (BHI) broth then serially diluted. These serial dilutions were then combined with Protein A antibody marked with a FITC fluorescent tag, specific for *S. aureus*. The specificity of the antibody was tested with *S. aureus* and against the other bacteria species. The findings of this part of the study showed that a 1:500 dilution of Protein A antibody was the optimal dilution for the flow cytometric assay. The study also showed that the antibody was highly specific for *S. aureus.* In the second phase of the study, milk samples were inoculated with *S. aureus* and washed with Triton X-100 and Proteinase K to remove the milk lipids and proteins prior to the addition of the Protein A antibody. Based on the fluorescence frequency it was difficult to discern the inoculated milk from the un-inoculated milk samples. In summary, it can be inferred that flow cyotmetry can be used to *detect S. aureus* in BHI broth, however detection of *S. aureus* directly from milk is hindered perhaps due to the inability of Protein A antibody to bind to *S. aureus* due to background interference of milk proteins and fat. It is recommended that a more effective washing step be developed to remove milk proteins and fat, in particular proteins that could bind to protein A, to improve the

diagnostic sensitivity of flow cytometry for detection of *S. aureus* directly from milk samples.

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Chapter 1

Introduction

Mastitis is a significant problem for the dairy industry and causes major economic losses (Kromker et al., 2012). Mastitis results in decreased productivity and in severe cases results in loss of function of the affected mammary gland and even death. In addition, the discarding of milk from the affected quarters and the treatment of cows with antimicrobial agents results in significant economic losses to dairy producers (Jayarao et al., 1991). The predominant bacteria species involved in mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and coliforms. (Watts, 1987).

Staphylococcus aureus is one of the most important mastitis pathogens in cattle, involving large economic loss (Halasa et al., 2007; Hogeveen et al., 2011). Researchers have focused extensively on the control of *Staphylococcus aureus* mastitis in dairy cows because of the contagious nature of this organism and its poor response to antibiotic therapy (Prescott, 1988). *Staphylococcus aureus* intra-mammary infection is typically diagnosed by bacteriological culturing. This procedure is both laborious and time consuming. Also for the conventional diagnostic methods, three consecutive samples are necessary to achieve a satisfactory diagnostic sensitivity (Sears et al., 1990). Due to these technical difficulties many cases of mastitis remain undetected, making the control of *S. aureus* mastitis difficult. These conventional methods must be carried out over many

days by trained technicians that know how to identify specific bacterial colony types and interpret the test results (Thornberg et al., 2000). Alternative diagnostic methods include immune-assays and PCR-based assays that have been developed for the detection of *S. aureus* in mastitis milk. Enzyme-linked immunosorbent assay (ELISA) has been developed for detecting *S. aureus* antibody in bovine milk (Matsushita et al., 1990) but there are considerable disagreements between the ELISA results and the culture results. Methods to purify *S. aureus* antigen, which can react with antibodies in the milk have been described (Adams et al., 1988). Also Real-time PCR methods have been developed for quantification of *S. aureus* in milk (Goto et al., 2007). This method targets the putative transcriptional regulator gene of *S. aureus*. However, the routine use of these assays has limited success.

The alternative diagnostic test explored in this study is flow cytometry (FCM). FCM is extremely sensitive, avoids the need for culturing or enrichment procedures, and can be both qualitative and quantitative (Attfiled et al., 1999, Shapiro et al., 1995). It provides quick and precise results with little effort from the technician. Holm et al. (2003) attempted to develop a preliminary diagnostic test that they created by gram staining bacteria, and using a flow cytometer to detect the results. However, their protocol only deciphered between gram negative and gram positive bacteria and further culturing would have needed to be done to determine the bacteria species. Gunasekera et al. (2000) have demonstrated that flow cytometry, coupled with fluorescence techniques for distinguishing between cell types, could potentially be employed in a wide variety of biological assays relevant to the dairy industry.

FCM can be used to detect bacteria in processed milk as well as raw milk (Gunasekera et al., 2000). FCM has also been used with fluorescence microscopy to detect viable but non-culturable bacteria in heat-treated milk (Gunasekera et al., 2000). It has been shown that by applying fluorescent antibody technology, it is possible to detect low numbers of specific microorganisms such as pathogens using FCM (Pinder and McClelland, 1994; Clarke and Pinder, 1998). Protein A is a surface protein found in the cell wall of *S. aureus*. Almost all clinical *S. aureus* isolates express protein A (Sorum et al., 2013). *Staphylococcal* protein is a key virulence factor that enables *S. aureus* to evade innate and adaptive immune responses (Cheng et al., 2009 and Kim et al., 2011). Protein A binds animal IgG with its five homologous Ig-binding domains.

The objective of this study is to develop and validate a FCM-based assay for the detection of *S. aureus* in raw milk. To accomplish this objective, *S. aureus* specific antibodies tagged with fluorescein isothiocyanate (FITC) dye will be suspended in raw milk and aliquots of the milk will be subjected to flow cytometric analysis. The study will be conducted in two phases.

- PHASE 1: To confirm the effectiveness of the protein A antibody to bind specifically to *Staphylococcus aureus* and determine its most suitable dilution which can be detected by a flow cytometer.
- PHASE 2: To standardize the milk treatment with Proteinase K and Triton X-100 to rid the samples of excess milk proteins and lipids.

It is anticipated that a successful FCM assay for detection of *S. aureus* in milk will allow development of similar protocols for detection of other important mastitis pathogens.

Chapter 2

Literature Review

Mastitis can be caused by many different types of bacteria, fungi and yeast. *Staphylococcus aureus* is one of the most common mastitis pathogens. *Staphylococcus aureus* is considered a contagious pathogen because it can be spread from cow to cow via direct contact, teat dips, milkers' hands or other fomites in the barn. It has been shown that *S. aureus* could be a resident flora of different parts of the body including the teat skin and teat ends. Fomites (e.g., wash towels, unclean milking units) can also be a significant source of *S. aureus*. The udder hygiene and sanitation of milking equipment play a crucial role in limiting the spread of *S. aureus* (Capurro et al., 2010).

