THESIS

A COMPARISON OF THE LIMULUS AMEBOCYTE LYSATE (LAL) AND RECOMBINANT FACTOR C (rFC) ENDOTOXIN BIOASSAYS: CHARACTERIZATION OF AEROSOLS AND SETTLED DUST ON A SHEEP FARM

Submitted by

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ABSTRACT

A COMPARISON OF THE LIMULUS AMEBOCYTE LYSATE (LAL) AND RECOMBINANT FACTOR C (rFC) ENDOTOXIN BIOASSAYS: CHARACTERIZATION OF AEROSOLS AND SETTELED DUST ON A SHEEP FARM

The goals of this study are: 1) To optimize an extraction protocol for bulk sheep dust analysis by rFC; 2) To compare the traditional chromogenic LAL to the newer fluorometric rFC assay; 3) To compare the effects of the two most widely used extraction media, pyrogen-free water (PFW) and pyrogen-free water with 0.05% Tween 20 (PFW-Tween 20), on the assay outcomes; and 4) To characterize endotoxin exposure in a sheep farm environment. Settled and airborne dust samples were collected from a sheep farm for analysis. Settled dust was analyzed with endpoint fluorogenic rFC and kinetic chromogenic LAL in two different laboratories. A total of 34 stationary airborne dust samples (32 matched) were collected with Institute of Occupational Medicine (IOM) inhalable dust samplers, using polyvinyl chloride (PVC) filters. Airborne samples were analyzed with endpoint fluorogenic rFC and endpoint chromogenic LAL in the same laboratory. The results of the bulk dust analysis showed a significant difference in

polystyrene (PS) tubes and polypropylene (PP) tubes used for extraction indicating that PS tubes yielded higher endotoxin levels ($t = 3.82$, $p = 0.000$). No difference in endotoxin levels was found for centrifugation and spike recovery was closest to 100% for extraction with PFW. The results of the airborne dust study showed a strong positive correlation between the rFC and LAL assays with PFW-Tween 20 extraction. All samples extracted in PFW-Tween 20 had a higher endotoxin recovery compared with those extracted in PFW for both the rFC and LAL assays.

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DEDICATION

To my mother, Shirley, for a lifetime of love, support, encouragement. Without your help, I would not have had the time, energy or confidence to complete this thesis. To my loving husband, Josh Moore, I cannot thank you enough for supporting me through this. I am ever grateful to have you in my life. And to my grandmother, Virginia, for always making me get my homework done before going out to play. I love you all so much!

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LIST OF ACRONYMS

3-OHFA	**Omega Hydroxy Fatty Acids**
CD14	**Cluster of Differentiation, human gene protein**
ECP	**Eosinophilic Cation Protein**
FDA	**Food and Drug Administration**
FEV 1	**Forced Expiratory Volume in First Second of Test**
FVC	**Forced Vital Capacity**
GC/MS	**Gas Chromatography/ Mass Spectrometry**
IL-8	**Interleukin, human chemokine**
IOM	**Institute of Occupational Medicine**
LAL	**Limulus Ameobocyte Lysate**
LBP	**Lipopolysaccharide Binding Protein**
LOD	**Limit of Detection**
LPS	**Lipopolysaccharide**
MD2	**Myeloid Differentiation, human glycoprotein**
MPO	**Myeloperoxidase**
NF-κB	**Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells**
ODTS	**Organic Dust Toxic Syndrome**
OSHA	**Occupational Safety and Health Administration**
PEL	**Permissible Exposure Limit**

PFW	**Pyrogen-Free Water**
PFW-Tween 20	**Pyrogen-Free Water with 0.05% Tween 20**
PMN	**Polymorphonuclear Neutrophils**
PNOR	**Particles Not Otherwise Regulated**
PP	**Polypropylene tube**
PS	**Polystyrene tube**
PVC	**Polyvinyl Chloride**
rFC	**Recombinant Factor C**
TLR4	**Toll-Like Receptor 4, endotoxin receptor**
TNF-α	**Tumor Necrosis Factor**

LIST OF UNITS

µl	**microliters**
EU	**Endotoxin Unit**
nm	**nanometers**
m^3	**cubic meters**
mg	**milli-grams**
ml	**milli-liters**
rpm	**revolutions per minute**

CHAPTER 1: INTRODUCTION

Endotoxins, the lipopolysaccharide (LPS) portion of the outer cell wall of Gram-negative bacteria, play an important role in respiratory disease in both occupational and non-occupational environments. Endotoxins appear to be a key constituent responsible for inflammation leading to respiratory symptoms and decrements in pulmonary function, particularly among agricultural workers.[1,2,3,4,5,6,7,8,9,10,11] Low levels of endotoxins have been associated with increased asthma severity [12,13], as well as with repeated wheeze in infancy [14,15]. However, endotoxin exposure early in life may be a protective factor that can reduce risk of childhood asthma [16,17,18,19]. Recent studies in animals and humans indicate that genetic factors also play an important role in LPS stimulation of inflammatory responses including production of TNF-α and recruitment of polymorphonucleocytes [16,20,21,22,23]. A clear understanding of the pathogenesis of LPS-induced lung disease and the ability to set exposure guidelines have been hampered by limitations of exposure assessment methods.

Currently no exposure limits have been set for airborne endotoxin in an occupational setting. Organic dusts may have highly variable components between different occupational environments, depending on the origin, making exposure limits difficult to establish. [24,25] Currently, the Occupational Safety and Health Administration (OSHA) does not address exposure to endotoxin alone, but has limits in place for exposure to organic dusts under the Particulates Not Otherwise Regulated (PNOR) heading. OSHA Permissible Exposure Limit (PEL) recommends a total dust limit of

15mg/m^3 and a respirable dust limit of 5 mg/m^3. [26] Since endotoxin concentration in organic dusts is highly variable depending on the occupation and the specific activity, the OSHA PEL will not ensure adequate worker protection from high levels of endotoxin.[27,28]

