THESIS

A COMPARATIVE ANALYSIS BETWEEN THE rFC AND LAL ENDOTOXIN ASSAYS FOR AGRICULTURAL AIR SAMPLES

Submitted by

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ABSTRACT

A COMPARATIVE ANALYSIS BETWEEN THE rFC AND LAL ENDOTOXIN ASSAYS FOR AGRICULTURAL AIR SAMPLES

Agricultural workers experience increased exposure to inhalable dust and endotoxins, which make up the outer membrane of Gram-negative bacteria species. Endotoxin has specifically been linked to an increased degree of pro-inflammatory symptoms from inhaled dust, leading to a variety of lung diseases. Because there is no standardized method of collection or analysis of endotoxin, there are paramount gaps in the knowledge of how best to collect and analyze samples. The aims of this study were to: (1) assess the recovery from PVC filters spiked with known endotoxin concentrations; and (2) compare two different biological endotoxin assay kits: Lonza rFC and Associates of Cape Cod Pyrochrome Chromogenic, in order to detect any significant variation in measured endotoxin concentrations and potentially establish a conversion factor for interstudy comparison purposes.

The LAL assay uses a component found in the blood of horseshoe crabs in order to detect and quantify endotoxin concentrations. This process poses some concern with variability, as the reactivity of lysate with endotoxin can vary greatly between individual horseshoe crabs. The newer rFC assay offers an additional option for endotoxin analysis that does not require the use of horseshoe crabs. Because all of the materials are produced in a laboratory, the consistency between kits is much higher.

In Aim 1, PVC filters in replicates of five were liquid-spiked with 5 levels of known amounts of endotoxin. To simulate effects of sampling and handling, each filter was then

desiccated for 24 hours and loaded into SKC Button Aerosol Samplers where air was pulled through them for 4 hours at a flowrate of 4 L/min to mimic field sampling conditions. Samples were then frozen at -80ºC, thawed, and extracted. Each sample was analyzed for endotoxins using the rFC assay. For Aim 2, a combination of personal, area, and field blanks were collected from two Colorado dairy farms from 2013-2014 in conjunction with a larger study for a total sample size of n=31. Samples were desiccated for 24 hours, frozen at -80ºC, thawed, and extracted. Each sample was then analyzed using the rFC and LAL assay and the results were compared.

Using the rFC assay, measurements for endotoxin concentrations were on average several magnitudes lower than the anticipated concentration. Spike recoveries ranged from 1-8%. It is likely that the hydrophobic properties of the PVC filters did not allow complete absorption of the liquid spikes, but rather evaporated into the air. For aim 2, there was no statistical difference found between the rFC and LAL assay for the total sample set (p-value 0.7146) using an alpha=0.10. There was also no statistical difference between assay types for the personal sample subset (p-value 0.3788). However, there was a statistically significant difference for the area sample subset (p-value 0.0698) and the lab and field blank sample subset (p-value 0.06638). Due to the small sample size, the power had to be adjusted to accommodate an alpha value of 0.10. The correlation between observations for all samples was found to be reasonably high with an r value of 0.867. The R^2 coefficient value was found to be 0.7524. This indicates that 75.24% of the variability in LAL assay data can be explained by rFC assay data.

The rFC assay serial dilution of standards gives a much more broad detection range of 0.005-5.0 EU/ml. The LAL standards only cover a fraction of this range, going from 0.005-0.04 EU/ml, 0.02-0.16 EU/ml, and 0.16-1.28 EU/ml. This dramatically decreases the chances of

correctly identifying the dilution factor on the first attempt, and can create additional costs to use extra kits for re-analysis. The rFC assay can also be a considerably more cost effective option when purchasing in bulk of 20-30 kits at a time; however, when purchased individually, the LAL assay is less expensive.

Overall, the development of the rFC assay greatly reduces the amount of horseshoe crabs harvested and bled for collection, reduces the costs of formulating the lysate enzyme, and most significantly, reduces the inconsistency in endotoxin measurement results.

ACKNOWLEDGEMENTS

I would like to thank my committee members, Dr. Stephen Reynolds, Dr. Joshua Schaeffer, and Dr. Robert Ellis for helping me to achieve this goal. Also, thank you to the High Plains Intermountain Center for Agricultural Health and Safety (HICAHS) and the National Institute for Occupational Safety and Health (NIOSH) Mountain and Plains Education and Research Center (MAP ERC) for the financial support for this project as well as my traineeship during my time at Colorado State University. I would also like to thank Dr. Ann Hess from the CSU Department of Statistics for invaluable statistical consultation and advice.

Last but not least, I would like to thank Amanda VanDyke for the many hours spent helping me in both the lab and office. I truly couldn't have done it without you.

DEDICATION

To my family. Thank you for the constant love and support. I would not be here without you.

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CHAPTER 1: INTRODUCTION

Agricultural workers are at an increased risk for exposure to endotoxins, commonly found in organic dust samples, and which are known to cause or exacerbate pre-existing respiratory conditions (1-4). Endotoxins, otherwise referred to as lipopolysaccharides (LPS), make up part of the outer membrane of most Gram-negative bacteria species (5-7). Endotoxin is a major inducer of neutrophilic airway inflammation, and thus can cause a number of acute and chronic respiratory diseases including: organic dust toxic syndrome (ODTS) and chronic obstructive pulmonary disease (COPD) (2, 4, 8, 9). Additionally, endotoxin exposure has been shown to contribute to the development or exacerbation of chronic asthma and bronchitis (2, 8, 10). There is a two-fold increase in the prevalence of lung disease among agricultural workers as compared to non-agricultural workers over a lifetime (10). Putatively, this increase is attributed to repeated high exposures to endotoxins and dust particulates (9, 10). Tasks involving grain handling, corn farming, rebedding stalls, animal movement, feeding, and milking cows during dairy and feedlot work have been linked with large amounts of dust and microbial exposure (2, 8, 11).

Currently, there is no threshold limit value (TLV) or permissible exposure limit (PEL) for endotoxins, however The American Conference of Governmental Industrial Hygienists (ACGIH) recognizes endotoxins as currently an agent "under study" (12). Biologically derived contaminants, such as endotoxins, are particularly difficult to quantify for compliance purposes as they can be detected using a variety of chemical, immunological, and biological assays (12). Although the United States has no enforceable exposure limits, countries such as the Netherlands have proposed health based occupational exposure limits of 90 EU/m^3 (13). The Occupational

Health and Safety Administration (OSHA) and ACGIH both recognize exposure limits for organic dusts, which can include, but are not limited to endotoxin. These limits are recognized as Particles Not Otherwise Specifed (PNOS) by ACGIH and Particles Not Otherwise Regulated (PNOR) by OSHA (14). The ACGIH TLV is 3 mg/m^3 for respirable dust, compared to the OSHA PEL of 5 mg/m^3. For total dust, the TLV is 10 mg/m^3, and the PEL is 15 mg/m^3 (12).

Decades of research have been done on dose-response relationships of dust exposures in the agricultural industry, which has resulted in several recommended occupational exposure limits. A study of personal aerosol exposure of swine production workers by Donham et al. suggests a maximum time weighted average (TWA) of total dust between 1.3 and 2.8 mg/m^3 (15). Several additional studies have supported the finding that exposures above 2.5 mg/m^3 of total dust was associated with significant cross-shift decrease in forced expiratory volume (FEV_1)(15-17).

There is no standardized method for endotoxin collection or analysis. Most often, endotoxin is collected on a filter using a three-piece closed-face cassette (CFC) in conjunction with a low flow pump (5). Glass fiber (GF) filters are commonly used; however, polycarbonate, Teflon, PVC, and cellulose-based media are all also acceptable media types (5). The difference in diameter and porosity vary for each type of media (5). There are two widely recognized methodologies for measuring endotoxin, through the use of biological assays and chemical analysis. The founding endotoxin biological assay, Limulus amebocyte lysate (LAL) assay quantifies the relative reactivity of the endotoxin with Limulus lysate (18). This process identifies endotoxin levels quickly with a high level of sensitivity (i.e., 0.001 EU/ml) (18-21). Additionally, there are several major limitations that may affect the performance of this assay, which include: differing extraction techniques, interference, collection media, sample

transportation, and thawing and freezing patterns (7, 18). Especially of concern is the interference between (1-3)-beta-D-glucan and Factor G, which is present in the LAL enzyme. This interaction can cause a false positive for endotoxin presence (7).

A study by Reynolds et al. compared endotoxin analysis of agricultural (chicken, swine, and corn) dust across six laboratories using the LAL assay. Samples were taken in bulk and randomly allocated to each laboratory. Three of the locations performed the analysis using an endpoint method, and three performed the analysis using a kinetic method (22). It was reported that all main effects including: assay method, dust type, laboratory location, and experiment were found to be statistically significant (22). For chicken dust samples, the endpoint method results reported higher endotoxin concentrations compared to the kinetic method. Conversely, for swine and corn samples, the kinetic method reported higher endotoxin concentrations compared to the endpoint method (22). There was also a high level of correlation across analysis locations with all three types of agricultural dust (22).

One of the most commonly used chemical analyses is Gas Chromatography Mass Spectrometry (GC-MS), where filters are extracted, and then re-suspended in anhydrous methanolic HCl and incubated at 100ºC for 18 hours. This produces methylesters of the 3-OHFAs present in the LPS of the samples, which is then quantified (23). This process is much more time intensive when compared to a biological assay, but as also shown to have lower likelihood of interference (24). It has been found that the correlation between LAL and 3-OHFA concentrations in dust samples have been variable (23-26).

Endotoxin detection and quantification is achieved through a cascading enzymatic reaction initiated by the binding of endotoxin to Factor C. Factor C is found in the LAL reagent

extracted from horseshoe crabs (27). However, the LAL reagent also contains Factor G, which can interact with (1-3)-beta-D-Glucans, and produce a false positive in some samples (27). The more recently developed recombinant factor C (rFC) biological assay works in a very similar fashion to the LAL assay, but utilizes a genetically engineered rFC that does not contain Factor G (27). Overall, the rFC assay has shown to produce results with less variability, as well as comparable sensitivity of 0.001 EU/ml (18, 27, 28).

The purpose of this study is to continue to bridge the gap in collecting and analyzing endotoxin in personal and area aerosol samples.

CHAPTER 2: LITERATURE REVIEW

ENDOTOXIN MECHANISM

Endotoxins are comprised of an O-specific chain, core oligosaccharide, and lipid A (7, 29). See Figure 2.1 for a diagram of the endotoxin portion of a Gram-negative bacteria cell structure. The actual structure and length of the fatty acid chains vary tremendously between bacterial groups (29-31). The O-specific chain is the outer most part of the cell, and is made up of numerous repeating polysaccharide units (7, 29). The oligosaccharide portion is in the middle of the endotoxin, and helps to bind the endotoxin together by connecting the O-specific chain and Lipid A (29). Finally, the Lipid A component, also known as the biologically active portion, is the inner most portion of the endotoxin and is responsible for activating innate immune responses (7, 29, 32). Studies have shown that variations of the chemical structure of the Lipid A portion have been associated with either increased or decreased biological activity. There is also much debate on the nature of the physical state of the biologically active Lipid A(32). In an aqueous environment, Lipid A forms a supramolecular aggregate structure (32). The chemical structure of the aggregate-forming molecules helps to determine the exact type of aggregate structure, and the level of endotoxin activity (32).