Clinical sign of mastitis include elevated somatic cell count, changes in milk color and composition, heat, pain or swelling of the udder and this may affect one quarter of the udder or multiple quarters. Subclinical signs of the disease may be irregular milk characteristics or elevated somatic cell count (Erskine, 2011). Due to its gram positive thick cell wall, *S. aureus* resists desiccation and increases its pathogenicity because it can survive off of the body, and can also adhere easily to the epithelial cells in the mammary gland (Capurro et al., 2010).

On entry into the teat canal, *S. aureus* is able to multiply in the presence of milk and later release toxins that cause necrosis of the epithelial cells, allowing *S. aureus* to infiltrate the sub-epithelial layer and colonize the udder. Many strains of *S. aureus* have

the ability to form biofilms that allow them to adhere to the epithelium within the teat and udder. These biofilms resist penetration of antibiotics to the core inner group of bacteria allowing them to persist. *Staphylococcus aureus* can easily become resistant to antibiotics making it very difficult to treat. This increases the virulence of *S. aureus*, usually leading to chronic, subclinical infections (Cucarella et al., 2004).

During the infection, the inflammatory process is initiated and capillaries become leaky to allow the immune cells, ions and proteins to reach the site of infection in an attempt to contain it. Leukocytes are one type of cell that enters the site of infection to phagocytize and kill the pathogens. These immune cells can infiltrate the milk and contribute to the somatic cell count. According to the "Grade A Pasteurized Milk Ordinance," the USDA requires that Grade A Pasteurized milk, have no more than 750,000 somatic cells per milliliter (Grade A Pasteurized Milk Ordinance Revision, 2009). Identification of pathogens is done using specialized media, diagnostic test kits and reagents, and requires at least 3-4 days to identify the species of bacteria; this process is tedious and time consuming (Thornberg et al., 2000).

A flow cytometer is a tool that is used to identify cell types and has been effectively used as a diagnostic tool to identify various pathogens using refined growth media. The cytometer filters cells through one cell at a time, while a laser is shown onto them. Once the light hits the cell, it is scattered based on its size which is referred to as forward scatter, and the complexity of the cell surface, which is referred to as side scatter. As the cell increases in size, the forward scatter increases, and as the cell surface increases in complexity, the side scatter increases. All of this data is recorded on a forward scatter versus side scatter dot plot graph. The flow cytometer can also detect

different fluorescent colors that may be emitted from the cells or fluorescently-tagged particles. There are multiple detectors within the cytometer to detect different colors. The FL-1 detector records green fluorescence (Veal et al., 2000). The FL-1 detector was used in this experiment to analyze the FITC fluorescence that was emitted from the Protein A antibody. The forward scatter and side scatter can be compared among species of bacteria to identify and gate the different species within a sample or across samples. Also, if fluorescently tagged antibodies are used, the level of fluorescence detected across samples is compared to determine whether or not the antigen bacteria species is present (Veal et al., 2000).

Flow cytometry has been used in various studies involving milk and mastitis. The methods for treatment of the milk were based on the protocol developed by Gunasekera et al. (2000) in their study, "A flow cytometry method for rapid detection and enumeration of total bacteria in milk." In this study Triton X-100 and proteinase K were used to treat the milk samples to breakdown the lipids and proteins in the milk that may interfere with the antibody. However, instead of utilizing a fluorescently tagged antibody, they used a fluorescent dye with DNA affinity and their goal was to count bacteria and to use forward and side scatter to determine what type of bacteria was present. In their study the importance of breaking down and cleansing the sample of lipids and proteins was to ensure that they were not mistaking those particles for bacteria cells (Gunasekera, et al., 2000).

For the present study the importance of removing the milk matrix was to prevent non-specific binding with the Protein A antibody and to prevent them from diluting the actual antigen-antibody binding and fluorescence. Antibodies are large immunoglobulins

that are created by an animal's B lymphocytes to detect an epitope, a specific molecular compound that is usually on the surface of a foreign cell. This enables the antibody to recognize that the cell is foreign, potentially a pathogen, and to initiate the inflammatory response. The use of an antibody to detect bacteria cells is highly effective because of the specificity of the antibody to the epitope on the bacteria's surface, even with other cellular particles present in the sample. IgG antibodies have a higher affinity for their epitopes than IgM antibodies and are a better choice for this experiment. The antibodies used were polyclonal which means that the sample purchased included antibodies that recognized different epitopes of the same Protein A antigen of *S. aureus*. The use of polyclonal antibodies does increase the chance of non-specific binding (Veal et al., 2000).

Chapter 3

Materials and Methods

3.1 Bacterial Isolates and Milk Samples

ATCC bacterial reference strains were used to test the specificity of the Protein A antibody in Phase 1 of the experiment and to standardize the milk treatment in Phase 2. The species utilized were *Staphylococcus aureus* (29213, 27644 and 19095), *Staphylococcus epidermidis* (14990), *Staphylococcus warneri* (27836) and *Escherichia coli* (23716). Reference strains of bacteria were stored at -80°C. Brain Heart Infusion (BHI) broth was used to culture the bacteria.

Aliquots of 20 raw milk samples were obtained from the Penn State Animal Diagnostic Laboratory, (PSU-ADL) and 4 samples from Farm A. All samples were run in the Coulter FC500 (Beckman Coulter, Brea, CA) at the Penn State Microscopy and Cytometry Facility in the Millennium Science Complex. The data was analyzed using FlowJo software (TreeStar Inc, Ashland, OR).