Bang first described a biological assay for measurement of endotoxin activity in 1956. [29,30] The assay was based upon activation of a protease catalytic coagulation cascade in a lysate from the horseshoe crab (Limulus polyphemus). The chromogenic Limulus ameobocyte lysate (LAL) assay most commonly used today to measure biological activity of LPS does not appear to respond to cell-bound endotoxin that may be important in eliciting responses in the human lung and respiratory disease. [31,32] Current LAL assays also exhibit some lack of specificity. [33] In particular, (1→3)-ß-D-glucans, carbohydrate polymers from fungi, are reported to activate the LAL assay. [34,35] Interlot variation is also possible since the lysate is derived from the hemolymph of living horseshoe crabs. [36] The new recombinant Factor C (rFC) endotoxin assay offers several potential advantages. [37,38,39,40] The production of assay via genetic engineering should greatly reduce lot-to-lot variability found with recovery of lysate. The protease reaction pathway utilizes only Factor C, eliminating Factor G which responds to glucans, thus improving specificity. Standard operating procedures for inter and intra-laboratory methods of sampling, extraction, and the type of biologic assay (LAL vs rFC) are necessary for establishing an internationally accepted endotoxin assessment protocol. [41] The focus of this study was to compare filter extraction media in pyrogen-free water (PFW) with pyrogen-free water with 0.05% Tween 20 (PFW-Tween 20), and to compare the widely used chromogenic LAL and rFC assays for inhalable and settled dust. This is

the first study that we know of to compare these assays on airborne samples from a sheep farm environment.

CHAPTER 2: LITERATURE REVIEW

Sheep Farming Industry

There are approximately 82,000 sheep farms in the United States, as of 2009. Sheep farming takes place in all 50 states and it is estimated that there are about 5.6 million head of sheep in the United States. Although sheep farming in the United States is not as prevalent as other species (cow, horse, swine), the industry is growing. Sheep farming is much more common and economically important in Australia, New Zealand, southern and central South America and Britain.[42] Sheep farm working conditions are much like any other farm environment which poses a variety of hazards to its employees. Injuries due to farm equipment and machinery, exposure to chemicals, pesticides, diesel fumes and organic dusts, long hours, six to seven day work weeks, and unfavorable weather are typical conditions. In 2008, approximately 850,000 workers were employed in the animal production sector. About 78% of these are small family-owned operations with 10 or fewer employees. Total employment in animal farming operations is very difficult to measure. There are approximately 2.5 million jobs available each year in agriculture and about 70% are filled by undocumented workers.[43] Exposures to dust, endotoxin and many other environmental conditions are largely unexplored in the sheep farming industry.

Occupational Exposures to Endotoxin

Endotoxins, also known as lipopolysaccharides (LPS), are cell-wall components of gram-negative bacteria. They are ubiquitous in the environment and are important

components of bioaerosols generated in occupational settings. Aerosolized endotoxin concentrations have been quantified in many occupational settings such as agriculture, livestock, sanitation, textile, wood processing and metal working [28] Endotoxin is found in both the inhalable and respirable dust fractions, usually more so in the inhalable fraction although the amounts may vary by dust type. [5] The ability to quantify endotoxin from dusts has been fundamental in our current understanding of respiratory disease due to occupational exposures. However, no standard operating procedure exists for endotoxin quantification and different methods yield different results. [36,41,44,45]

Many health complications have been reported to be associated with occupational exposure to endotoxin. Agricultural workers are exposed to very high levels of bioaerosols containing endotoxin. Adverse respiratory effects have been described for poultry workers, workers in dairies, pig farmers and grain and seed industries. Symptoms range from wheeze and cough to chronic bronchitis and organic dust toxic syndrome (ODTS). [28,46,47,48,49,50,51] Smit et al. (2006) indicated that extremely high endotoxin exposures seen in the grass seed industry may be responsible for ODTS. [49] Rylander and Fernanda (2006) showed poultry workers to have a higher incidence of airway inflammation, toxic pneumonitis and chronic bronchitis. [46] Senthilselvan et al. (2007) reported that workers in large scale swine operations are at greater risk of usual cough and phlegm and shortness of breath. Shortness of breath was found more pronounced in male workers, and other respiratory symptoms were more severe in female workers. [52] Pulmonary function decrements have also been associated with endotoxin exposure. Reductions in forced vital capacity (FVC), forced expiratory volume in 1 second (FEV1), and the FVC/FEV1 ratio have been reported for animal feed workers, poultry workers

and cotton textile workers. [5,46,47,53,54] Christani et al. showed a longitudinal loss of FEV1 in a 15-year follow up study of cotton textile workers and Vogelzang et al. (2000) showed longitudinal decrements in FEV1 and FVC for pig farmers. [54,8]

More research is needed to establish dose-response relationships with symptoms and endotoxin levels found in occupational dusts. Industry specific exposure limits are needed due to the highly variable nature of organic dusts amongst the different agricultural environments. Possible synergy with other dust components cannot be discounted. [23,28,55]

The Immune Response to Endotoxin

In order to understand the pathogenesis of endotoxin, a complete picture of the human immune response to its presence in the body is needed. The presence of endotoxin stimulates the innate immune system. The immune response of the individual is dependent on the dose of endotoxin, the duration of exposure and genetic factors. [48,56,57] After inhalation, endotoxins, also termed lipopolysaccharides (LPS), interact with macrophages in the airway. LPS binds to the lipopolysaccharide binding protein (LBP). The LBP-LPS complex is delivered to the cell surface protein CD14. CD14 then transfers the LPS to the myeloid differentiation protein 2 and toll-like receptor 4 (MD2/TLR4) complex. The activation of TLR4 initiates the signal transduction cascade within the cell that leads to activation of NF-κB. This signals the cell to start production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukins 1 and 8 (IL-1, IL-8). [28,56,59] The Lipid A portion of LPS has different degrees of acylation, or number of fatty acids attached. Aggregates of LPS molecules, containing hexa-acylated Lipid A, have the ability to activate the TLR4 receptor and start