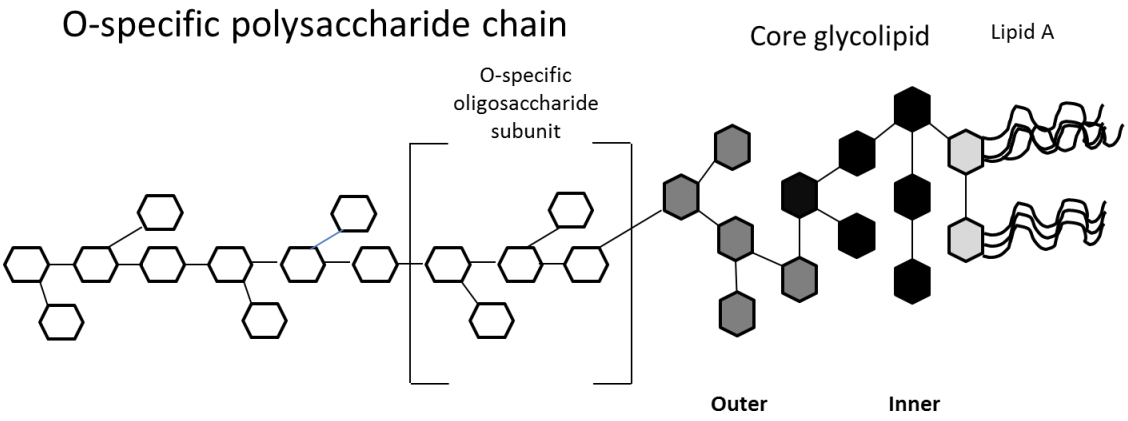

Figure 2.1 Diagram of the "LPS" or endotoxin part of an outer Gram-negative bacteria cell (33).

The endotoxin portion of the Gram-negative cell makes up the largest part of the cell, comprising up to three-quarters of the surface area in organisms such as *E. coli*. The main purpose of this LPS component is for cell survival, integrity and viability (31). Pathophysiological effects only occur when the endotoxin portion of the cell is disrupted, and ultimately released into the environment (31). During the cell cycle, as well as when Gram-negative cells die and lyse, the "total LPS" is also released (31, 34). The endotoxin component of cells then mediates the interaction with host organisms through nutrient transport and multiplication processes (31). If inhaled, LPS stimulates the release of chemoattractants known as cytokines, which initiate inflammation from the alveolar macrophages and epithelial cells (34, 35). Endotoxin is difficult to get rid of in both the body and environment because inactivation only occurs at 160° C for at least 4 hours (34, 36). This adaptability allows it to survive much longer than viable bacteria (34, 36).

At a cellular level, LPS binds to the lipid binding protein (LBP) in liquid serum. LPS is then transferred to CD14, which is known as a cluster of differentiation 14 (CD14) (7, 37). This

gene is expressed as protein on the cell membrane of macrophages. Finally, LPS is transferred to a TLR4/MD2 immune signaling complex (7, 37). MD2 is a novel human gene protein molecule that provides a link between the TLR4 and LPS. The protein Toll like-receptor 4 (TLR4) is a pattern recognition receptor associated with the innate immune system. TLR4 has the ability to recognize microbes that break physical barriers in the human body such as skin or intestinal tract mucosa (7). Once the LPS has been bound to the TLR4/MD2, the TLR4 goes through a signaling process which activates specific immune responses (7, 36, 38). The activation of the alveolar macrophages then leads to the production and distribution of proinflammatory cytokines, chemokines, and adhesion molecules (36, 39, 40). Known endotoxin cytokines include: TNF-α, interleukin (IL) 1-β, IL-6, and IL-8 (36, 39).

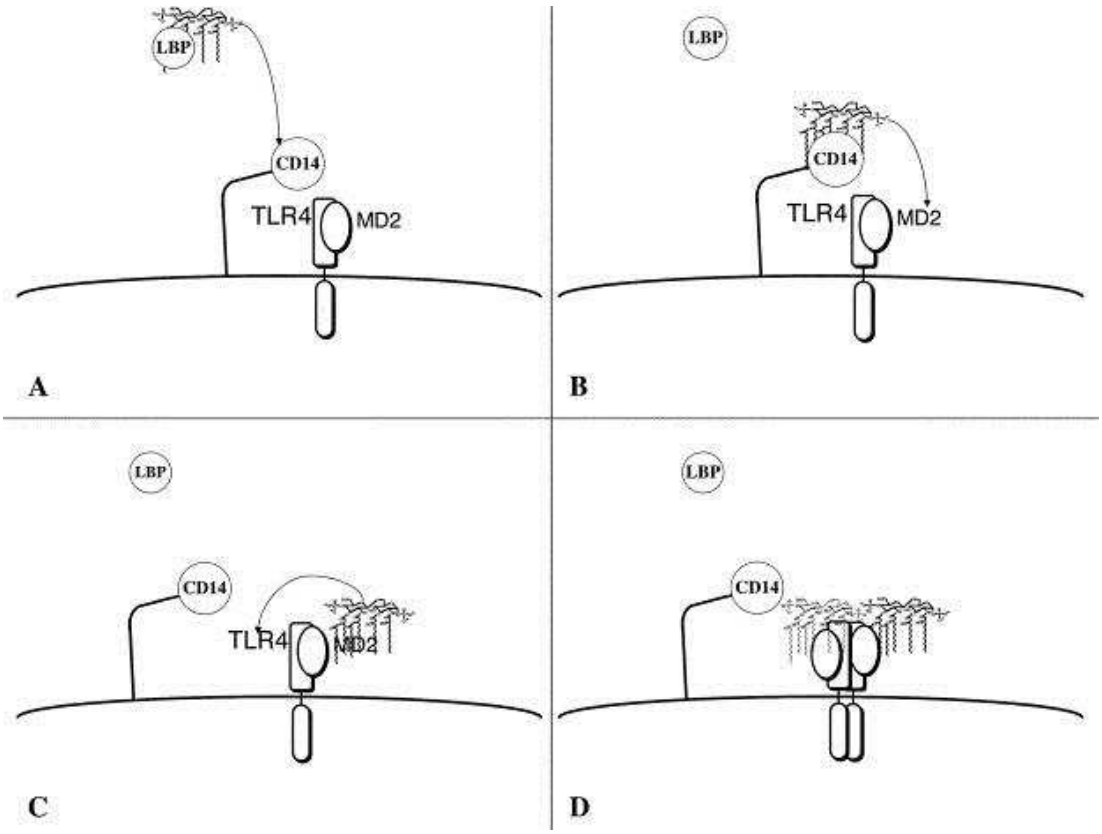

Figure 2.2 Signaling pathway of A. toll-like receptors, B. Lipid Binding Protein (LBP), C. Toll-like Receptor 4 (TLR4), and D. Cluster of Differentiation 14 (CD14) involved in process of LPS binding to TLR4 (38).

DAIRY ENVIRONMENT

During daily operations, both the dairy cows and employees are exposed to indoor and outdoor environments. Most operations allow lactating cows to spend the majority of the day outside in a pen or pasture, but they are moved three times a day into the parlor for milking (41). During these tasks, workers are exposed to varying levels of dust and microorganisms re-suspended by animal and worker activity, including inside the milking parlor. Flooring surfaces for dairy cow enclosures are generally either grass pasture or dirt. Although concrete is easier to clean and may be associated with lower dust concentrations, it is typically not used because it

has been associated with higher levels of lameness, injuries and general discomfort among the cows (41). It is also important to keep the flooring surfaces dry, despite causing more environmental dust, to minimize the rates of infectious hoof diseases (41).

The use of technology has had a significant impact on dust exposure. The transition to modern dairy farms, which typically includes more mechanized work tasks, including the installation of automated feeding systems, may help decrease the occupational exposure to inhalable hazards (11). Handling of animal feed is associated with a higher risk of bronchial symptoms and accelerated decline in expiratory flow (42). A study by Thaon et al. found that farmers that handle or had handled animal feed, such as straw or hay were at an increased risk of respiratory symptoms such as: wheezing, coughing, and morning phlegm. Feed handlers were also found to have a lower peak expiratory flow (PEF) and FEV_1 when compared to controls (11, 42).

Modern dairy operations have increased usage of mechanical and natural ventilation, which are important to remove moisture, manure gases, excess heat and bioaerosols (aerosols with a biological origin), which can contribute to maintaining both herd and worker health (41, 43, 44). There are many different types of ventilation systems utilized in animal housing, each with different air mixing, exhaust, and supply systems (43). In an air mixing system, fans are placed above stalls of cows in the barns; air is thus well distributed throughout the building, but is not exchanged. Hence, dust and other particles may become easily trapped in the air (43). Alternatively, two types of exhaust ventilation are commonly used, tunnel ventilation and wall exhaust. Tunnel ventilation works the air in a single direction, where it is pushed through the building with fans and then exhausted at the opposite end. While tunnel ventilation moves air very effectively, a large portion of the air travels through the parlor without contact with the

cows. Especially in the summer, this can cause heat exhaustion for the animals. This does not make it a popular choice among producers who want to protect the wellbeing of their animals. Wall exhaust involves exhaust fans on multiple walls of the structure, and will exchange a similar amount of air, but often times, the rate of airflow is not measurable (43, 45).

The increase in herd sizes on modern dairies has also increased generation, accumulation, and storage of animal waste products (46). The average manure production by a lactating dairy cow ranges from 69 to 103 kg/day per 1000 kg of body weight (47). Both pathogenic and non-pathogenic microorganisms are present in high concentrations in manure (46, 48). Daily work practices that can result in exposure for workers include: moving animals on manure, and storage, maintenance, and removal of manure (34). There are three main types of manure removal including: immediate disposal by transporting manure into a spreader, collecting manure and spreading monthly, and collecting manure into a lagoon and pumping it out later (49). The size of a manure lagoon can vary depending on herd size, space, and environmental regulations, but removal should occur on average about every 8 weeks, with a thorough clean-out every 6 years (49).

Aerosolization of endotoxin is inevitable during these processes. Fecal particles can become aerosolized, and have been shown to exacerbate atopic asthma in some individuals (34, 50). Additionally, animal waste products can also release high concentrations of gas by-products, which are associated with a variety of health effects. Hydrogen sulfide is one by-product which at high levels may cause death by asphyxiation, and at low levels may cause pulmonary edemas, dizziness, or unconsciousness (51, 52). Ammonia is also typically found at agricultural facilities, and causes irritation to the mucous membranes and upper respiratory

airways, as well as sinusitis, and COPD (51). Carbon dioxide and methane gas are also asphyxiants that are primary constituents of animal waste by-products (51).

During the milking process, workers are also exposed to organic and inorganic compounds. With the modernization of dairies and increase in herd size, the switch to automatic milking methods has been applied on a global scale (53, 54). A study by Basinas et. al found that automatic milking and manure handling were both strongly associated with personal inhalable dust and endotoxin exposure (53). Workers on farms using milking robots were also exposed to an average of 2-fold higher levels of dust compared to those using parlor or pipe milking (53). This was primarily because parlor milking workers spent more time actually milking cows, whereas robotic milking allows workers to spend more time on other tasks, where they may increase their dust exposure elsewhere (53).

AGRICULTURAL OCCUPATIONAL RESPIRATORY ILLNESSES

Endotoxin exposure for agricultural workers in grain handling, hog confinement, poultry handling, and similar tasks has been found to be between 60 and 1000 ng/m^3 (55-58), which is incredibly high. Personal inhalable inlet dust samples measured at modern dairy farms have been found to contain endotoxin concentrations between 25 and 35,000 EU/m^3 (11, 59). Compared to the suggested exposure limits of 90 EU/m^3, many of these measurements would result in an extreme overexposure. In states such as California, Colorado, Iowa, and Texas, as well as parts of the UK, organic dust concentrations and endotoxin have been highly variable, with many workers considered overexposed by the suggested occupation exposure guideline of 2.4 mg/m^3 and 90 EU/m^3 (11, 13, 60-64). Endotoxin exposure related to inflammatory responses may be more severe for workers with pre-existing respiratory diseases such as asthma (34, 65).

OSHA estimates that more than 120,000 workers in the agriculture, forestry, and fishing industries are affected annually by respiratory illness (14). The following subparts provide detailed information on some of the most prevalent respiratory diseases associated with inhalable exposures to endotoxin.