3.2 Phase 1: Confirming the effectiveness of the Protein A antibody to bind specifically to *Staphylococcus aureus* and determine its most suitable dilution which can be detected by a flow cytometer.

Bacterial reference strains were grown in Brain Heart Infusion (BHI) broth at 37°C for 24 hours. Following the incubation period, serial dilutions in 0.15% NaCl solution were performed with each sample. Three different titers of the antibody, 1:250, 1:500 and 1:1000, were tested with 10^{1}, 10^{-2}, 10^{-4} and 10^{-6} dilutions of antigen to determine the most effective antibody dilution. Samples with bacteria and without antibody served as controls. The antibody/antigen mixture was incubated at room temperature for 30-45 minutes to allow the antibody to bind to the *S. aureus* protein A antigen, if present. Prior to being analyzed by the flow cytometer the sample was incubated with 1% formalin for 15-30 minutes.

Each bacterial species was visualized on the forward scatter versus side scatter graph in a different area due to differing surface complexities and sizes. Therefore, different gates were identified for each species to ensure relevant data points were being analyzed for fluorescence. These gates were established in Phase 1 and used for those species throughout the rest of the trials in BHI broth and NaCl solution. The gates were determined under the guidance of Ruth Nissly, a Penn State Cytometry Research Technologist. The data points within the gate were assumed to be the bacteria species of that specific gate.

The data points within the species gates were further analyzed on a fluorescence histogram for the FITC fluorescence. The presence of FITC fluorescence indicated the

binding of the Protein A antibody and the antigen, suggestive of the presence of *S. aureus*. The measurement of fluorescence called 'fluorescence frequency' was recorded as a percentage representing the amount of data points fluorescing within the original population that was gated on the forward scatter versus side scatter graph. A 'positive gate' threshold was determined upon comparison with the FITC FL-1 (x-axis parameter) between *S. aureus* samples and other bacteria species samples. This threshold was used for the remainder of the experiment to attempt to distinguish between *S. aureus* and other bacteria species or milk particles in samples. The percentage of data above the threshold was used to compare different species and is referred to as the frequency of fluorescence. The fluorescence frequency will be used as the diagnostic marker to determine whether or not *S. aureus* is in the milk sample because it is the measure of the FITC fluorescence detected from the *S. aureus* and Protein A antibody binding. The greater the percentage indicated that more binding of antibody and antigen occurred, indicating the presence of more *S. aureus* bacteria.

3.3 Phase 2: Establishing the Protocol in Milk: Standardization of the milk treatment with Proteinase K and Triton X-100 to rid the samples of excess milk protein and fat.

ATCC reference strains of *S. aureus* (29213, 27644 and 19095) and 20 raw milk samples from PSU-ADL and 4 raw milk samples from Farm A were used in this part of the study. The bacteria were grown in the BHI broth at 37°C for 24 hours and then used

to inoculate the milk. Milk samples without bacteria were utilized to serve as a control, as well as milk samples with non-specific IgG antibody. The serial dilutions were performed with 0.15% NaCl. The milk was treated with 0.2% Triton X-100 and 2 mg/mL Proteinase-K to digest the milk lipids and proteins (Gunasekera et al., 2000). After both treatements the samples were centrifuged for 10 minutes at 14000 gs, then the milk matrix was pipeted off of the bacteria pellet. The pellet was then suspended in 0.15% NaCl solution and vortexed. Following this treatment, 1:500 dilution of antibody was added to the milk samples and incubated at room temperature for 30-45 minutes. Prior to being run in the flow cytometer the sample was incubated with 1% formalin for 15-30 minutes at room temperature.

A new milk gate was drawn on the forward scatter versus side scatter graph to encompass the *S. aureus* gate as well as the milk particles due to the possibility that some *S. aureus* bacteria could adhere to the leftover milk particles and appear in a different area on the graph. The previously determined fluorescence threshold and frequency from Phase 1 were used to determine if there was any non-specific interaction between milk and the antibody and if *S. aureus* could be detected in a milk medium.

Chapter 4

Results

4.1 Phase 1: Confirming the effectiveness of the Protein A antibody to bind specifically to *Staphylococcus aureus* and determine its most suitable dilution which can be detected by a flow cytometer.

The goal of Phase 1 was to confirm the specificity of the Protein A antibody and to determine what dilution of antibody should be used in the trials with milk in Phase 2. Trials with *Staphylococcus aureus* as well as other bacteria species, including *Escherichia coli* and other *Staphylococcus* species including *S. warneri* and *S. epidermidis* were done to test the specificity for *S. aureus* in Phase 1. Different concentrations of the antibody, included 1:250, 1:500 and 1:1000, were tested to determine the ideal dilution that would show the greatest fluorescence with *S. aureus* and the least fluorescence with other bacteria species.

The flow cytometry data was collected and displayed on a side scatter versus forward scatter graph. Then the gates were drawn individually around each species. The fluorescence threshold was established after witnessing a dramatic difference between *S. aureus* fluorescence levels and that of the other species which determined where the positive gate was drawn on the fluorescence histogram.

The gates that were used can be seen in Figure 1 for *S. aureus*, *S. warneri*, *S. epidermidis* and *E. coli*. These sample graphs were chosen due to their representation of the average population graph for bacteria grown in broth. Within the species gates, the data was then displayed on a fluorescence frequency histogram. This graph can be seen in Figure 2. This graph contains each of the bacteria species grown in broth that were from their respective side scatter versus forward scatter graphs from Figure 1. Figure 2 shows the positive gate that was created to compare the absence or presence of FITC fluorescence. The Protein A antibody was at 1:500 dilution and the antigens were at 10^{-2} dilutions in Figures 1 and 2. As seen, the *S. aureus* population has 98.9% of its data points within this gate, confirming the extensive binding to the antibody. The rest of the populations were below the positive gate on the x-axis and had minute levels of fluorescence with less than 0.5% detected in the FITC range. Therefore, it was confirmed that the Protein A antibody was specific for *S. aureus*. The data and analysis of all of the samples were analyzed in this manner and the fluorescence frequency percentage comes directly from the fluorescence histograms of each sample.