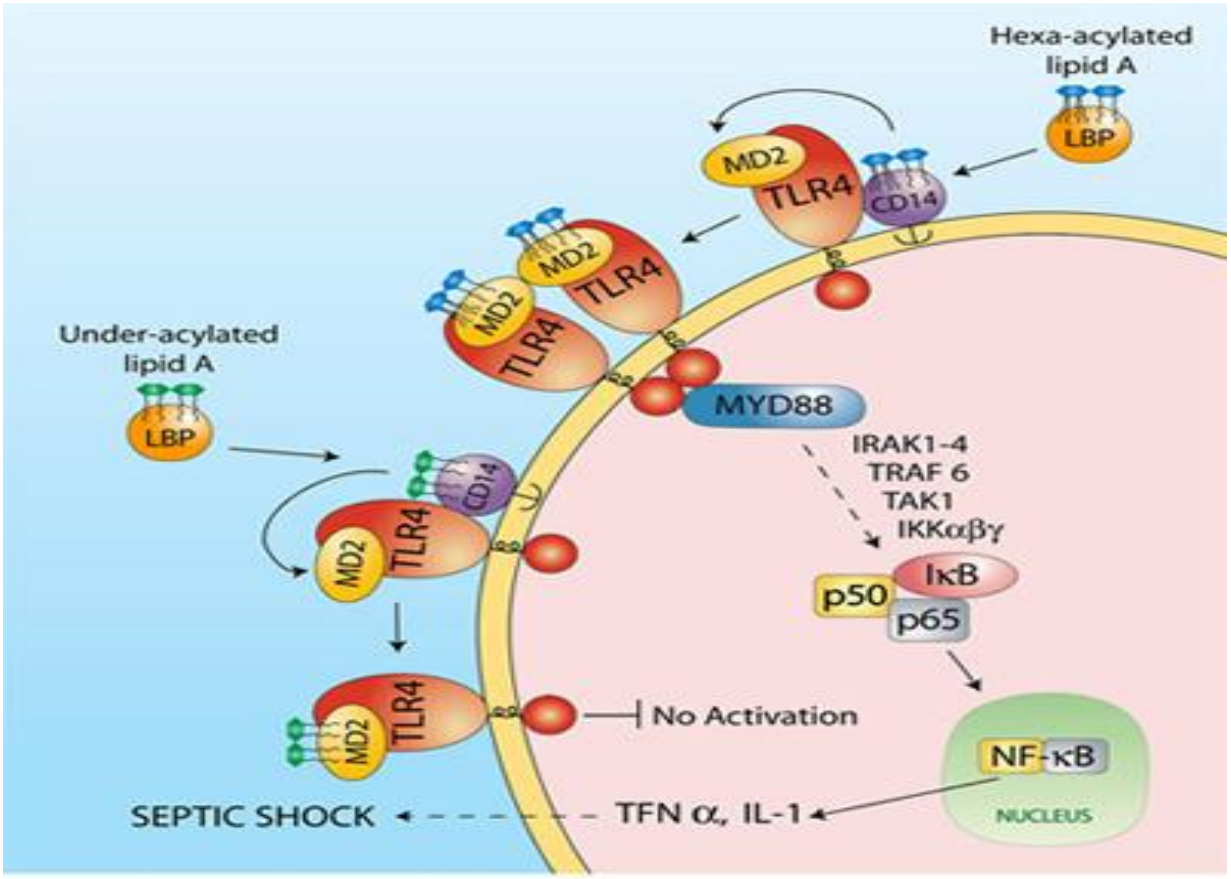
the signal cascade within the cell. Aggregates are biologically active, not monomers of LPS. [59]

Wilson et al. (2008) demonstrated a correlation between the production of TNF-α and increased monocyte production following exposure to swine barn dust, indicating this occupational exposure initiates the immune response associated with LPS exposure.[51] Burch et al. (2010) found elevated IL-8, PMN (polymorphonuclear neutrophils), MPO (myeloperoxidase) , and ECP (eosinophilic cation protein). These inflammation markers were found to be correlated with even-numbered carbon chains of 3-OHFA (three-hydroxy fatty acids) rather than odd-numbered chains which were quantified by GC/MS. [55] Elevated neutrophils, denedritic cell maturation and reduction in neutrophil apoptosis play important roles in airway remodeling and increased collagen production which leads to LPS-induced respiratory disease and exacerbation of asthma. [57,60,61,62]

Exposure to endotoxin has other immune implications as well, some of which the cellular mechanisms are not as clear. There have been many studies showing that exposure to endotoxin early in life is protective against the development of atopic asthma. [19,50,63,64] Eder et al. (2005) showed that farm animal rather than pet exposure during childhood is protective of asthma in children with the same genetic polymorphism in CD14/-260, therefore controlling for one of the genetic components related to asthma and indicating phenotypic plasticity related to different environmental exposures. [63] LeVan et al. (2005) studied carriers of the CD14/-159T allele which have more CD14 in circulation. Homozygotes TT and -1619 GG homozygotes had significantly lower lung function than C or A carriers, also showed increased prevalence of wheeze and decreased pulmonary function. [57] In a study of Italian dairy cattle farmers, endotoxin exposure

was found to be correlated with a decreased incidence of cancer indicating a possible protective effect. The study also showed that the protective effect diminished when the exposure was removed. [65] More research is needed to uncover the genetic factors relating to endotxin exposure and possible protection from or increased risk of disease.

Figure 2.1 Immune response to LPS in human monocyte [58]



Agonist and antagonist activities of lipid A.
Hexa-acylated but not under-acylated lipid A bound to MD-2 induce oligomerization of TLR4 leading to the production of inflammatory cytokines.

Bioassays for Endotoxin

The *Limulus* Ameobocyte Lysate (LAL), obtained from the blood cells (amebocytes) of the horseshoe crab *Limulus polyphemus*, contains the clotting factor coagulogen which clots in the presence of endotoxin. This discovery was reported by

Frederick Bang in 1956 and has been the basis for endotoxin bioassays. The LAL assay was later developed by Frederick Bang and Jack Levin in 1964, after Levin joined the Marine Biological Laboratory at Johns Hopkins. In 1977 the FDA approved the use of the LAL assay to test for pyrogenicity, eliminating the use of rabbits as endotoxin detectors. [66]

The LAL assay only reacts with biologically active endotoxin, cell bound endotoxin is not detectable. The LAL assay is the most widely used assay in endotoxin research. [28,44,48] Quantitative measurements of endotoxin can be done with the turbidimetric and chromogenic assays, while gel clot assays are used for qualitative measurement. The kinetic version of the chromogenic assay is very sensitive, detecting 0.01 endotoxin units (EU) per milliliter of sample. [41,48]

The LAL assay is reported to have interference with (1→3)-ß-D-glucans, carbohydrate polymers from fungi, since they may also activate the clotting protein coagulogen by an alternative pathway via Factor G (Fig. 2). Also, lot-to-lot and interlot variability with respect to sensitivity has been reported due to seasonal horseshoe crab lysate variability. [34,66,67]