Hypersensitivity Pneumonitis (HP)

"Farmer's Lung", a type of hypersensitivity pneumonitis (HP), is an allergic respiratory disease caused by sensitization and recurrent exposure to specific organic dusts (66). HP is caused by several *Actinomyces* species (e.g., *Saccharaopolyspora rectivirgula* and certain *Aspergillus* fungi in particular (51). These microorganisms are commonly found in old and moldy hay, straw, and animal feed (51). Also known as extrinsic allergic alveolitis, this condition is characterized by a non-Immunoglobulin E facilitated inflammation (67). Symptoms begin quickly within 4-6 hours after exposure and may include: fever, chills, malaise, and cough (66). In order to be diagnosed, all following criteria must be met: 1) physical findings and pulmonary function test indicate interstitial disease; 2) X-ray is consistent with this finding; 3) there is a reasonable exposure pathway; and 4) there is antibody in the body for the antigen (51). In some cases, HP can progress so severely that the result is a debilitating or even fatal lung disease (67). Preventive measures are important to ensure that HP is not contracted by workers (51, 66).

Organic Dust Toxic Syndrome (ODTS)

Organic dust toxic syndrome (ODTS) is a systemic inflammatory reaction. Symptoms typically resemble that of influenza and can include: fever, myalgias, chills, chest tightness, and cough, and can occur within 4-8 hours of exposure (36, 51). ODTS is often mistaken for

farmer's lung if proper testing is not done. In order to diagnose ODTS, tests such as a pulmonary function test, oxygen saturation test, and chest radiography are required (51). Rales and interstitial pattern from the chest x-ray are commonly observed with ODTS (68). ODTS is likely a toxic reaction rather than a direct immune reaction, where endotoxin is the cause of the inflammation (51, 69). OTDS is similar to HP, but is associated with approximately 10 times greater dust concentrations (50, 51). Diagnosis of ODTS is often associated with high exposures to grain dusts in animal feed or around the farm (7, 8). Madsen et al. studied the effects of endotoxin exposure on grass seed plant workers. It was found that many workers having symptoms of OTDS had very high exposures to endotoxin, up to 3x105 EU/m^3 (7, 8, 69).

Chronic Bronchitis

Chronic bronchitis is a type of chronic obstructive pulmonary disease (COPD). Unlike HP and OTDS, symptoms exclusively affect the respiratory system and include: bronchial irritation, mucosal secretion, and harsh cough lasting up to three months (7, 70). There are four additional symptoms which typically begin after age 35; difficult breathing, persistent cough, excessive sputum production in airways, and discolored sputum (7, 70). Chronic bronchitis can take years to develop following exposure to irritant constituents as the mucous naturally produced begins to block airflow through the bronchi region of the lungs over time. This creates an advantageous environment for infection to occur (7, 70).

Occupational Asthma

Occupational asthma is associated with airway hyper-responsiveness, airway inflammation, and bronchoconstriction (7, 71). Exposure to endotoxin is known to intensify this inflammation and breathing obstruction (36, 72). The prevalence, incidence, and severity of

asthma symptoms have been on the rise in the United States (72), as over 250 asthmagens and counting have been recognized in various work (7, 71). Exposure to endotoxin in individuals already suffering from allergic asthma, where airway inflammation is already present, has been shown to elicit an additive response (72). Additionally, the inhalation of endotoxin by individuals without preexisting allergic asthma has also been shown to cause characteristic features of the disease (72). Some symptoms, such as wheezing and bronchial hyperactivity, have been reported as more severe in agricultural workers when compared to other physician-diagnosed asthma cases (51). Occupational asthma is difficult to truly diagnose, as it has many clinical subtypes affected by a wide variety of environmental exposures, but continued research has shown it to have a significant effect on agricultural workers (72, 73).

ENDOTOXIN SENSITIZATION

Recent literature suggests that at some dose levels, exposure to endotoxin at an early age may offer a protective immune response. Most studies have used household dust measurements to represent environmental endotoxin exposure to children. Homes with pets are typically associated with higher endotoxin levels, and farm homes have correlations of similar endotoxin concentrations to some barn dust exposures (74). Studies have shown a strong negative association between allergen sensitization and animal exposure for children living in rural areas or on farms (74, 75). Dust and endotoxin levels have also been found to be higher in lower socioeconomic housing neighborhoods, but still not nearly to the levels in rural and farm homes (74, 76).

Studies have shown variation in results comparisons for specific types of asthma (77). The protective effect has been proven against atopic asthma, whereas there has only been a

minimal protective effect against non-atopic asthma symptoms (77). The exact advantages of exposure at a young age are still very unknown, as well as what age creates the greatest immunity (77). There is still significant evidence that shows endotoxin exposure may both worsen asthma symptoms in diagnosed cases, as well as induce new cases of both allergy and non-allergy related asthma in previously healthy adults (77, 78).

Similarly, exposure to bioallergens at a young age has also been shown to reduce the incidence of asthma, whereas exposures during adulthood may increase the risk of an asthma diagnosis (34, 79).

MEASUREMENT OF ENDOTOXIN

Limulus Amebocyte Lysate (LAL) Assay

Limulus amebocyte lysate (LAL) is an aqueous component of the blood of Atlantic horseshoe crabs (*Limulus polyphemus*) *(80)*, which is extracted to provide a quantitative analysis method for measuring endotoxin levels (7). Atlantic horseshoe crabs are one of only four remaining species of horseshoe crab, and are predominantly found along the east coast of the United States between New Jersey and Virginia. Horseshoe crabs are a prehistoric species, surviving the mass extinction of the dinosaurs 65 million years ago. They have been around for over 450 million years, and are commonly known as "living fossils" due to their lack of evolution from the trilobites, a close (and now extinct) relative. (81, 82).

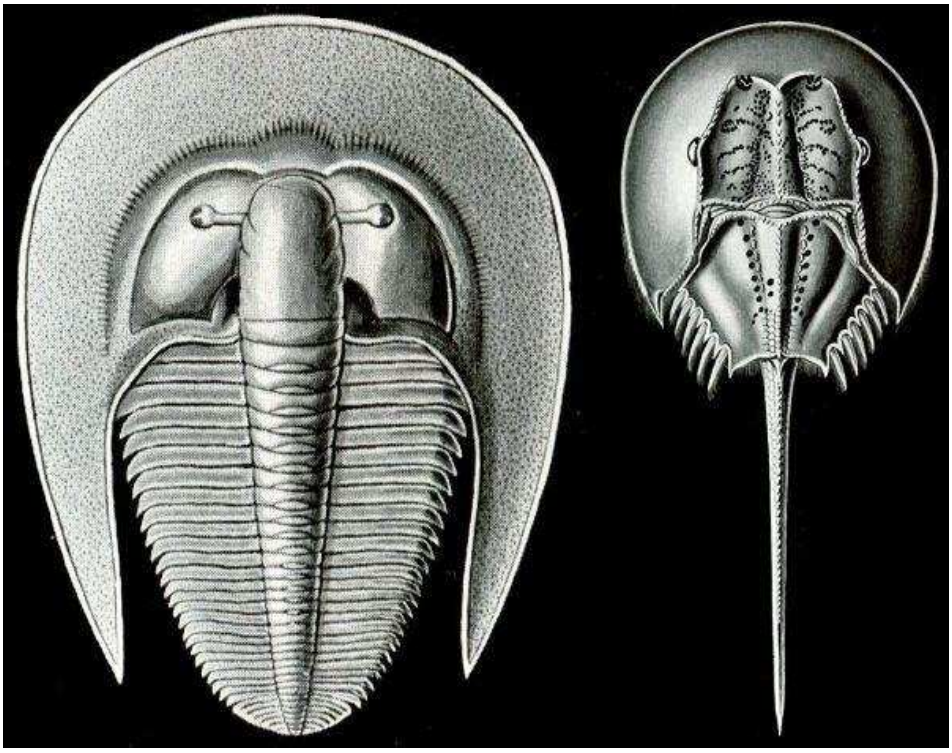

Figure 2.3 Left: trilobite fossil; Right: modern horseshoe crab fossil (83).

The LAL assay is based on an enzymatic reaction, activating the *Limulus* proenzyme isolated from the amebocyte (84, 85). The amebocyte cells in horseshoe crab blood have a particular clotting protein called coagulogens, which help prevent Gram-negative bacteria infections from infiltrating and spreading. This activation then catalyzes the splitting of a peptide chromogen substrate added as part of the procedure. Once the p-nitroaniline is cleaved from the complex, a yellow color is produced. This color is then measured using a spectrophotometer at 405-410 nm (7). The measured absorbance directly corresponds to the amount of endotoxin detected (86). Concentrations are specifically calculated from a generated standard curve (86). The step-by-step mechanism for the LAL assay is described in Figure 2.4 below.

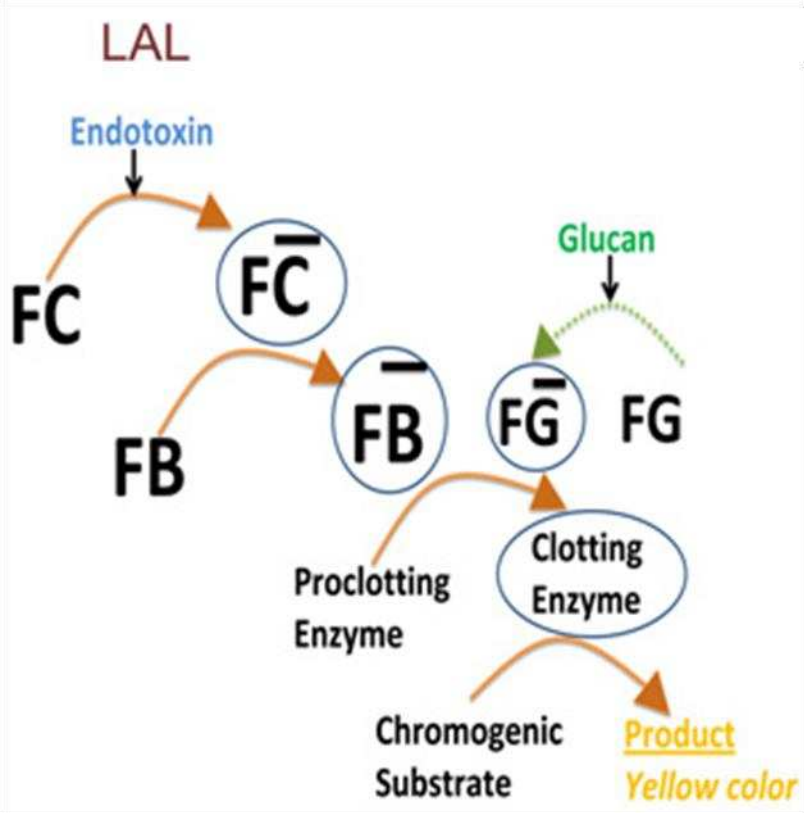

Figure 2.4 Cascading enzymatic reaction for endotoxin detection and quantification for the LAL system (87).

There are three types of LAL endotoxin testing methods including: gel-clot, turbidimetric, and chromogenic (80). These assays are used for evaluation of many prescription medications and vaccinations (7, 80). The gel-clot method uses a clotting protein which is cleaved by the activation of a clotting enzyme. The resulting reaction yields a gel like substance that can be analyzed. This is thought to be the most accurate method, but is also the most complex and time consuming (80). The turbidimetric method can determine endotoxin concentrations in several ways by measuring optical density and comparing it to a standard for either: the rate of increase in turbidity, the duration of time until desired turbidity is reached, or the extent of turbidity after a set time of incubation (80). Finally, the chromogenic method was

developed after discovering that endotoxin activated LAL would cleave amino acid sites that contained chromogenic peptides. The chromogenic method also has multiple measurement options: kinetic and endpoint. In kinetic measurement, the time required for the sample to reach an absorbance of 405 nm is correlated to the concentration of endotoxin. In the endpoint method, the amount of p-nitroaniline produced is quantified after a set incubation time (80). Chromogenic provides the most user-friendly and time efficient method for analyzing endotoxins (80).