The fluorescence percentage describes the percent of data points, or bacteria cells, that are attached to the Protein A antibody and fluorescing due to its FITC activation upon bonding. These data points are above the previously determined positive *S. aureus*/FITC threshold and are within the gate that was drawn around the bacteria species population on the forward scatter vs. side scatter graph. Therefore, the higher the percentage, the more fluorescence that is detected specifically from FITC and the antibody-antigen binding of the Protein A antibody and *S. aureus*. Fluorescence

frequency equaling or below 0.5% was determined to be insubstantial and due to background fluorescence.

To prove that the bacteria itself did not give off a fluorescence that may be mistaken for antibody-antigen binding, samples were tested containing the different bacteria species without antibody. The non-*S. aureus* species averaged a fluorescence frequency of 0.013% indicating an insubstantial level of fluorescence which could be described as background fluorescence due to the ability of all particles or cells to give off a very slight, insignificant amount of fluorescence. The *S. aureus* trials indicated that there was an average fluorescence of 0.05% collected which also is an insubstantial level of fluorescence.

When testing the 1:250 antibody dilution the non-*S. aureus* bacteria species fluorescence frequency was averaged at 5.28%, which was higher than expected, but may be due to background binding or the excess of antibody. The *S. aureus* average fluorescence frequency was 96.45% which is expected and shows that the antibody binds well and at high levels to *S. aureus* because it shows that well over 90% of cells within the species gate. The 1:250 dilution does show an ideal result for *S. aureus*, however the fluorescence with non-*S. aureus* species was too high to be considered background fluorescence.

The best results came from the trials with the 1:500 antibody dilution. In this trial with the non-*S. aureus* species there was a low fluorescence frequency at an average of 2.40%. The *S. aureus* trials averaged at 93.07% fluorescence frequency. The 1:500 antibody dilution showed that about 93% of the cells in the *S. aureus* gate were bound to antibody which is a very strong result. There was minimal reactivity with the non-*S.*

aureus species at 2.40%. This exhibited the combination of a maximized reactivity between *S. aureus* and the antibody, which is desired, and a minimized reactivity between the antibody and non-*S. aureus* species.

The last trial of Phase 1 tested the antibody dilution of 1:1000. In the trials with the non-*S. aureus* species there was an average of 0.60% fluorescence frequency which is another minimal amount of fluorescence and is an expected result indicating insubstantial fluorescence and reactivity levels. However, the *S. aureus* average fluorescence frequency was 75.32% with the 1:1000 antibody dilution which is not an ideal level of fluorescence for the specific antigen with its antibody, perhaps due to a low amount of antibody present in regard to a high amount of antigen.

In conclusion for Phase 1, the 1:500 dilution of the antibody was determined the most effective based on its low reaction with the non-*S. aureus* bacterial species and substantial reaction with *S. aureus* and was selected to be used for the remainder of the experiments in Phase 2.