As an answer to the issues of lysate variability and glucan interference, an alternative genetically engineered assay has been developed by Ding and Ho in 1995 by cloning Factor C cDNA from the Singapore horseshoe crab *Carcinascorpius rotundicauda*. The recombinant Factor C (rFC) zyomogen is activated by endotoxin and cleaves a synthetic fluorogenic substrate. The fluorescence units are converted to endotoxin units, giving a quantifiable estimate of endotoxin. The rFC assay is specific to endotoxin only, eliminating Factor G. The rFC zymogen, created by cell culture, should

improve specificity and reproducibility, reduce lot-to-lot variation and help save a dwindling horseshoe crab population. [39,67]

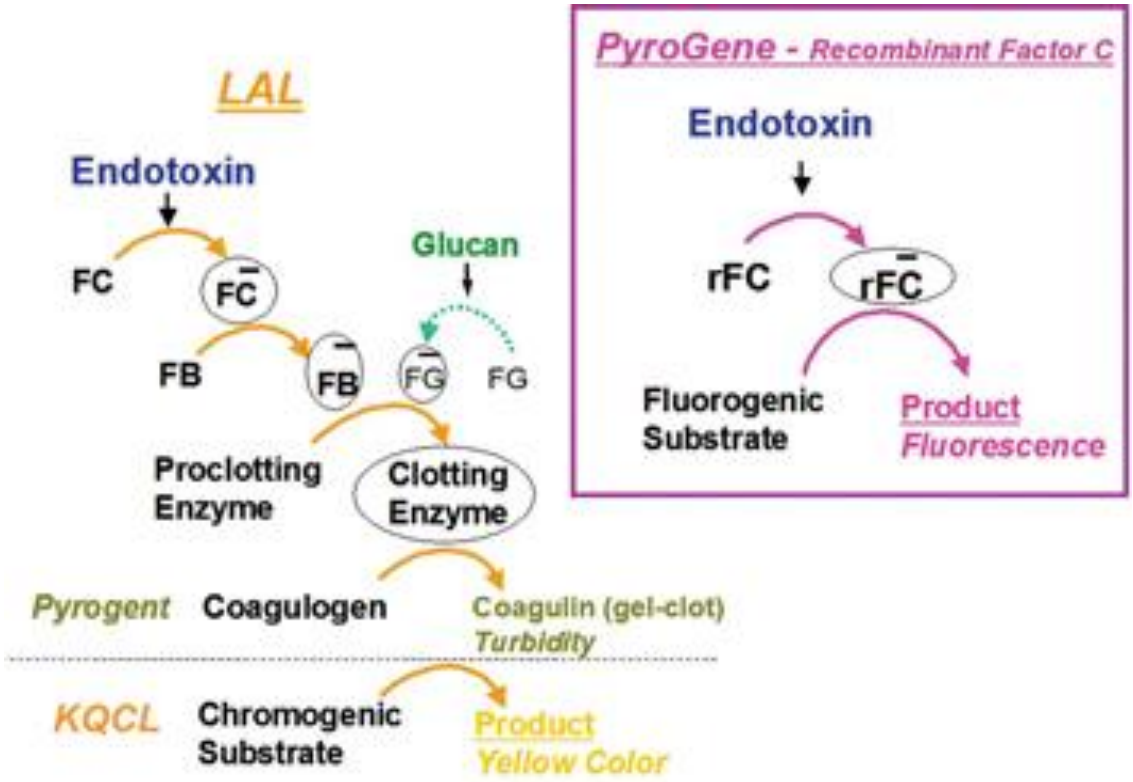


Figure 2.2 Serine protease catalytic coagulation cascade activated by endotoxin in both the LAL and rFC endotoxin assays [67]

Comparison of Analysis Methods

Analysis of airborne endotoxin from different environmental and occupational settings has been accomplished in a variety of ways. There are no formal procedures or guidelines for analysis of endotoxin samples from agricultural environments. Different methods and different laboratories have lead to inconsistency in reporting of endotoxin exposure levels. Nonuniform methodology clearly allows for variability in factors that clearly affect results of the endotoxin assay.[69,70] Many studies have been conducted to try to optimize a standard protocol for endotoxin analysis. Generally, the studies have

shown potential sources of assay variability to be extraction media, storage conditions, type of filter, type of assay, and source of dust. [41,69,71] In a recent study by Spaan et al., the effect of adding 0.05% Tween 20 to the pyrogen-free water (PFW) extraction media, significantly increased the endotoxin recovery. This result is consistent with previous studies. Other effects noted to be significant were the use of Teflon filters and refrigeration rather than freezing of samples, both resulted in significantly lower endotoxin recovery.[69] In a study by Liebers et al., the addition of 0.05% Tween 20 to PFW in the extraction of sample filters significantly increased endotoxin levels, while increasing the extraction volume lowered the endotoxin recovery. Centrifugation and tube type did not contribute to the differences. The differences between laboratories analyzing the same or similar samples are significant, usually related to the extraction method and assay used. The differences vary depending of source of dust as well. These differences in methods contribute to the overall inconsistency in endotoxin measurement.[71

CHAPTER 3: PURPOSE AND SCOPE

There are three purposes of this study. The first purpose was to optimize a standard protocol for extraction of bulk dust samples from a sheep farm for endotoxin analysis. In this study, the bulk samples were analyzed by kinetic and endpoint fluorogenic rFC and kinetic chromogenic LAL assays. The study also compared differences in the extraction protocol for the rFC assay such as extraction media (PFW and PFW + Tween 20), type of extraction tube (polypropylene and polystyrene), and centrifugation versus non-centrifugation. These data were then incorporated into the study of the inhalable dust samples collected from a sheep farm, which is the second purpose. In this part of the study, the inhalable samples were compared using the endpoint fluorogenic rFC and the endpoint chromogenic LAL , and were compared by extraction media (PFW and PFW-Tween 20). The tube type and centrifugation variables were left out of the second part of the study because they did not show a significant difference. Other study variables were also controlled. Only one type of filter, polyvinyl chloride, for inhalable dust collection was used. Sample collection and analysis was performed by only one analyst in the same laboratory setting. Also, only one lot of QCL-1000 (Lonza, Inc) lysate for LAL was used to reduce possible lot-to-lot variability. Thrity-four airborne samples were collected and 32 were matched at the site of collection for comparison of the extraction media. All samples were analyzed with the rFC and the LAL assays. The third purpose of this study is to characterize the potential exposure to

inhalabe endotoxin for workers on a sheep farm, and to compare to other agricultural environments.