Recombinant Factor C (rFC) Assay

The recombinant factor C (rFC) assay is a more recent development utilizing cloned rFC from the *Carcinoscorpius rotundicauda* species of horseshoe crab, typically found along the coast of Asia (27). This laboratory-made assay has both advantages and disadvantages when compared to the LAL assay. First, the rFC assay does not contain Factor G, which has shown to cause significant interference from (1→3)-Beta-D-Glucans in the LAL method. In addition, because the rFC assay is synthetic, the lot-to-lot variation is much less than with the LAL assay. Finally, because the rFC assay contains Factor C, it is able to recognize endotoxin and react with a fluorescence compound directly. This makes the mechanism much shorter, providing less opportunities for error or interference (27). The step-by-step mechanism of the rFC assay is illustrated in Figure 2.5 below.

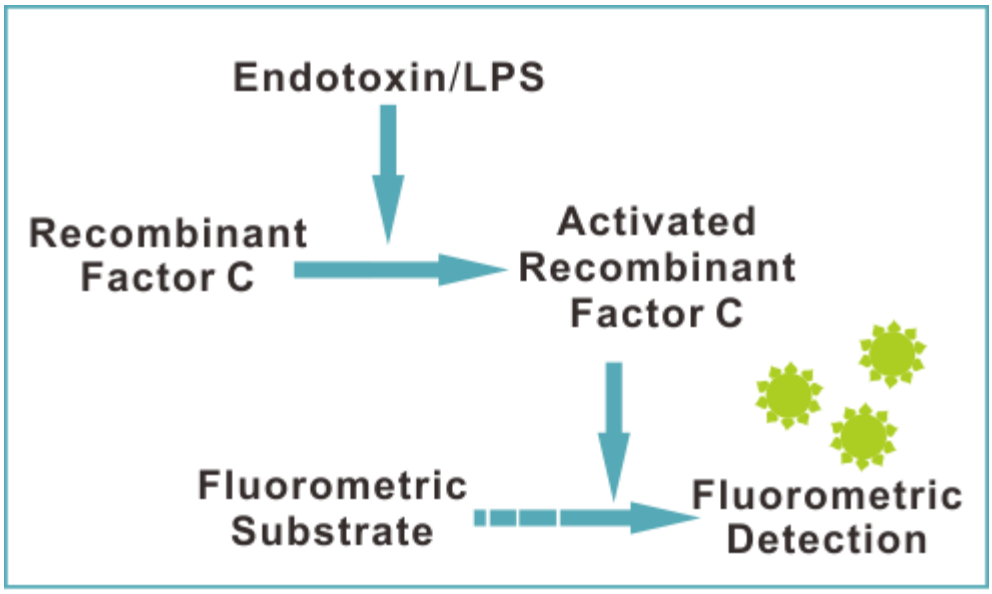

Figure 2.5 Endotoxin detection mechanism for the rFC system where endotoxin activates the recombinant Factor C to produce a fluorometric substrate (88)

There have been several studies comparing the rFC and LAL endotoxin assays. Most recently, Thorne et al. performed air monitoring at 10 livestock facilities, and ran paired sample analysis using the rFC and LAL assay. Excellent agreement was found between the two assay types with an r value of 0.86 (89). Additionally, correlation between type of facility or type of dust ranged from 0.65 to 0.96 (89). Another study done by Alwis and Milton used house dust samples to compare the rFC and LAL assay. It was found that the LAL assay endotoxin measurements were higher than the rFC, but the two assays were still highly correlated (27). For the total sample size for this study, an r value of 0.86 was calculated (27). One final study also looked at a comparison of the rFC and LAL assay in three different animal environments: dairy farm, animal research facility, and a feed mill. Statistically significant correlations were found between the dairy farm samples and animal research facility samples with r values of 0.82 and 0.39 (7).

CHAPTER 3: PURPOSE AND SCOPE

The purpose of this study was to: 1. Evaluate filter recovery from spiked endotoxin samples and 2. Compare two different biological endotoxin assay kits: Lonza rFC and Associates of Cape Cod Pyrochrome Chromogenic. The Bland-Altman statistical analysis will be used in order to detect any significant variation in measured endotoxin concentrations. A linera regression will be used to establish a conversion factor for interstudy comparison purposes between the rFC and LAL assay. This study specifically compared these differences using personal and area air samples collected at dairies across Colorado. The extraction solution from each filter was then used for analysis with each endotoxin assay.

The null hypotheses for this study were as followed:

(1) There is no difference between the rFC and LAL kit analyses.

(2) The conversion factor of the rFC to LAL assay is equal to 1.

This work is important because although both of these endotoxin assays have been used for many years, there are still considerable gaps in knowledge and the literature comparing them. There are several advantages as well as limitations with this study. One advantage was the degree of variability in the environmental conditions under which these samples were collected, and therefore variability in endotoxin analysis levels are expected to vary as well. Samples were collected seasonally, so we expect dust levels to vary between months, and therefore contain different levels of endotoxin. Samples were also collected inside and outside the milking parlor, which can have extremely different dust levels. Samples were also collected in worker's personal breathing zones, so depending on their tasks and work environment for the day, overall dust and endotoxin levels may vary greatly.

One limitation of this study includes the sample processing and handling. These samples have been collected over the period of approximately two years, and stored in a freezer at -80°C after collection. This may affect the integrity of the filter, extraction solution, and overall endotoxin level.

CHAPTER 4: MATERIALS AND METHODS

AIM 1: SAMPLE PREPARATION

This was a laboratory based study to assess the filter extraction recovery of the rFC endotoxin assay at different concentrations on PVC filters. The filters were liquid-spiked with known concentrations of endotoxin in the amounts of: 0.1, 1, 10, 100 and 1000 EU. Analysis was done in replicates of five for each concentration plus one lab blank for a total of 26 samples.

A Reference Standard Endotoxin (RSE) of 10,000 EU/ml of *Escherichia coli* (*E. coli*) (Lonza, Walkersville, MD) was used for the liquid spiked filters. Serial dilutions used for the standards were made from the reconstituted 10,000 EU/ml RSE and 0.05% Tween-20 LAL water. The 10,000 EU/ml RSE was diluted with 0.3 ml of the RSE into 2.7 ml of LAL water to produce the 1000 EU spike. A volume of 0.3 ml of the 1000 EU spike was diluted with 2.7 ml of LAL water to produce the 100 EU spike. A volume of 0.3 ml of the 100 EU spike was diluted with 2.7 ml of LAL water to produce the 10 EU spike. A volume of 0.3 ml of the 10 EU spike was diluted with 2.7 ml of LAL water to produce the 1.0 EU spike. A volume of 0.3 of the 1.0 EU spike was diluted with 2.7 ml of LAL water to produce the 0.1 EU spike.

All filters were stored in a sterile desiccator for at least 24 hours prior to sample preparations. All work was completed in a sterile biosafety cabinet on pieces of sterile foil, baked at 150° C for three hours in order to ensure no endotoxin contamination. Each filter was loaded with 100 µl of the appropriate spiked concentration, pipetted onto filters in 50 µl increments, and complete absorption was observed visually.

Figure 4.1 Biosafety cabinet setup during filter spiking process

Filters were then desiccated for 24 hours. The next day, filters were loaded into SKC Button Aerosol Samplers, and connected to SKC Aircheck 5000 pumps, with a flowrate calibrated to 4.0 L/min in order to best mimic field practices and the dynamics between the filter and endotoxin during air sampling. Each pump was run for four hours inside the biosafety cabinet. Filters were then stored in a 50 ml Falcon conical, polystyrene tube (BD, Bedford, MA) and frozen at -80° C. Once frozen for 24 hours, samples were removed from the freezer, extracted in 50 ml tubes, using 10 ml of sterile, pyrogen-free water (PFW) containing 0.05% Tween-20 and placed in the shaker for 1 hour at 100 rpm at 22° C. Endotoxin concentrations were measured in triplicates containing 100 µl of sample.

RECOMBINANT FACTOR C (rFC) ENDOTOXIN ASSAY

The endotoxin standard used to create the standard curve was reconstituted using 0.05% Tween-20 solution in pyrogen-free water to a volume described in the Lonza certificate of analysis based on the lot number. This reconstituted solution yielded a 5 EU/ml standard. Dilutions were then made to create a 5.0, 0.5, 0.05, and 0.005 EU/ml standards. Additionally, some samples were diluted in order to ensure a readable level during the test. The 96 well-plate was then loaded with 100 µl of each solution in triplicates. Loaded samples included: blank 0.05% Tween-20 LAL water, 5.0 EU/ml standard, 0.5 EU/ml standard, 0.05 EU/ml standard, 0.005 EU/ml standard, 1000, 100, 10, 1.0, and 0.1 liquid standards, and 26 extracted filter samples. The liquid standards were also spiked with 10 µl of the 5 EU/ml endotoxin standard for quality control assurance.

After the plate was loaded, it was inserted into the BioTek FLX800 Fluorescence Reader and was incubated for 10 minutes at 37°C. During this time, the working reagent was prepared by combining 5.50 ml of fluorogenic substrate, 4.40 ml of rFC assay buffer, and 1.10 ml of rFC enzyme solution. The fluorogenic substrated provides the fluorescence source, which is used to measure the relative endotoxin concentrations. The assay buffer helps to optimize the conditions of the solution, and the enzyme solution is always added last sequentially because it begins the reaction.

After incubation, the plate was removed from the reader and 100 µl of the reagent was added to each well. The plate was returned to the reader and readings were taken at sensitivities 35, 45, and 55 at time “0” minutes and again at time “60” minutes. The results were then based

off of the sensitivity which provided the relative fluorescent units closest to the standard as provided by the manufacturer. All data was processed with BioTek Gen5 software.

The use of a standard curve creates the ability to measure endotoxin levels relative to the standards. The scale for the rFC assay ranges from 0.5-5 EU/ml, as mentioned above.

AIM 2: SAMPLE COLLECTION

Samples were collected from two dairy farms in Colorado. All data were collected seasonally between winter 2013 and winter 2014 in conjunction with a larger study. Both personal and area samples were collected on 25 mm PVC filters using the SKC Button Aerosol Samplers. Samples were collected over an eight-hour work shift at a flow rate of 4 L/min. Additionally, a lab blank and two field blanks were also included during each sampling trip. Personal samplers were affixed to the collar of workers to monitor their breathing zone. Tasks associated with personal monitoring occurred in both indoor and outdoor environments and included: parlor milking, feeding, directing and moving cattle, veterinary care, calf care, and machinery work. Area samples were collected in duplicate inside the milking parlor, as well as upwind and downwind of the parlor. A subset of 30 samples was selected to represent different tasks, and seasons, and dust and endotoxin concentrations.

Figure 4.2 Fully assembled SKC Button Aerosol Sampler with 25 mm PVC filters (90) and SKC AirChek XR5000 Pump (91).

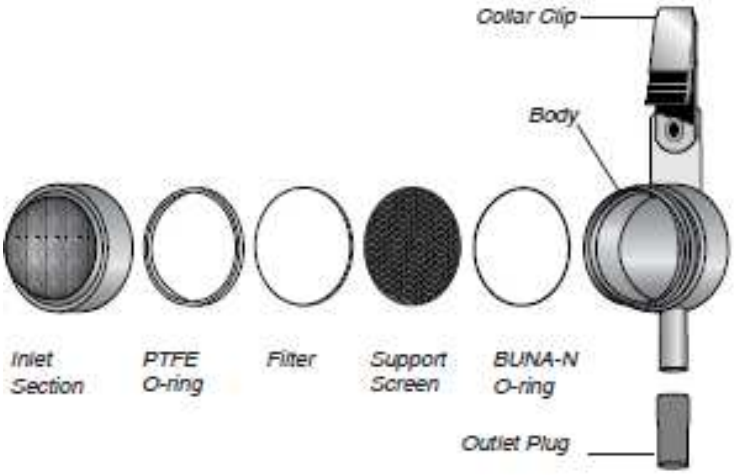

Figure 4.3 Chain of assembly of SKC Button Aerosol Sampler (92).