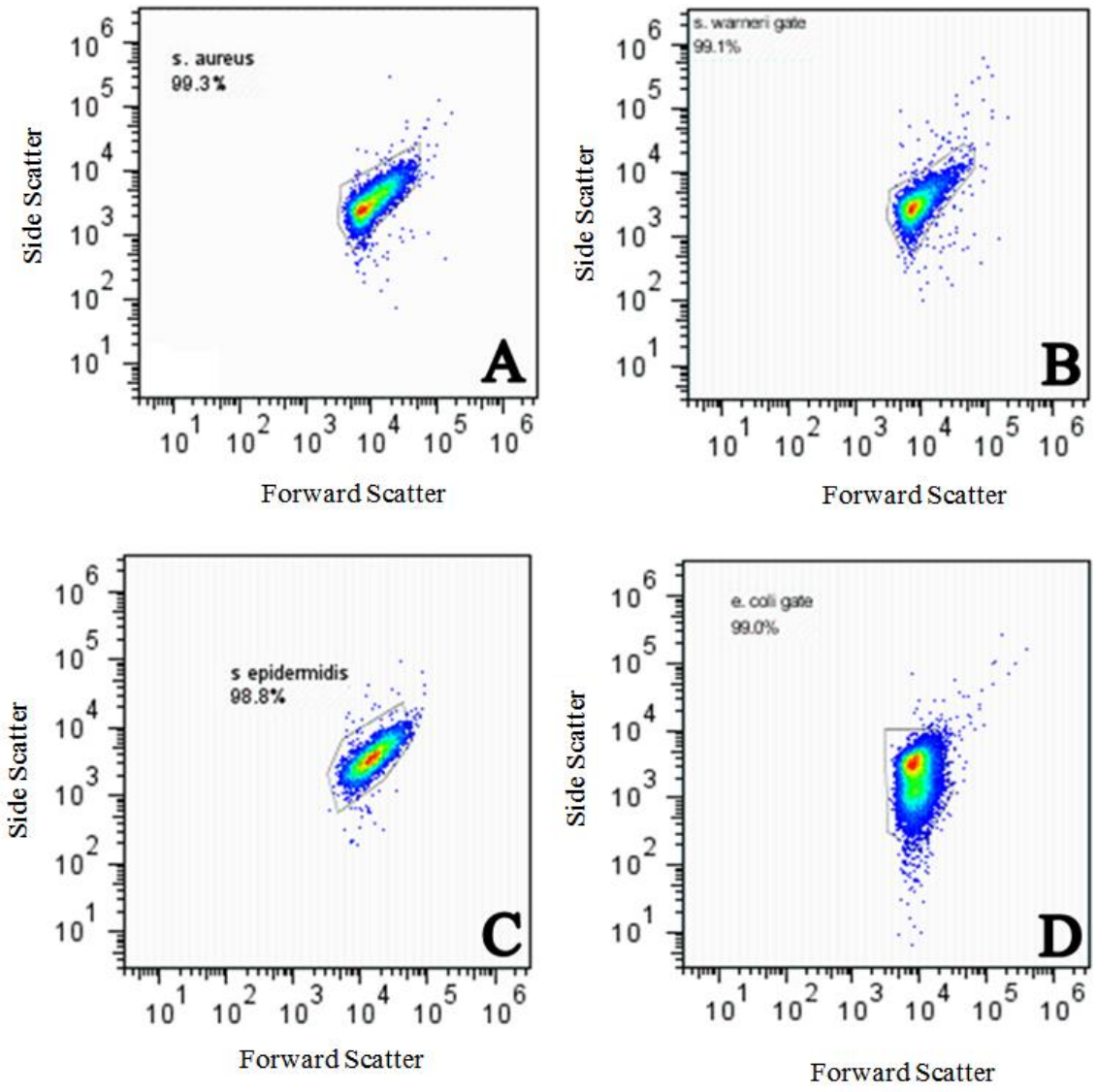

Figure 1: Forward scatter versus side scatter graphs of *S. aureus* (A), *S. warneri* (B), *S. epidermidis* (C) and *E. coli* (D). These graphs illustrate the differences in size (forward scatter), and surface complexity (side scatter) between the different bacteria species. The colors in the graph demonstrate the density of cells in that area, blue is the least dense and red is the densest. For the purpose of this study the colors can be disregarded.

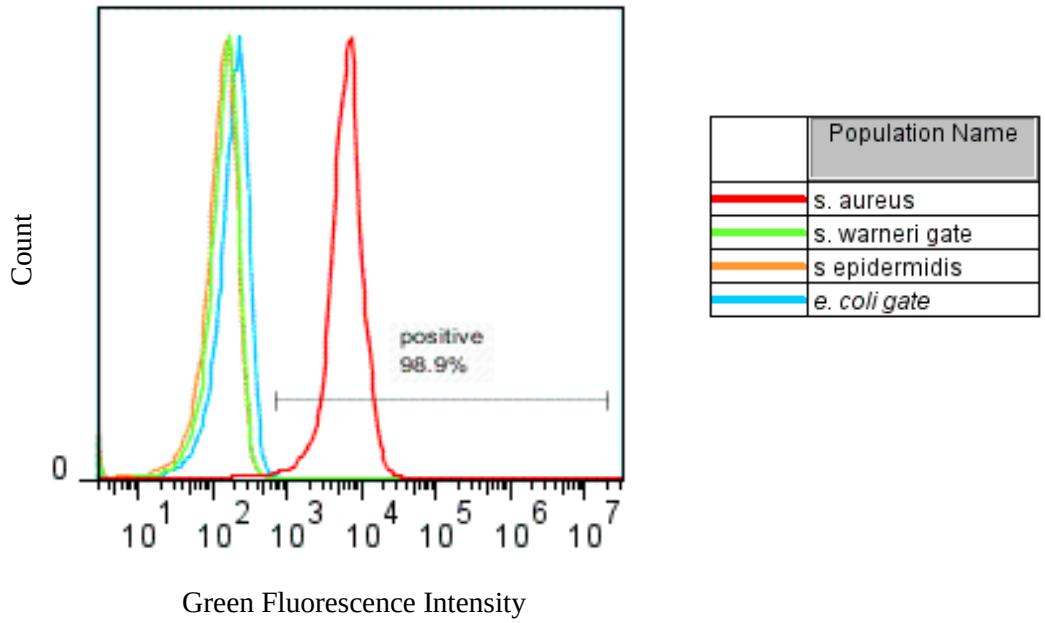

Figure 2: Histogram of fluorescence of *S. aureus, S. warneri, S. epidermidis* and *E. coli* with the positive fluorescence gate. The graph shows that 98.9% of the data points, or cells in the *S. aureus* gate, fall above the positive gate threshold whereas the other bacteria species all reside lower on the x-axis. This difference is due to the FITC-green color detected from the binding of the *S. aureus* group that is absent in the other bacteria species. This confirms the specificity of the Protein A antibody to *S. aureus*.

Species	*S. aureus*	*E. coli*	*S. warneri*	*S. epidermidis*
Antibody Dilution	Mean Fluorescencey Frequency (%)			
0	n=3	n=2	n=2	n=2
	0.05	0.00	0.01	0.03
1:250	n=8	n=8	n=5	n=5
	96.45	1.28	10.30	4.26
1:500	n=8	n=8	n=5	n=5
	93.07	1.19	3.36	2.64
1:1000	n=8	n=8	n=5	n=3
	75.32	1.36	0.17	0.27

Table 1: Summary of mean fluorescence frequency of bacterial species at four antibody dilutions. The fluorescence frequency of *S. aureus* on binding to Protein A antibody is generally >90% while other bacteria species displayed lower levels of fluorescence, on average ranging from about 1-10% for the different Protein A antibody dilutions. The number of samples in each group is represented by the ‘n’ value.

4.2 Phase 2: Establishing the Protocol in Milk: Standardization of the milk treatment with Proteinase K and Triton X-100 to rid the samples of excess milk protein and fat.