The null hypotheses tested are:

1) There is no difference between the rFC and LAL assays.
2) There is no difference between sample extraction in water and water with 0.05% Tween 20.
3) There is no difference between polypropylene and polystyrene tubes used for extractions.
4) There is no difference between centrifuging and not centrifuging samples prior to assay analysis.

There a few limitations to this study. First, about 8 years has lapsed between the collection and analysis of the bulk dust and the collection and analysis of the airborne dust. This allows for many different environmental conditions to change. Although, the livestock is the same, the types and amounts of bacteria present in the environment may have evolved significantly and may change the bioassay results. Another limitation is the kinetic chromogenic LAL assay was used for comparison on the bulk dust and the endpoint chromogenic LAL assay was used on the airborne samples. The kinetic version is much more sensitive and capable of a broader standard curve than is optimized for the endpoint version. The endpoint LAL was chosen in this study to eliminate the need for analysis in a different laboratory. Also, the sample size is small compared to other method comparison studies, making statistical significance more difficult to find.

CHAPTER 4: MATERIALS AND METHODS

Bulk Dust Collection and Preparation

Settled dust was collected from a sheep farm using sterile whirlpack bags. The bulk samples were taken from sheep pens, feeding area, various locations within the barn where shearing and medical care activity takes place. Bulk dust was transported back to the laboratory on ice. Bulk dust samples were sieved with a #140 mesh sieve and shaken to homogenize. One milligram aliquots of dust were weighed into polystyrene and polypropylene centrifuge tubes (BD Falcon, Franklin Lakes, NJ) using a Mettler MT-5 balance (Mettler Instruments, Westerville, OH). Samples were then extracted using 10 ml of sterile water (Cambrex, Walkersville, MD) or 0.05% Tween (EM Science, Gibbstown, NJ) by shaking at 100 rpm for 1 hour at 20° C.

Three dilutions of 1:25, 1:50, 1:75 or 1:100 were prepared from each sample using PFW, PFW with 0.05% Tween, and buffer solution. The buffer solution was prepared by baking monobasic and dibasic potassium phosphate salts at 180°F for four hours then dissolving the salts in 100 ml PFW. Also, a 1% stock solution of triethylamine (TEA) was prepared with PFW. The working buffer was prepared in a separate pyrogen-free bottle by combining the following: 3.9 ml of 1.0 M dibasic potassium salt solution, 5.1 ml of 0.2 M monobasic potassium salt solution, 90 ml of PFW, and 1 ml of 1% TEA. The endpoint and kinetic versions of the rFC assays were performed. First analyses were performed without centrifugation. Samples were then stored at -4°C. Original sample extracts were then centrifuged, and the dilutions and

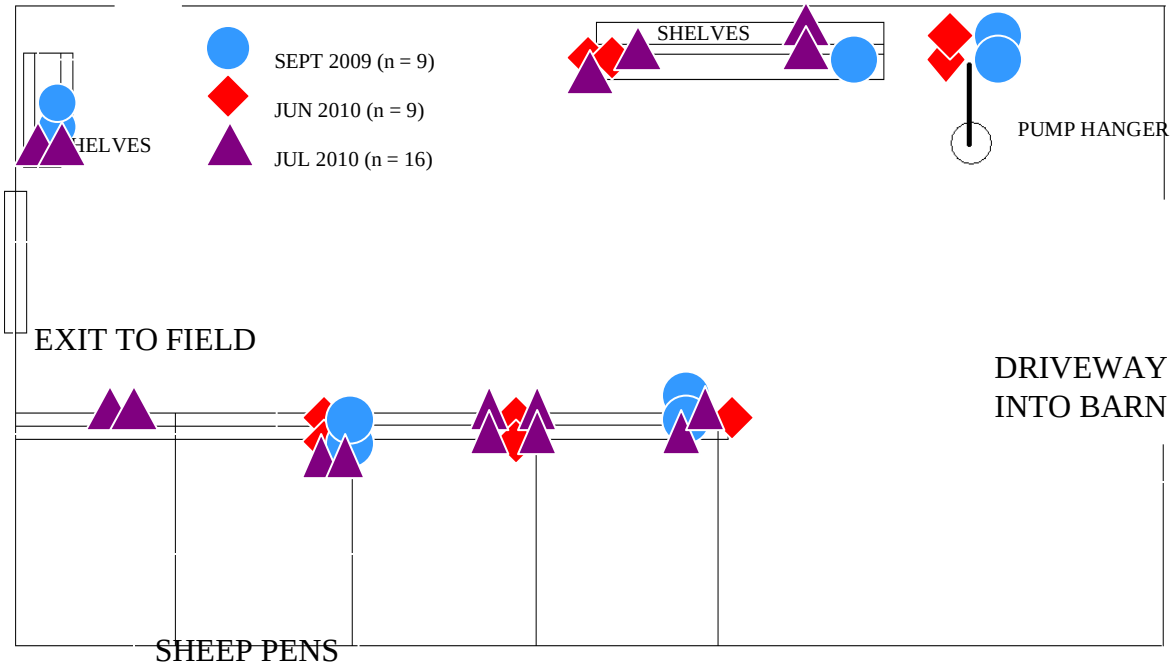
endpoint and kinetic assays were repeated. A comparison of extractions using polystyrene or polypropylene centrifuge tubes (BD Falcon, Franklin Lakes, NJ) was also conducted. A subset of aliquots of extractions were stored at 4° C and shipped to Dr. Chen at Cambrex for analysis by rFC and KQCL. The total number of bulk samples analyzed was 100.