Approval and consent to collect personal samples was secured through the Institutional Review Board (IRB). All participants were selected on a volunteer basis and were compensated for their time. Each participant was also given a thorough consent form to fill out with the stipulation that they could remove themselves from the study at any time.

EQUIPMENT STERILIZATION

The SKC Button Aerosol Samplers, as well as tweezers, were sterilized between each sampling visit. First, buttons, O-rings, support screens, and tweezers were all washed thoroughly with warm water and antimicrobial soap. The buttons, support screens, and were then wrapped in aluminum foil and baked at 150° C for 3 hours. The O-rings are submerged in 70% ethanol and sonicated for 60 minutes.

GRAVIMETRIC ANALYSIS

Analysis of inhalable dust amounts were measured by weighing each individual filter pre and post sampling in duplicate using a Mettler MT5 balance (Mettler-Toledo, LLC, Columbus, OH). The difference was then used to determine the net weight gain of inhalable dust. All filters were desiccated for 24 hours prior to pre-weighing and post-weighing. Each filter was placed on a Plutonium neutralizer strip for 10 seconds prior to being placed on the balance.

SAMPLE STORAGE

All sterile filters were stored in a sealed sterile desiccator until use. After post-sampling desiccation and weighing, filters were transferred individually to sterile 50 ml falcon tubes and stored frozen at -80° C.

FILTER EXTRACTION

Samples were extracted in 50 ml conical, polystyrene tubes, using 10 ml of sterile, pyrogen-free water (PFW) containing 0.05% Tween-20 and placed in the shaker for 1 hour at 22° C. Aliquots used for the assays were taken directly from the 50 ml falcon tube, and were then used in both the rFC and LAL assay.

PYROCHROME® CHROMOGENIC LAL ENDOTOXIN ASSAY

The endotoxin standard was reconstituted using 3.6 ml of 0.05% Tween-20 LAL water as described on the Associates of Cape Cod certificate of analysis. This reconstituted solution yielded a 50 EU/ml standard. Dilutions were then made to create one of three separate set of standards. The first range consists of: 1.28, 0.64, 0.32, and 0.16 EU/ml standards. The second range consists of: 0.16, 0.08, 0.04, and 0.02 EU/ml standards. The third range consists of: 0.04, 0.02, 0.01, and 0.005 EU/ml standards. Samples were diluted based on previous results in order to provide a readable output for the test and assay was run with appropriate standard selection.

The 96 well-plate was then loaded with 50 µl of each solution in triplicates. Loaded samples included: blank 0.05% Tween-20 LAL water, liquid standards, and samples at appropriate dilutions. Select samples were also spiked with 10 µl of the highest EU/ml endotoxin standard for quality control purposes. After the samples were loaded, the Pyrochrome reagent was reconstituted with 3.2 ml of Glucashield Buffer. Using a multi-channel pipette, 50 µl of the Pyrochrome reagent was added to each well and the plate was inserted into the BioTek FLX800 Fluorescence Reader for incubation at 37°C. The incubation times for the 1.28-0.16 EU/ml, 0.16-0.02 EU/ml, and 0.04-0.005 EU/ml are 22 minutes, 37 minutes, and 61 minutes respectively. After being incubated for the appropriate amount of time, the plate was transferred to the BioTek PowerWaveXS Microplate Spectophotometer and absorbance was read at 405 nm. All data were processed with BioTek Gen5 software

RECOMBINANT FACTOR C (rFC) ENDOTOXIN ASSAY

The rFC assay was carried out using the same methodology described under aim 1. Many samples were run in serial dilutions to ensure readability and as a quality control measure for the dilution accuracy.

SAMPLE SIZE

Sample size was determined by performing a paired t-test based on previous rFC versus LAL assay data collected at Colorado State University in 2009. This study compared endotoxin levels in air samples between dairy farm, feedlot, and lab animal research facility environments. It was found that a total sample size of 22 was required to yield a power of 90% to detect a significant difference in endotoxin concentration between assay types.

STATISTIAL ANALYSIS

Data were statistically analyzed in December of 2015 with the assistance of Dr. Ann Hess from the Department of Statistics consultation program at Colorado State University. All statistical analysis was completed using R Statistical Programming version 3.2.3. Aim 1 data were graphically represented using five bar charts. Each bar chart represents one expected concentration range and includes the actual measured concentrations for each sample. In addition, ranges, means, standard deviations, and coefficients of variations were calculated for each subset.

For Aim 2, data distributions were analyzed and represented in ranges, means, standard deviations, and coefficient of variations. These data sets were presented for the total data set as well as broken down by personal, area, and lab/field blanks. Data was tested for normality and it

was determined that a log transformation would be appropriate. Using the log transformed data, a statistical difference between the rFC and LAL assay was analyzed using an agreement analysis technique called the Bland-Altman analysis by recommendation of Dr. Hess. An alpha value of 0.10 was determined to be appropriate based on the total sample size. Box plots with error bars showed the graphical variation between the rFC and LAL assays between the three types of samples. Additionally, correlations were done between the rFC and LAL assays comparing each sample type, and a linear regression was used to compute a conversion equation.

CHAPTER 5: RESULTS

OVERVIEW

The purpose of Aim 1 was to determine the recovery efficiency of the rFC assay using pre-loaded PVC filters with known concentrations. Recoveries were analyzed using five different concentrations, each in replicates of five. Statistical descriptives for each concentration including: ranges, means, standard deviations, and coefficients of variations can be found in Table 5.1. Tables presenting descriptives for each individual sample can be found in the Appendices (Tables A.1-A.5). Additionally, a graphic representation of comparisons of replicates for each concentration can be found below (Figure 5.1).

The purpose of Aim 2 was to use previously collected agricultural air samples and compare the measured endotoxin concentrations of the rFC and LAL assays. All data collection sites were dairy farms located in Northern Colorado and samples were collected between winter 2013 and winter 2014. Summary statistics including: ranges, means, and standard deviations can be found in Tables 5.2-5.3. A boxplot with error bars illustrates the distributions of endotoxin measurements in Figure 5.2. These data are separated by assay type as well as sample type.

AIM 1 RESULTS

It was anticipated that the total measured mean EU/filter would be close to the expected concentration for each set of samples; however, the results indicated much lower concentrations. For the filters theoretically preloaded with 1000 EU/filter, there was a sample recovery of 5% with a total mean of 50.37 EU/filter (standard deviation: 7.34). Similarly for the 100 EU/filter samples, there was a 2% sample recovery with a total mean of 1.93 EU/filter (standard deviation:

1.30). Both the 10 and 1.0 EU/filter samples had a 1% sample recovery with total means of 0.13 EU/filter (standard deviation: 0.13) and 0.010 EU/ml (standard deviation: 0.010) respectively. Finally, the 0.1 EU/filter samples had a sample recovery of 8%, with a mean of 0.0081 EU/filter (standard deviation: 0.0081).

Table 5.1 Summary of descriptive statistics for spiked filter recovery (n=25)

Expected Concentration	Range (EU/filter)	Mean (EU/filter)	Standard Deviation	Coefficient of Variation
1000 EU/filter	46.17-62.33	50.37	7.34	43.12%
100 EU/filter	0.65-4.02	1.93	1.30	16.14%
10 EU/filter	0.07-0.22	0.13	0.060	4.39%
1.0 EU/filter	0.007-0.011	0.010	0.0025	2.70%
0.1 EU/filter	0.004-0.013	0.0081	0.0043	4.98%

Figure 5.1 graphically demonstrates the variability between replicates for each concentration subset. Especially high variability was observed between samples from the 100, 10 and 0.1 EU/filter concentrations.

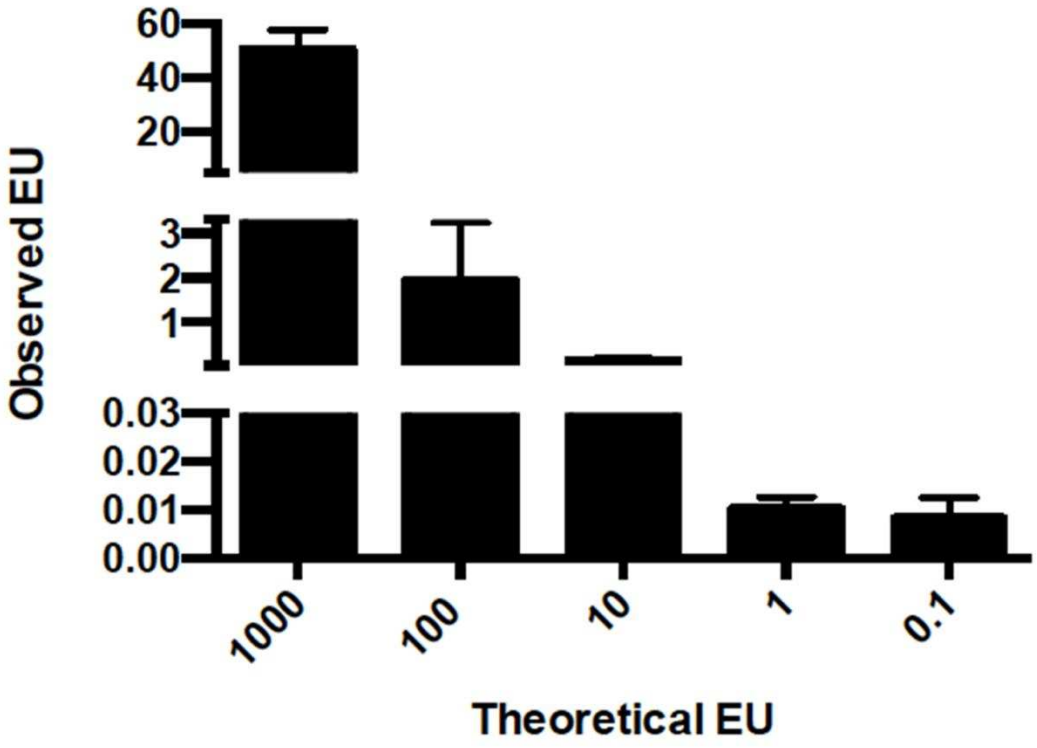

Figure 5.1 Theoretical vs. Observed endotoxin EU/filter per concentration

AIM 2 RESULTS

In order to normalize the results, data were log-transformed. No statistical difference was observed between assays for the total sample size (p-value 0.7146). Additionally, there was no statistical difference between assays for the personal sample subset (p-value 0.3788). However, there was a statistically significant difference found for the area sample subset (p-value 0.0698) and the lab and field blank sample subset (p-value 0.06638) with an alpha=0.10. Comparative descriptive data between the rFC and LAL assay results can be found in Table 5.2, while Table 5.3 includes the same information broken down by sample subsets.

Table 5.2 Summary of descriptive statistics comparing the rFC and LAL assays (n=31)

Assay	Range (EU/ml)	Mean (EU/ml)	Mean with dilution factor (EU/ml)	Standard Deviation
rFC	0.0035-5.01	0.096	2263.13	1.50
LAL	0.0060-1.21	0.29	2207.10	0.40

Table 5.3 Summary of descriptive statistics for the rFC and LAL assays by sample type (n=31)

	rFC Assay			**LAL Assay**		
	Range (EU/ml)	Mean (EU/ml)	Standard Deviation	Range (EU/ml)	Mean (EU/ml)	Standard Deviation
Personal	61-10027.33	8913.91	5225.06	15-18784	9016.62	7286.97
Area	0.11-3312.33	484.92	1062.54	0.017-2584	312.09	712.49
Lab/Field Blank	0.0035-0.31	0.11	0.11	0.016-177	38.80	72.42

A side-by-side boxplot distribution comparison between the rFC and LAL assay for each type of sample is illustrated in Figure 5.2. Each subset had similar means and variation between the rFC and LAL assay with the exception of the lab and field blanks. The variation was exceptionally high for the LAL assay. The means for the log transformed data were most closely related for the field and lab blank (FB/LB) subset, despite the fact that it was also the subset with the largest degree of variability. For both the area and personal sample subsets, the rFC assay reported higher average endotoxin concentrations when compared to the LAL assay.