In Phase 2, raw milk was inoculated with bacteria to test the strength and specificity of the Protein A antibody and FITC fluorescence in milk medium. The goal of Phase 2 was to determine if *S. aureus* could be identified in milk samples that were inoculated with bacteria, treated with Triton X-100 and Proteinase K and then treated with the Protein A antibody. A new gate was drawn for the milk samples to include the leftover milk proteins and lipids with the possibility that *S. aureus* could adhere to these molecules and appear on the side scatter versus forward scatter graph in a different location than normal. This new gate can be seen in Figure 3. Ideally there would not have been the amount of leftover protein and lipid molecules that there were in this experiment, but it was decided to adhere to the protocol and not add additional wash steps that could have caused variation in the protocol as well as substantially decreased the bacteria load. The milk data within the milk gate was plotted on a fluorescence histogram with the positive fluorescence threshold described previously. The histogram in Figure 4 was one of the ideal results with little positive fluorescence in the milk with the Protein A antibody. Overall, there were mixed results in the milk samples.

Milk samples were run without antibody to establish the background fluorescence for milk and determine if it would create any fluorescence that could be mistaken for antibody-antigen interactions. This data is seen in Table 3, and the average fluorescence of the milk was 0.08%, which is an insubstantial amount of fluorescence.

Next, milk samples were treated separately with Protein A antibody as well as a non-specific IgG antibody. The purpose of these tests was to rule out the possibility of cross interactions between the antibodies and milk. The data for this portion of Phase 2 can be seen in Table 2. When the milk was combined with the non-specific IgG antibody, there was an average fluorescence frequency of 0.095% indicating an insignificant amount of fluorescence. However, when the milk was combined with the Protein A antibody, there was an average fluorescence frequency of 5.14%. When compared to the *S. aureus* result in the broth this number is minimal, but when compared to the level of fluorescence of milk without antibody which was 0.0768%, this is indicative that something was binding to the Protein A antibody in the milk. This inconclusive result also occurred throughout the milk trials when individual colonies of ATCC strains of *S. aureus* were actually added to the milk. The data from these samples is recorded in Table 3. The average fluorescence frequencies for the milk dilutions of 10^{1}, 10^{-2}, 10^{-4} and 10^{-6} were 10.67%, 5.52%, 10.03%, 16.59%, respectively.

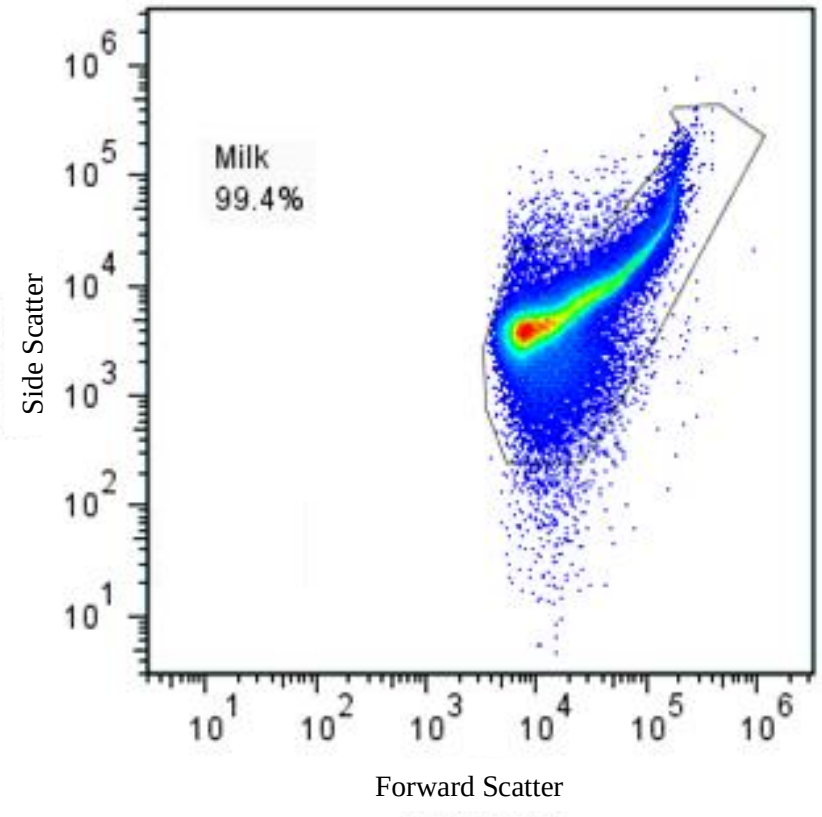

Figure 3: Milk sample data and gate on side scatter vs forward scatter graph. Leftover milk protein and fat can be seen in the upper right hand corner of the graph that were not removed during the washing process. This could dilute the bacteria in the assay. The *S. aureus* gate was modified to account for the extra milk particles present that it could bind to and show up in the graph in a different area than what is normal for the bacteria alone. The graph shows that 99.4% of all data points on this graph, or cells and particles, reside within the milk gate drawn. The colors in the graph demonstrate the density of cells in that area, blue is the least dense and red is the densest. For the purpose of this study the colors can be disregarded.

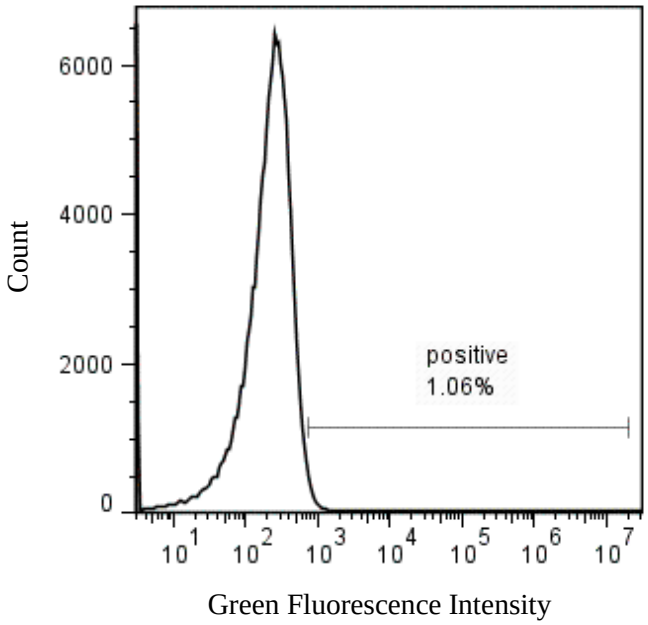

Figure 4: Histogram of the milk data with Protein A antibody from within the milk gate. This graph serves as the control for the milk-based experiments (fluorescence at 1.06%).