IOM (Inhalable) Dust Sample Collection and Preparation

A total of 34 stationary breathing zone samples were collected using Institute of Occupational Medicine (IOM) inhalable samplers were collected from a single sheep farm in Colorado in 2009 – 2010 during primary operation in the summer season. The IOM inhalable samplers were paired and placed in various locations inside the main barn on the sheep farm, where most activities take place. Figure 4.1 shows the locations of the samplers placed around the barn. The barn has four internal pens for shearing and medical activities, and a large breeze way through the middle for vehicles to pull into the barn. The two large entrances (one for sheep and one for trucks) are at the east and west sides of the barn, respectively, and allow for a reasonable cross-breeze. The floor is concrete and the gates, pens and support beams are made of wood. The IOM buttons were placed between 63 and 72 inches above the ground to capture dust in the breathing zone of the employees. The IOM inhalable samplers used 25mm polyvinyl chloride filters (SKC Inc.). SKC, MSA Escort ELF and Escort LC pumps were set at a flow rate of 2.0 liters per minute (Lpm) over a 6 hour period during typical working hours. The pumps were calibrated before and after sampling using the DryCal DC-Lite (BIOS International, Butler, NJ). Two field blanks were taken to each site visit. All samples and

blanks were weighed using a Mettler MT balance (Mettler Toledo Inc.) The filters were put into 50 ml conical polypropylene centrifuge tubes and frozen at -4° C until extraction.

FIGURE 4.1 IOM Sampling locations within sheep barn by collection date



Inhalable Dust Sample Extractions

Samples were extracted in pyrogen-free water (PFW) (Lonza, Walkersville, MD) and PFW with 0.05% tween for 1 hour at room temperature at 20°C, with shaking at 70 rpm. The tubes were then centrifuged for 10 minutes at 1000 rpm. Prior to dilution, all samples were vortexed for two minutes. The samples were diluted to 1:10 and 1:25 to bring them into the range of the assay standard curve. The sample dilutions were put into 10 ml conical polypropylene tubes. Extracts were analyzed by rFC and LAL within 24 hours of extraction and were stored at 4°C between assays. Assays were performed in Dr. Stephen Reynold's Industrial Hygiene Laboratory at Colorado State University in the Environmental Health building during August and September of 2010. Endotoxin

concentrations in EU/m^3 of dust were calculated based on the stationary air sampling as previously described. [36]

Recombinant Factor C (rFC) Assay

Sample extracts were assayed for endotoxin using the PyroGene Recombinant Factor C Endotoxin Detection System provided by Lonza (Walkersville, MD). The standard endotoxin was *E. coli* 055:B5 supplied with the PyroGene assay. The endotoxin standard was reconstituted with PFW or PFW with 0.05% tween, respective to the samples extracted with the same media. The standards were serially diluted from 20 EU/ml to 0.01 EU/ml. The highest standard used for the standard curve was 10 EU/ml per the assay instruction manual (Lonza). One hundred microliters (µl) of blanks (PFW or PFW with 0.05% tween), standards, samples and sample dilutions were loaded into a sterile 96-well plate inside a biosafety hood. Samples were spiked with 10 µl of the 10 EU/ml standard dilution for quality assurance. The plate was then pre-incubated for 10 min. at 37°C in the fluorescence micro-plate reader (BioTek FLX800IU, Winooski, VT). During the pre-incubation period, the rFC working reagent was prepared by mixing rFC enzyme solution, fluorogenic substrate, and assay buffer (ratio 1:5:4). After the 10minute pre-incubation, the 96-well plate was placed inside a biosafety hood and 100µl of the rFC working reagent was dispensed into each well. For endpoint analysis, the 96-well plate incubated in the fluorescence micro-plate reader for 60 minutes at 37°C. Fluorescence readings were taken by the micro-plate reader at one hour as the final result with the initial (Time 0) reading subtracted as baseline. Reading wavelengths were set at excitation 380/20nm emission 440/30nm. Shaking was set at intensity 3 for 15 second durations. A polynomial regression was used for the standard curve. The gain or

sensitivity settings of the plate reader were set at 35, 40 and 45. Endotoxin concentrations (EU/ml) of samples and sample dilutions were calculated according to the standard curve using KC4 software (Bio-Tek Instruments, Inc. Winooski, Vermont).

Limulus Ameobocyte Lysate (LAL) QCL-1000 Assay

The samples were also analyzed for endotoxin using the Endpoint Chromogenic Limulus Ameobocyte Lysate QCL-1000 provided by Lonza (Walkersville, MD) for comparison with the endpoint rFC assay. The endotoxin reference standard, *E. coli* 0111:B4, was provided in the LAL QCL-1000 kit by Lonza, Inc. The standards were prepared with PFW and PFW with 0.05% tween, respective to the extraction media of the samples. The standards were prepared by serial dilution at the following concentrations per the LAL QCL-1000 instruction manual: 0.1 EU/ml, 0.25 EU/ml, 0.5 EU/ml, and 1.0 EU/ml. The same extraction media used for standards and samples were used for blanks. Serial dilutions of 1:10 and 1:25 were made for each sample just prior to assaying. Blanks, standards and samples were dispensed in the sterile, 96-well microplate in 50 µl aliquots, in triplicate. At time zero (T = 0), 50 µl of the Limulus Ameobocyte Lysate is added to each well with an 8 channel pipette, and then incubated at 37°C in the microplate reader (PowerWave XS, North Star Scientific Limited, United Kingdom). At T = 10 minutes 100 µl of the substrate solution was added to the mircroplate in the same manner as the previous step. The plate is then put back in the plate reader to incubate at 37°C. At T = 16 minutes, 100 µl of the stop reagent (25% glacial acetic acid) was added to each well in the same manner as the previous step. The microplate is then returned to the plate reader and the absorbance was read at 405 -410 nm. The final concentrations were adjusted for the blank readings and calculated according to the standard curve using

KC4 software (Bio-Tek Instruments, Inc. Winooski, Vermont). The lysate and substrate solutions were reconstituted with the LAL pfw provided with the kit. The stop reagent was made with pfw provided by Lonza Inc.

Statistical Analysis

Statistical analyses were performed using MINITAB Student Version 14.11.1. The Ryan-Joiner test for normality was used to evaluate the distribution of data. The dust concentrations for the IOM (inhalable) samples was found to be normal, while all other data for bulk and inhalable was found to be log-normally distributed. All other data were natural log transformed before proceeding with the analysis. The geometric mean (GM) and geometric standard deviation (GSD) were calculated for the endotoxin concentrations. For the bulk dust samples, means between each subset were tested for significant differences using the two sample T test. For the inhalable samples, Paired T-tests were performed to detect differences in the means. Pearson correlations were also calculated to determine if there were positive or negative relationships between the data sets.