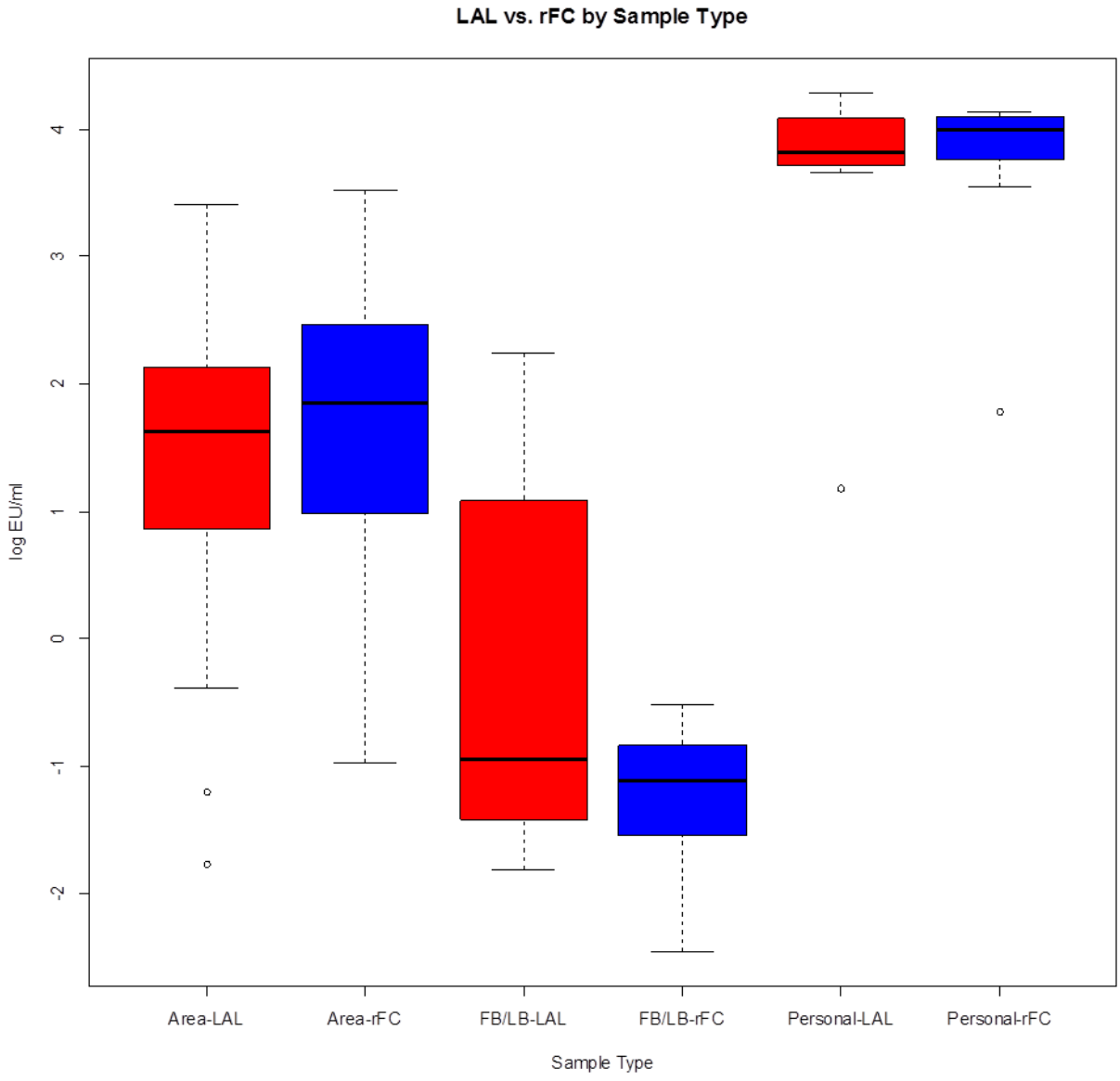

Figure 5.2 rFC vs. LAL assay boxplot distribution by sample type

A correlation graphic showing the correlation between the log transformed data of the rFC assay results and the log transformed data of the LAL assay can be found in Figure 5.3. The correlation between observations for all samples was found to be r=0.867 (relatively high). The R^2 coefficient value was found to be 0.7524. This indicates that 75.24% of the variability in LAL assay data can be explained by rFC assay data. Correlations were also conducted for the

rFC vs. LAL assay data broken down by sample type. This can be found in Figure 5.4. As mentioned previously, the correlation was the weakest between the field and laboratory blanks subset. Conversely the strongest correlation was seen between the personal sample subsets.

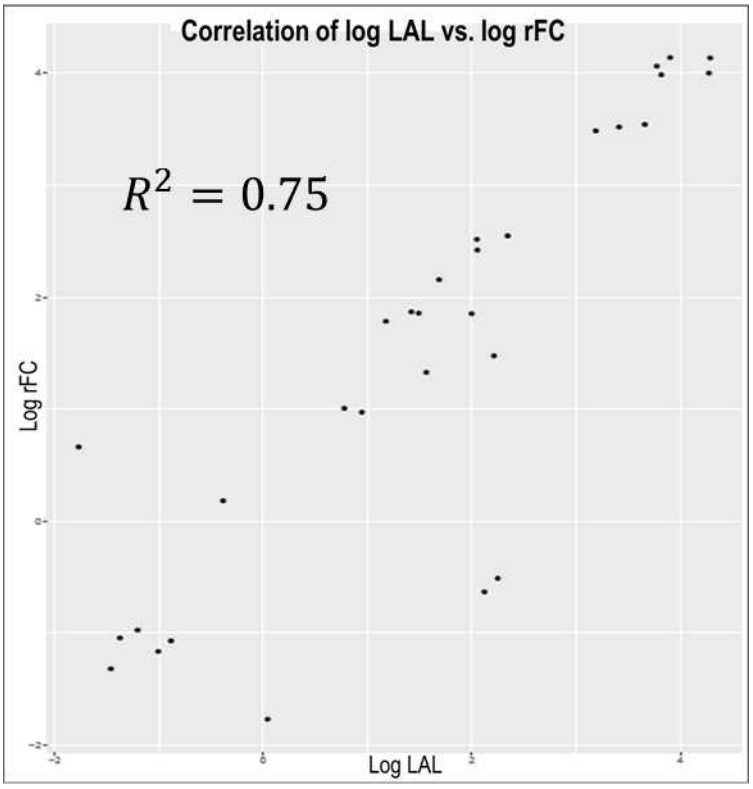

Figure 5.3 Scatterplot of log transformed endotoxin concentration means by assay type (rFC vs. LAL)

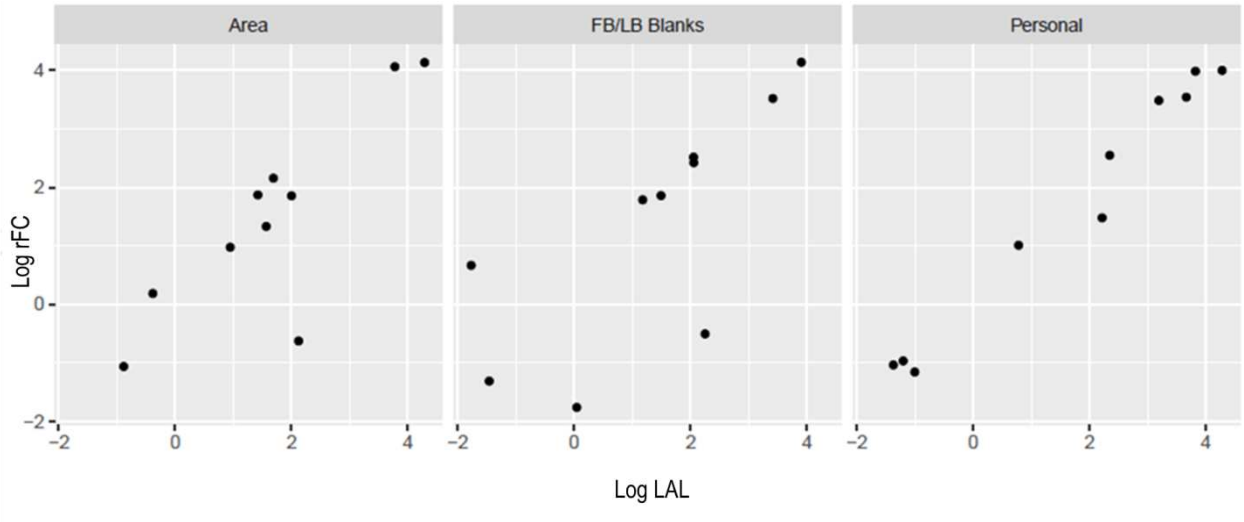

Figure 5.4 Scatterplot of log transformed endotoxin concentration means by assay type (rFC vs. LAL) broken down by sample type

In addition to correlations, a linear regression was performed to develop a conversion factor in order to convert between rFC and LAL endotoxin measurements. The equation was found to be: $y=0.89312x + 0.121$, where y=the log transformation of rFC and x=the log transformation of LAL. Additionally, it was also found that the intercept was not significant (p-value=0.605), whereas the slope was found to be significant (p-value<0.0001). This further confirms that there was no significant difference found between the rFC and LAL assay.

There were several methods of quality control during this study. Table 5.4 illustrates the quality control data collected including: field and lab blank endotoxin measurements, spike recoveries and standard curve correlation coefficient (r) values. Three randomly selected samples per plate were spiked with 10 µl of 5.0 EU/ml endotoxin standard in addition to being run for endotoxin measurement. Using a comparison between the original sample and the spiked sample, a spike recovery percentage was calculated.

The lab and field blank sample concentrations were considerably higher for the LAL assay, which was likely due to contamination during sample handling and transfer after the rFC assay was completed. Furthermore, the mean standard curve correlation coefficient for the LAL assay was lower than the rFC, and did not meet the recommended value from the manufacturer (Table 5.4). Two sample curves for comparison of the rFC and LAL assay can be found in the appendix (Figure A.1-A.2). All trials for the rFC assay resulted in a standard curve correlation coefficient which exceeded the manufacturer recommendation.

Table 5.4 Quality control and standard curve results by assay (n=31)

Quality Control	rFC	LAL
Lab/Field Blanks	0.11 EU/ml	38.80 EU/ml
Spike Recovery %	94.05%	79.49%
Standard Curve Results		
Recommended (r) values	>0.98	>0.98
Observed mean (r) values	0.965	0.998

CHAPTER 6: DISCUSSION AND CONCLUSIONS

In this study, PVC filters were preloaded with known amounts (EU) of endotoxins in replicates of five. Each filter was then extracted and measured for endotoxin recovery using the rFC assay. Additionally, inhalable air samples, which were collected from two different dairies, were measured for endotoxin concentrations and compared using the rFC and LAL assays.

Endotoxin recovery from preloaded filters was on average several magnitudes lower than the anticipated amount. The endotoxin was reconstituted in LAL water and liquid spiked on a PVC filter. It took approximately 15 minutes for the liquid spike to fully absorb into the filter based on visual observation. The solution consisted of a mixture of reconstituted endotoxin and 0.05% Tween-20 LAL water. This was determined as the most effective method for delivering known amounts of endotoxin to filters after a series of different solutions that were preliminarily tested including ethanol and isopropyl alcohol. PVC filters have certain hydrophobic properties, which make them a popular choice to collect dust particles, but generally samples are desiccated for 24 hours before analysis to ensure that water vapor in the air does not affect the gravimetric results.