Milk Sample Source	Protein A Antibody 1:500 Dilution	Non-Specific IgG Antibody 1:500 Dilution
	Mean Fluorescence Frequency (%)	
Animal Diagnostic Lab n=12	4.67	0.07
Farm A n=4	5.61	0.12

Table 2: Data of milk trials using Protein A antibody compared to control milk trials with non-specific IgG antibody. The average fluorescence percent from the non-specific IgG data was between 0.07-0.12% which is below 0.5% indicating that this amount of fluorescence is insubstantial. This concludes that there should not be cross reactivity in the study. However, there was fluorescence present at low but not insubstantial levels when the Protein A antibody was added to the milk samples. The number of samples in each group is represented by the ‘n’ value.

	No Antibody	Protein A Antibody 1:500 Dilution			
Sample Dilution	10^{-2}	10^{1}	10^{-2}	10^{-4}	10^{-6}
	Fluorescence Frequency (%)				
S. aureus	0.05	4.84	3.12	3.62	27.00
	0.05	4.03	3.17	5.76	15.90
	0.26	19.00	3.36	2.83	16.00
	0.09	9.08	19.70	30.50	8.67
	0.00	7.04	0.66	13.80	0.32
	0.01	6.93	2.64	14.50	17.00
	--	12.60	6.56	5.08	20.10
	--	8.56	3.77	11.60	27.70
	--	5.28	1.22	7.13	--
	--	11.00	0.77	0.25	--
	--	10.00	3.86	7.97	--
	--	29.70	17.40	17.30	--
Mean	0.08	10.67	5.52	10.03	16.59

Table 3: Milk samples inoculated with *S. aureus* and Protein A antibody: Twenty milk samples were inoculated with *S. aureus* and Protein A antibody then diluted to different concentrations. There were 6 samples without antibody to serve as controls. Each value in the table is a different sample. The sample dilution groups with dashed lines in some of their cells have fewer samples than the other groups.

Chapter 5

Discussion

The objective of this study was to test the use of a flow cytometer to detect *Staphylococcus aureus* in milk samples. The study was done in two phases. The goal of Phase 1 was to confirm the specificity of the Protein A antibody for *S. aureus* and to determine the best concentration of the antibody to use in Phase 2 with milk. The goal of Phase 2 was to verify the established protocol of Phase 1 in a milk medium, as well as standardize the treatment of Triton X-100 and Proteinase K to remove the milk fats and proteins.

Phase 1 was successful in providing a protocol that utilized the Protein A antibody at the most effective dilution of 1:500, to distinguish between *S. aureus* and other bacteria species that included *Staphylococcus warneri*, *Staphylococcus epidermidis* and *Escherichia coli*. This was achieved by setting a threshold for fluoresce and measuring fluorescence frequency within BHI broth. *S. aureus* samples showed substantially higher fluorescence frequencies beyond the threshold than other bacteria species. Methicillin-resistant *S. aureus* and non-methicilin-resistant *S. aureus* can be detected by measuring the fluorescence intensity emitted by the binding of the fluorescent-tagged Protein A antibody and *S. aureus*. This study also was able to successfully differentiate between the two varieties of *S. aureus* by their location on a forward scatter versus side scatter graph (Shrestha et al., 2011). In the present study,

different bacteria species were able to be distinguished in the same manner on a forward scatter versus side scatter graph. From these results it can be confirmed that through the use of forward scatter versus side scatter graphs to gate different strains or species of bacteria, it is possible to further analyze the data within those gates through the measurement of fluorescence frequency and intensity from the Protein A antibody and *S. aureus* complex. Rüger et al. (2014) has shown that species-specific cell concentrations can be monitored by flow cytometry. They have developed a flow cytometric method which can assess the species-specific viability in defined three-species mixed cultures namely *Pseudomonas aeruginosa, Burkholderia cepacia* and *S. aureus.* Our results show that fluorescence from Protein A antibody could be used as a diagnostic indicator of *S. aureus* and that the method of flow cytometric analysis within the current study matches similar studies.

The results of Phase 2 of the study were inconclusive. It was not possible to distinguish between milk without *S. aureus* and milk that had been inoculated with *S. aureus* based on fluorescence. There are a few theories as to why this has occurred. First, it was difficult to obtain a true control in the milk with the Protein A antibody because raw milk is not a sterile substance and microbes are normally present, potentially even *S. aureus*. Therefore, it is difficult to differentiate between background fluorescence from the milk and fluorescence from the antibody-antigen complex. Another study used ultra-pasteurized milk samples. This process may have removed any existing bacteria; however some of the Protein A antigens could have still remained present within the milk for the antibody to detect (Gunasekera et al., 2000).

Also, there was the possibility that there was cross-reactivity between the Protein A antibody and the milk particles themselves, however, this was considered unlikely due to the lack of interaction between the non-specific IgG antibody and the milk. It is known that fibrinogen is present in milk from dairy cows with different grades of mastitis (Tabrizi et al., 2008) and it has been shown that protein A has adjacent fibrinogen and fibronectin binding sites (Stemberk et al., 2014). This explains the detected presence of protein A in pasteurized milk and the possible cross reactivity. Also there is a possibility that antibodies could bind to ligands similar to their epitope, even if the sources of the ligands are completely different. Mechanisms of antigen mimicry have been explored as possible causes of antibody cross-reactivity, but little about the cross-reactivity process is presently known (James et al., 2003).