Quality Control and Quality Assurance

For each 96-well plate, space was set aside for samples to be spiked with a known concentration of endotoxin in order to check the recovery rates for each extraction and assay method. Samples were spiked in the wells after all samples were dispensed into the 96-well plate. For the bulk dust, controls were spiked with 10 µl of the 1 EU/ml standard for a maximum recovery of 0.1 EU/ml. For the IOM (inhalable) samples, the spiking concentration differed for each assay. The LAL assay samples were spiked with 10 µl of

the 1 EU/ml standard for a maximum recovery of 0.1 EU/ml. The rFC assay samples were spiked with 10 µl of the 10 EU/ml standard for a maximum recovery of 1 EU/ml.

CHAPTER 5: RESULTS

Bulk Dust Samples

No significant differences were found between endpoint and kinetic rFC assays (values not shown). Values from the endpoint analysis are shown in Table 5.1. Spike recoveries were closest to 100% for extraction in PFW and extractions in PFW-Tween-20 and buffer ranged broadly (Table 5.2). Two sample t-tests were performed to detect differences in the means for each assay protocol (Table 5.3). Extractions in PFW-Tween 20 yielded the highest concentrations for rFC assay, and was found to be higher than water $t = 2.16$ ($p = 0.036$) and significantly higher than buffer $t = 2.98$ ($p = 0.005$). The PS tube extractions were found to be significantly higher than the extractions in PP tubes $t = 3.82$ ($p = 0.000$). No difference was detected between samples that were centrifuged and samples that were not. Reproducibility was best for extraction in PFW-Tween 20 (CV = 41%) and was worst for extraction in buffer (CV = 122%). Direct comparison of the rFC and KQCL in the same lab (Cambrex) found very high correlation for extraction in water ($r = 0.93$) as shown in Figure 5.1.

Table 5.1. GM and GSD endotoxin concentrations for bulk dust samples by extraction protocol.

Extraction Media	Tube Type	Centrifugation	n	Endotoxin per dust (EU/mg)	
				GM	GSD
PFW	PS	Yes	8	6.86	2.178
		No	8	6.922	2.791
	PP	Yes	10	5.205	2.238
		No	10	5.024	1.684
PFW-Tween 20	PS	Yes	4	6.726	1.295
		No	4	6.64	1.601
	PP	Yes	10	6.233	1.445
		No	10	6.328	1.442
Buffer	PS	Yes	9	5.791	3.019
		No	9	5.859	3.402
	PP	Yes	9	5.527	3.926
		No	9	5.548	2.828

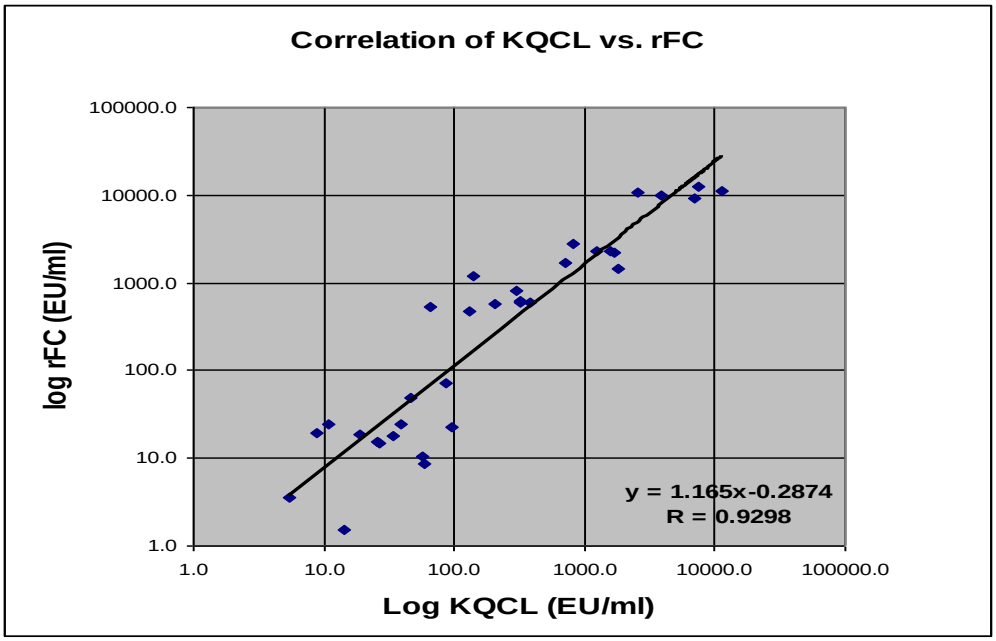
Table 5.2. Spike recoveries for bulk dust analysis.

Extraction Method			# of wells spiked	Avg. % Recovery
Water	polypropylene	centrifuged	7	95.14
		not centrifuged	7	97.6
	polystyrene	centrifuged	3	136.3
		not centrifuged	3	226
Buffer	polypropylene	centrifuged	9	303.8
		not centrifuged	2	334.65
	polystyrene	centrifuged	5	27.7
		not centrifuged	8	330.3
Tween	polypropylene	centrifuged	5	171.9
		not centrifuged	5	5.5
	polystyrene	centrifuged	3	324.1
		not centrifuged	3	102.5

Table 5.3. T-tests for differences in population means for bulk dust samples by extraction parameter

	T-Test (μ1 - μ2) (df)	t-value	p-value
Extraction Media	PFW-Tween 20, PFW (44)	2.16	0.036
	PFW-Tween 20, Buffer (45)	2.98	0.005
	PFW, Buffer (69)	0.58	0.565
Tube Type	PS, PP (81)	3.82	0.000
Centrifugation?	No, Yes (97)	-0.03	0.975

Figure 5.1. Correlation of rFC and KQCL with PFW extractions of bulk sheep dust (Dr. Lin Chen, Cambrex)