Due to the hydrophobic nature of the PVC filters, the preloaded amount of the water-based endotoxin solutions were likely not fully absorbed onto the filters, skewing the results. Some of the liquid spike may have evaporated inside the biosafety cabinet or partially absorbed, but was then removed by the desiccation process. This resulted in a much lower yield of endotoxin than expected. It is possible that there was an error with the rFC assay, but this is unlikely as the individual liquid spikes were also tested. Each liquid spike was found to be within 10% of the anticipated concentration, compared to the only 1-8% recoveries from the

filters. Additionally, the endotoxin could have been so deeply imbedded in the filter that it was not extracted properly.

Based on the results from a variety of previous studies, some have indicated that there is an overestimation associated with the rFC assay, and an underestimation with the LAL assay, while other cases have found the opposite effect (7, 27, 29, 89). Results in present study found that on average, the rFC assay measured higher levels of endotoxin for area samples. The LAL assay measured higher levels of endotoxin for both the personal and lab and field blank samples. Since each sample was analyzed with the rFC assay first, the samples underwent one additional freeze-thaw cycle during a storage period before the LAL assay was conducted. The number of freeze-thaw cycles has been thought to affect the concentration of endotoxin in some cases, which may partially explain why some of the LAL results were higher. A study done by Tiscornia et al. demonstrated that over the course of 30 freeze-thaw cycles, endotoxin levels in spring wheat and oat dust increased and decreased in a wave like pattern (Figure 6.1). There are a number of aspects that affect sample vitality during freeze-thaw cycles including: duration spent thawed, duration spent frozen, temperature at which the sample was frozen, and number of freeze-thaw cycles completed. Unfortunately, the true consequences of this process are still unknown, and there is not currently a better alternative to maintaining sample integrity while concurrently running multiple analyses.

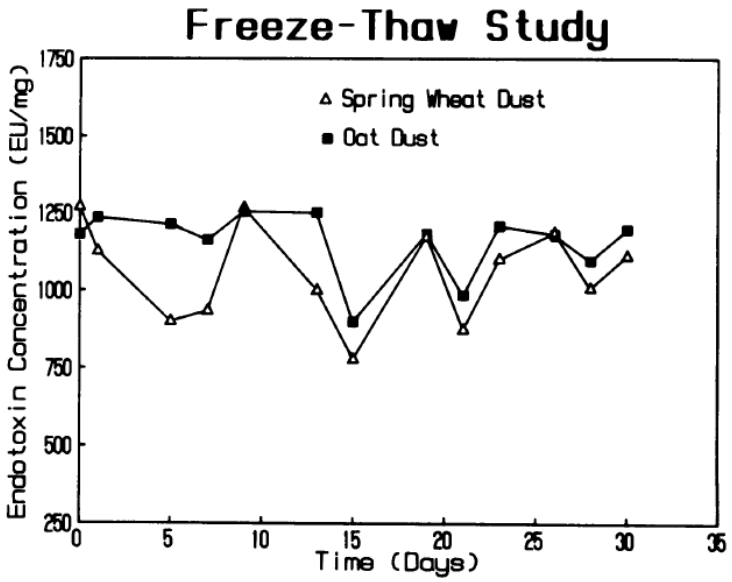

Figure 6.1 Endotoxin measurement (EU/mg) vs. days of freeze-thaw cycles

The protocol for each assay is also quite different, leading to some unique challenges. The rFC and LAL assay both require accurate pipetting of the individual samples into a 96 well plate. It is important to pipette quickly and accurately into the correct well to avoid sample contamination. Both protocols also require time sensitive steps. After 10 minutes of incubation during the rFC assay, it is critical that the enzyme mixture is pipetted as quickly as possible using a multi-channel pipette in order to ensure that minimal reaction has taken place before it is mixed with the samples. This requires pipetting each of the three reagents into the reservoir, removing the 96-well plate from the reader, pipetting the reagent into each well, and placing the plate back into the reader as swiftly as possible. The transfer of the plate between the biosafety cabinet and reader also poses a threat of contamination to the samples. The process is similar for the LAL assay when adding the Pyrochrome reagent and Glucashield Buffer mixture; however, there is no pre-incubation before adding the reagent so the element of transferring the plate is removed.

One major difference between the rFC and LAL assay is the method by which endotoxin concentrations are measured. The rFC assay uses fluorescence, whereas the LAL assay uses absorbance. This requires the use of different equipment in many cases. The rFC assay was able to be performed solely with the use of the BioTek FLX800, because it has the capacity to incubate and read fluorescence. It lacks, however, the ability to measure absorbance. This presented a challenge for the LAL assay, as it required the use of a BioTek Powerwave XS to read absorbance, but the samples still needed to be incubated. In order to accommodate these requirements, a custom protocol was developed for both the FLX800 and Powerwave XS. In the first step, the plate was loaded into the FLX800 and incubated for the appropriate time based on the manufacturer's incubation table. Next, the plate was quickly transferred to the Powerwave XS and the protocol for reading at an absorbance of 405 nm began.

Although there is equipment that will both incubate and read absorbance, it was not financially feasible for the scope of this project. The transfer of samples partway through the analysis process could potentially cause contamination from particles in the air. To minimize the chances of the occurrence, the two machines were placed side by side on the laboratory bench. Additionally, this transfer likely caused a slight drop in temperature of the samples so the ideal temperature may not have been met when endotoxin units were quantified. On average the transfer was completed in less than 20 seconds to minimize the effects mentioned above.

COST ANALYSIS

Aside from the differing procedural techniques, the supplies required are actually quite similar. Both assays require 2 boxes of sterile, pyrogen-free 100-1000 µl pipette tips for one 96-well plate containing 24 samples. For both assays in this study, one box of 1000 µl pipette tips

was used for dilution preparation and sample loading. An additional box of 200 µl pipette tips was used in conjunction with the multi-channel pipette to load the reagents. The 200 µl pipette tips purchased from Fischer Scientific cost $77.84 for a case of 10 boxes. The 1000 µl pipette tips purchased from Fischer Scientific cost $84.70 for a case of 10 boxes. The most significant cost difference is the price of the assay kit itself. The rFC assays were purchased from Lonza in bulk, which did decrease the price per kit. Table 6.1 demonstrates the exact side-by-side cost comparison between the two assays. The rFC assay is much less expensive when ordered in bulk, which includes the supplies to run 30 plates. However, the LAL assay is significantly cheaper if the rFC assay is purchased at the individual price of $504/kit. These prices are also subject to change based on availability and cost of resources.

Table 6.1 Cost comparison between rFC and LAL assay for a single 96-well plate

Supply	rFC assay cost-in bulk	rFC assay cost-individual	LAL assay cost
Assay Kit	$229	$504	$440
Pipette Tips	$16.25	$16.25	$16.25
Reagent Reservoirs	$3.76	$3.76	$3.76
Total Cost	$249.01	$524.01	$440.01

Beyond offering insight into the difference in cost, this study also provided information regarding the technical aspects of endotoxin measurement. Each plate prepared used a freshly made set of serial dilutions of endotoxin standard. For both the rFC and LAL assays, the respective manufacturers recommend that the standard curve (r) values should be greater or equal to 0.980. This requirement was only consistently met for the rFC assay. A number of the

LAL assay (r) values were below the recommended 0.980, and as a result, so was the mean between all the trials. This lower standard curve (r) value does increase the concern with the validity of the results. Because endotoxin is measured on a scale based on the standards, this could indicate a reason for differences between results for the two assay types. Based on these quality control measures, it is likely that the rFC assay results may be closer to "the true" concentration of endotoxin in each sample.

Based on this information, it seems apparent that the rFC assay offers a more cost efficient and at least equally as accurate option for endotoxin analysis. As discussed previously, the LAL assay utilizes an enzyme found in the blood of horseshoe crabs. This enzyme is incredibly sensitive to the presence of endotoxin. Although this reaction has the ability to detect very finite amounts of endotoxin, the variability between organisms is a concern. Horseshoe crabs have proven to have great variability throughout their single species (93). Research has shown that some lineage of horseshoe crabs have especially excitable blood cells, while others do not (93). Armstrong et al. has a study specifically providing research-based proof that the American Horseshoe Crab species has vast variability from animal to animal in blood clotting capability, which is what makes the LAL assay function properly (93).

The processing of materials for the LAL assay also includes the harvesting of live horseshoe crabs. The lysate is collected using a 14 gage needle inserted into the circulatory system of the horseshoe crab. The blood is then centrifuged and the amebocytes are freeze dried for preservation and storage (93). One horseshoe crab can provide anywhere from 50-400 ml of blood in a procedure that takes less than 10 minutes (93). Currently horseshoe crabs have a stable population; however, with their recent use in biomedical industries, many conservation programs have been developed to provide both protection to the species as well as information

and education to the public. One major collaborative group is the Mid-Atlantic Sea Grant Network, which is comprised of: the University of Maryland, the University of Delaware, North Carolina State University, and the State University of New York. This consortium helps to bring together government, industry, research, and the general public to provide better understanding of the use of resources regarding lysate.

The use of live animals for research always presents unique challenges and limitations. Studies show that the harvesting of horseshoe crabs and daily blood extractions have no known negative health effects on the animal (93). However, it is likely that living in overpopulated harvesting operations and having roughly 1/3 of their blood drained poses some stress to the horseshoe crabs.

The use of the rFC assay offers several advantages over the LAL assay for endotoxin analysis. As mentioned previously, it is a much cheaper alternative as long as it is purchased in bulk. It is estimated that 400 ml of horseshoe crab blood is valued around $6,300 (93), which gives insight into why the LAL assay kits are so expensive. The rFC assay removes the aspect of using an animal product, and instead offers a fully synthetic and laboratory made kit. The laboratory process allows for a much more uniform product, as there is no concern in variability between individual horseshoe crab. Although the rFC kits do have varying lot numbers which correspond to a specific reconstitution volume of the standard, it is likely that the results across various kits will still be equivalent. The fluctuating reconstitution volume simply ensures that all of the endotoxin standards contain the same concentration for the serial dilutions.

The rFC assay also decreases the number of samples requiring re-analysis due to "out of range" results. One difficulty in endotoxin measurement is the "guess and check" method of

dilutions. The rFC assay provides a broader detection range of 0.005-5.0 EU/ml. The LAL standards only cover a fraction of this range, going from 0.005-0.04 EU/ml, 0.02-0.16 EU/ml, and 0.16-1.28 EU/ml. This dramatically decreases the chances of correctly identifying the dilution factor on the first attempt, and can create additional costs to use extra kits for re-analysis.

Overall, the development of the rFC assay greatly reduces the amount of horseshoe crabs harvested and bled for collection, reduces the costs of formulating the lysate enzyme, and most significantly, reduces the inconsistency in endotoxin measurement results.

It is difficult to truly interpret endotoxin concentrations, especially for personal samples, as there are currently no enforceable standards in the United States. Having more than one widely used method makes inter-study comparison difficult between research groups. The ability to more accurately detect and/or report endotoxin concentrations will allow researchers to gain insight into occupational exposures and the pathogenicity of respiratory symptoms caused by endotoxins. This type of research will also provide more information on environmental endotoxin concentrations in order to potentially develop exposure limits in the future.

STUDY LIMITATIONS

There were several limitations throughout the course of this study. For aim 1, the most feasible way of pre-loading the filters with a known concentration of endotoxin was to directly pipette a liquid mixture. Because the PVC filters have hydrophobic qualities, they likely would have absorbed more endotoxin in an airborne form, but it would have been very difficult to know the true concentration captured on the filter. Additionally, all samples were analyzed in a laboratory at Colorado State University. Validating the results with an external laboratory would

have helped to confirm the findings. All samples were collected over the past two years and have been stored in a -80℃ freezer. Unfortunately, the number of freeze-thaw cycles was not recorded, and may be different for each sample depending on when it was collected and its use in other analyses. Since the LAL assay was performed second to the rFC, all samples had one additional freeze-thaw cycle from the storage in between assays.

Another major limitation of this study was the use of two separate machines for incubation and absorption reading during the LAL assay. If resources were available, it would be a better option to have an incubating absorption reader to eliminate the concern of contamination during the transfer. This would also ensure that the temperature stayed constant throughout the entire process.