Another difficulty in analysis was that there was background fluorescence observed in most milk samples, which made it difficult to determine a threshold of fluorescence that could be considered diagnostic. These levels could have been diluted by the excess of milk matrix that remained even after treatment and washing steps. Also, the actual cause of this fluorescence could not be completely confirmed or ruled out as *S. aureus* or milk due to the presence of florescence from the relatively pathogen-free milk samples and the Protein A antibody. In addition to this, when samples had irregularly high fluorescence frequencies in 10^{-6} dilution samples, it was misleading because these samples were so dilute that they had very few particles and any fluorescence was disproportionately inflated.

Simultaneous identification of antibodies to *Brucella abortus* and *S. aureus* has been shown in milk samples by flow cytometry (Iannelli et al., 1998). Similarly,

improvements and further assessment of this protocol and data could be done to experiment the use of the Protein A antibody in detecting *S. aureus* in milk samples. There was little idea about the amount of *S. aureus* bacteria present in the milk samples due to the plethora of bacteria and different species that grow in milk. Attempting to standardize this part of the protocol could improve the results, such as collecting the samples from the same cow and isolating the different bacteria. Having nearly sterile milk may improve the results.

Another improvement could be in the steps taken to remove the proteins and lipids from the milk. Bacteria could have been lost in the wash steps, yet still there were milk particles in the samples when run through the flow cytometer. It was found that through the ultra-pasteurization process, proteins and lipids underwent denaturation that could make them more susceptible to the proteinase enzymes and detergents during the milk treatment process. By heating the milk, it may be easier to chemically and enzymatically breakdown the milk matrix and remove it during wash steps to eliminate the dilution of bacteria during flow cytometer analysis. In that study there was a small amount of milk matrix present on their flow cytometry graphs and it was possible to easily distinguish between the milk matrix and the bacteria (Gunasekera et al., 2000). However, in the present study, there was a large amount of milk matrix leftover after treatments and it was difficult to distinguish between the milk matrix data points and the bacteria data points on the forward scatter versus side scatter graphs. If these groups could be distinguished, the dilution of fluorescence from the Protein A antibody and *S. aureus* complex by the milk matrix could be avoided in the present study's fluorescence

histograms. This elimination of dilution in the fluorescence histogram could potentially give significant results in the milk trials.

Summary

Protein A antibody tagged with FITC was tested for specificity for *Staphylococcus aureus* in BHI broth and in milk samples. A 1:500 dilution of Protein A antibody was found to be the optimal dilution for detection of *S. aureus*. The Protein A antibody was highly specific for *S. aureus*, and other test strains examined in this study were not detected using this antibody. With respect to milk samples inoculated with *S. aureus*, it was difficult to discern the inoculated milk from the un-inoculated milk samples based on the fluorescence frequency. It can be inferred that flow cyotmetry can be used to *detect S. aureus* in BHI broth, however the detection of *S. aureus* directly from raw milk using a flow cytometry method is hindered perhaps due to the inability of Protein A antibody to bind to *S. aureus* due to background interference of milk proteins and fat. It is recommended that a more effective washing step to remove milk proteins and fat, in particular proteins that could bind to protein A, could considerably improve the diagnostic sensitivity of flow cytometry for detection of *S. aureus* directly from milk samples.

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ACADEMIC VITA

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Education

Pennsylvania State University
Schreyer Honors College
Bachelor of Science in Veterinary and Biomedical Science — Anticipated 5/14
Minor in Sociology

Volunteer Experience

Penn State Reproduction Research Team — 1/14 - present
- Perform health and heat checks on the dairy cow research herd weekly

Dr.Bhushan Jayarao's Veterinary Medicine Laboratory — 11/11 - present
- Honors thesis work with bacteria and flow cytometry

Haymarket Veterinary Service — 5/13 - 8/13
- Shadowed equine veterinarians on ambulatory visits

Straley Veterinary Associates — 1/13 - 4/13
- Shadowed and assisted a large animal veterinarian

Shaver's Creek: PSU Veterinary Extension — 9/13/12
- Administered Ivermectin injections to rescued birds of prey

Penn State Dairy Expo — 4/12 - 5/12
- Trained, groomed and showed a Holstein heifer

Fort Washington Veterinary Hospital — 5/11 - 8/11
Veterinary Technician Assistant
- Shadowed and assisted small animal veterinarians

University of Pennsylvania VETS Undergraduate Program — 7/11/11 - 7/15/11
- Attended lectures and labs

Professional Experience

The Limited, Fairfax, VA — 5/13 - 8/13
Sales Associate
- Assisted customers and arranged products

Fort Washington Veterinary Hospital — 5/12 - 8/12
Small Animal Veterinary Technician
- Restrained animals for examination and assisted veterinarians
- Performed blood work, fecal & urinalyses tests

Pets Plus Pet Store — 8/09 - 8/10
Cashier
- Operated cash register along with other customer service duties

Activities

Small and Exotic Animal Club

Pet Meet and Greets Officer 8/11 - present

- Organize pet therapy visits twice a month to nursing homes

General club member 2010 - present

Pre-Vet Club member 2010 - present

Awards and Achievements

Dean's List 8/10 - 12/13

Lawrence Kahan Memorial Scholarship in Animal Health 2012 - 2014

Horace T. Woodward Scholarship in the College of Agriculture 2012 - 2013

College of Agriculture Shigley Memorial Pre-Veterinary Scholarship 2011 - 2012

College of Agriculture Paul R. and Ethel S. Guldin Scholarship 2011 - 2012