Additionally, because the two reader system had to be used, this also limited the LAL assay to an endpoint mechanism, rather than the option of a kinetic assay. Each type of assay has pros and cons. The kinetic assay has a longer incubation time, making it a more time intensive process; however, it is also subject to less operation error (94). In contrast, the endpoint assay takes a shorter amount of time to complete, but has a more operator intensive procedure (94). When a chromogenic method is used, the endpoint assay measures the endotoxin concentration after a set period of time, whereas the kinetic assay is an absorbance method. The kinetic assay relies on the amount of time required for the sample to reach a set absorbance level, at which point the endotoxin concentration is measured (80).

CONCLUSIONS AND FUTURE STUDIES

During Aim 1, PVC filters were spiked using a mixture of LAL water and a known concentration of endotoxin standard. Unfortunately, despite testing a variety of solutions for

their adhering ability, the hydrophobic nature of the filter did not allow for complete absorption. Due to this limitation, the rFC endotoxin assay results were much lower than anticipated based on the known preloaded concentrations. In Aim 2, endotoxin concentrations were not found to be statistically significantly different for the rFC versus the LAL assay for the total sample size. When compared by sample type, there was no statistical difference observed between assay types for the personal sample subset. There was a statistically significant difference observed for the area sample subset and the lab and field blank sample subset. Based on the research done for this project, it can be concluded that the rFC assay offers a more cost efficient option for endotoxin analysis. Because the rFC assay is a newer technology that does not require the use of animal product, the rFC assay also has lower variability and higher consistency among readings. The standard endotoxin serial dilutions provide a broader range of detection for the rFC assay when compared to the LAL assay. This minimizes the amount of reruns required due to results outside the limit of detection.

Three samples were spiked during each endotoxin assay run to determine the specificity. It was found that the rFC assay, which had an average spike recovery of 94.05%, is more specific to endotoxin detection when compared to the LAL assay, with an average spike recovery of 79.49%. According to Lonza, an acceptable spike recovery is between 50-200%. According to Associates of Cape Cod, the range of acceptable spike recovery is 50-200% for the LAL assay. Based on the manufacturers' requirements, both assays were well within the acceptable range for spike recovery; however, the rFC assay did average 14.56% higher recovery on average at 94.05%. Despite these differences, the rFC and LAL assay were highly correlated with an R^2 value of 0.7524, demonstrating that roughly 75% of the variability within in the LAL assay data can be explained by the rFC assay data.

This research study has provided a framework for evaluating the rFC endotoxin assay using preloaded PVC filters. There are several ways in which further research could provide potentially more successful ways of confirming the detection capability of the rFC assay.

- Conduct additional research to determine if there is a better liquid medium to transfer known endotoxin concentrations onto PVC filters.
- Explore the option of adhering a known endotoxin concentration to PVC filters using an aerosol form in an enclosed chamber.
- Repeat experiments with a different type of filter such as Polytetrafluorethylene (PTFE) or GF, which may absorb liquid more effectively.
- Expand the evaluation of recovery to include a comparison of the rFC and LAL endotoxin assay.

Aim 2 of this research study has presented information on the comparison of the endotoxin detection and quantification between the rFC and LAL assay. Data collected was from two different dairies in Colorado during two different seasons. There are many areas in which more in-depth research could benefit not only research with environmental samples, but also with biomedical research and pharmaceuticals.

- Conduct further environmental air sampling at other agricultural locations and analyze endotoxin concentrations using the rFC and LAL assay methods.
- Analyze samples for endotoxin concentrations comparing the rFC and LAL assays with fresh samples to avoid the concern of freeze-thaw cycles.
- Analyze environmental air samples before and after lengths of time in the freezer for endotoxin concentrations to determine the true effects of freeze-thaw cycles.

- Perform additional testing between the rFC and LAL assay in partnership with an external laboratory to confirm the validity of the results.
- Conduct further research with the LAL assay using an incubating absorbance reader.
- Continue to develop alternative methods for the use of lysate in endotoxin analysis, to decrease the strain put on the horseshoe crab species, and increase the consistency in the results.

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APPENDIX

Table A.1 Raw descriptives for 1000 EU/ml data

Sample	Mean (EU/ml)	Standard Deviation	Coefficient of Variation
A	62.33	0.0035	35.50%
B	52.33	0.0045	23.21%
C	44.12	0.0025	35.10%
D	46.33	0.0015	60.66%
E	46.67	0.0015	61.10%

Table A.2 Raw descriptives for 100 EU/ml data

Sample	Mean (EU/ml)	Standard Deviation	Coefficient of Variation
A	4.02	0.0015	52.59%
B	0.65	0.0020	6.50%
C	1.13	0.0029	7.85%
D	2.15	0.0044	9.86%
E	1.70	0.0087	3.90%

Table A.3 Raw descriptives for 10 EU/ml

Sample	Mean (EU/ml)	Standard Deviation	Coefficient of Variation
A	0.070	0.001	14.0%
B	0.082	0.0076	2.16%
C	0.12	0.0171	1.42%
D	0.14	0.0191	1.43%
E	0.22	0.0150	2.95%

Table A.4 Raw descriptives for 1.0 EU/ml

Sample	Mean (EU/ml)	Standard Deviation	Coefficient of Variation
A	0.0098	0.0032	2.90%
B	0.011	0.0078	1.35%
C	0.013	0.0031	4.36%
D	0.007	0.0	0.0%
E	0.007	0.0035	2.18%

Table A.5 Raw descriptives for 0.1 EU/ml

Sample	Mean (EU/ml)	Standard Deviation	Coefficient of Variation
B	0.013	0.0015	8.29%
C	0.004	0.0	0.0%
D	0.008	0.0049	1.66%
E	0.004	0.0	0.0%

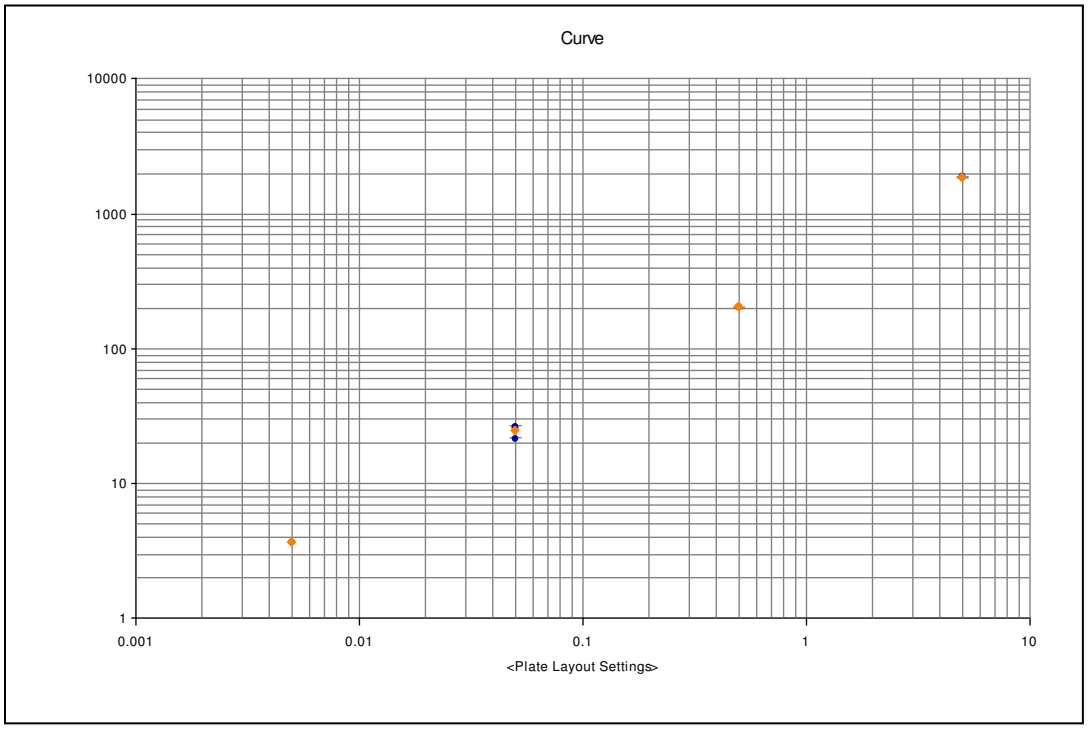

Figure A.1 Endotoxin standard curve for the rFC assay

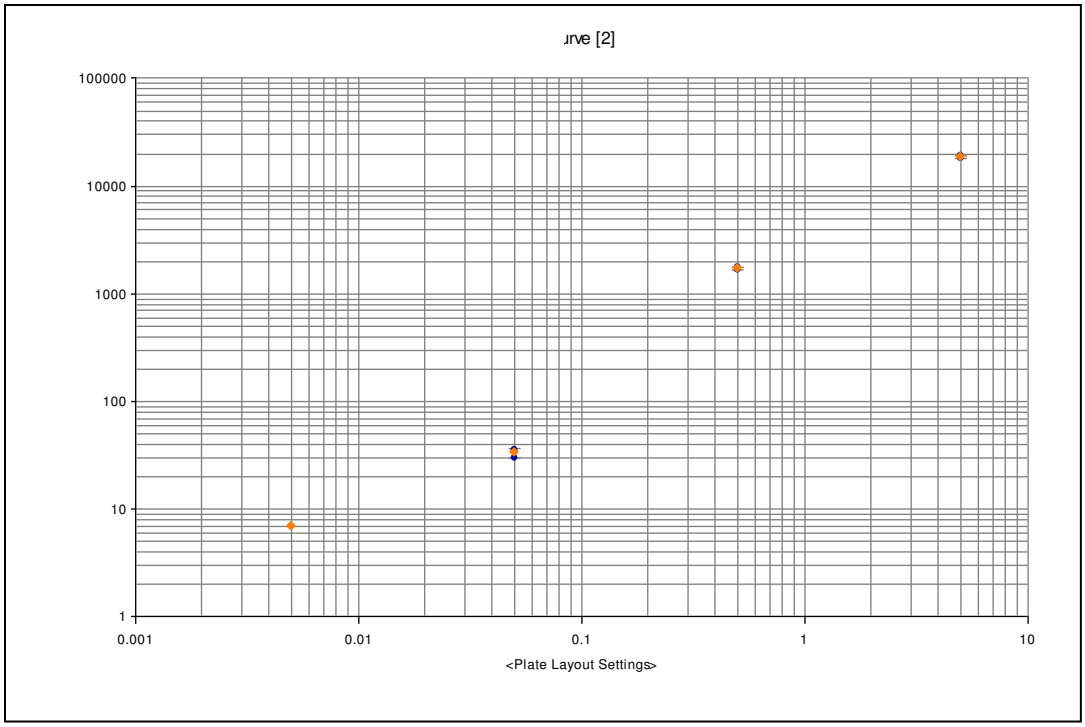

Figure A.2 Endotoxin standard curve for the LAL assay

LIST OF ACRONYMS

ACGIH	American Conference of Governmental Industrial Hygienists
CD14	Cluster of Differentiation 14
CFC	Closed Face Cassette
COPD	Chronic Obstructive Pulmonary Disease
EU/ml	Endotoxin Units per milliliter
FEV_1	Forced Expiratory Volume
GF	Glass Fiber
HP	Hypersensitivity Pneumonitis
LAL	Limulus Amebocyte Lysate
LBP	Lipid Binding Protein
LPS	Lipopolysaccharide
ODTS	Organic Dust Toxic Syndrome
OSHA	Occupational Safety and Health Administration
PEF	Peak Expiratory Flow
PEL	Permissible Exposure Limit
PFW	Pyrogen Free Water
PNOR	Particles not otherwise regulated
PNOS	Particles not otherwise specified
PTFE	Polytetrafluroethylene
PVC	Polyvinyl Chloride Filter
rFC	Recombinant Factor C

RSE	Reference Standard Endotoxin
TLR4	Toll-Like Receptor 4
TLV	Threshold Limit Value
TWA	Time Weighted Average