IOM (Inhalable) Dust Samples

The airborne dust samples showed a high degree of variability within the four groups and between the four groups. Inhalable endotoxin exposures were found to be between 12 EU/m^3 and 3354 EU/m^3. All 34 samples were analyzed by endpoint rFC and endpoint LAL assays. The geometric mean and geometric standard deviation for dust endotoxin concentrations are shown in Table 5.4. Each of the 16 pairs were extracted with PFW and PFW-Tween 20 (one of each). Figure 5.2 shows the box-plot of endotoxin concentrations of the four groups. The four groups of data are: 1) rFC/ PFW extraction; 2) LAL/ PFW extraction; 3) rFC/ PFW-Tween 20 extraction; 4) LAL/ PFW-Tween 20 extraction. Only two of the comparisons showed significant differences: 1) rFC/ PFW-Tween 20 is significantly higher than rFC/ PFW ($p = 0.000$); 2) LAL/ PFW-Tween 20 is significantly higher than LAL/ PFW ($p = 0.032$). Comparisons of rFC/ PFW and LAL/ PFW and rFC Tween-20 and LAL/ PFW-Tween 20 showed no significant difference in the means (Table 5.8). Pearson correlations (Figure 5.3) showed strong positive correlations for LAL/ PFW-Tween 20 and rFC/ PFW-Tween 20 ($r = 0.910$) and rFC/ PFW and LAL/ PFW ($r = 0.761$). rFC/ PFW and rFC/ PFW Tween 20 were moderately positively correlated ($r = 0.523$). No correlation could be found with statistical significance for LAL/ PFW and LAL/ PFW-Tween 20. Spike recoveries were closest to 100% for rFC/ PFW and LAL/ PFW-Tween 20 (126% and 75%, respectively). The spike recoveries for LAL/ PFW were significantly lower than the other 3 groups, at 30% (Table 5.5). Reproducibility for all groups was relatively similar. LAL/ PFW-Tween 20 had the best reproducibility (CV = 17%) and LAL/ PFW had the most variation among replicates (CV = 29%). Standard curves for the assays, calculated by polynomial regression, had R^2

values of 0.984 or higher. The rFC assay had R^2 values closest to 1.0. The dust levels captured by the IOM cassettes were relatively small, compared to other agricultural environments (Table 5.6). The airborne sample concentrations of endotoxin (EU/mg) were found to be not significantly different from the concentrations of the bulk samples, although they were slightly lower. The endotoxin concentrations for samples collected on June 15, 2010 were slightly higher than the other samples collected on the other two dates.

Table 5.4. GM and GSD endotoxin concentrations for IOM (inhalable) dust samples by extraction media.

Extraction Media	Assay	n	Endotoxin per air (EU/m3)		Endotoxin per dust (EU/mg)	
			GM	GSD	GM	GSD
PFW	rFC	17	4.61	2.39	5.913	2.578
	LAL	17	4.531	1.965	5.807	2.379
PFW-Tween 20	rFC	16	5.869	3.705	7.025	3.109
	LAL	10	5.056	2.25	6.22	1.838

Table 5.5. Endotoxin spike recovery for IOM (inhalable) dust samples.

	rFC Assay	LAL Assay
PFW	**125.63 %** (n = 6)	**29.75 %** (n = 9)
PFW-Tween 20	**166.85 %** (n = 6)	**74.7%** (n = 1)*

*All other spiked samples for this group were found to be above the Limit of Detection (LOD).

Table 5.6. Basic statistics for dust concentrations of IOM (inhalable) samples in mg/m^3 by collection date.

		Dust per air (mg/m^3)		
Date of Collection	**n**	**Mean**	**Standard Deviation**	**Range**
Sep 19, 2009	9	0.4277	0.0757	(0.272, 0.526)
Jun 22, 2010	9	0.3147	0.2221	(0.038, 0.698)
Jul 15, 2010	16	0.3809	0.3614	(0.089, 1.699)

Table 5.7. Endotoxin concentrations by date of IOM (inhalable) sample collection.

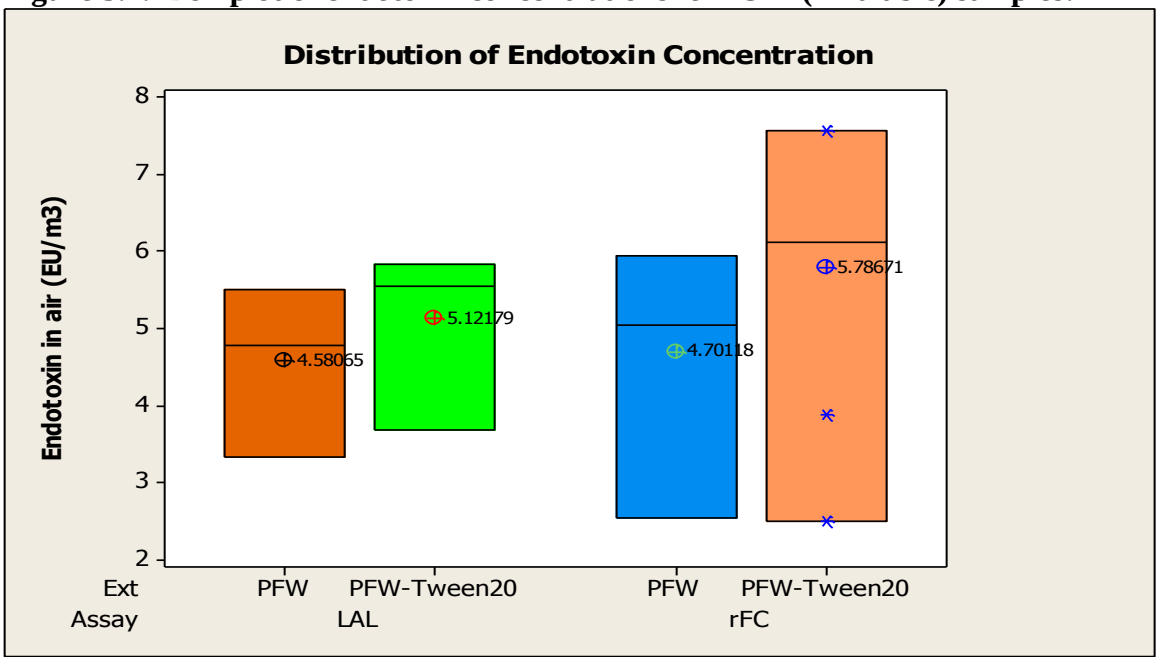
		Endotoxin per air (EU/m^3)	
Date of Collection	**n**	**GM**	**GSD**
Sep 19, 2009	9	5.36	1.81
Jun 22, 2010	9	5.89	2.96
Jul 15, 2010	15	4.75	2.98

*These values were obtained by averaging the LAL and rFC result for the sample.

Table 5.8. Paired T-test for difference in population means for IOM (inhalable) samples by extraction and assay.

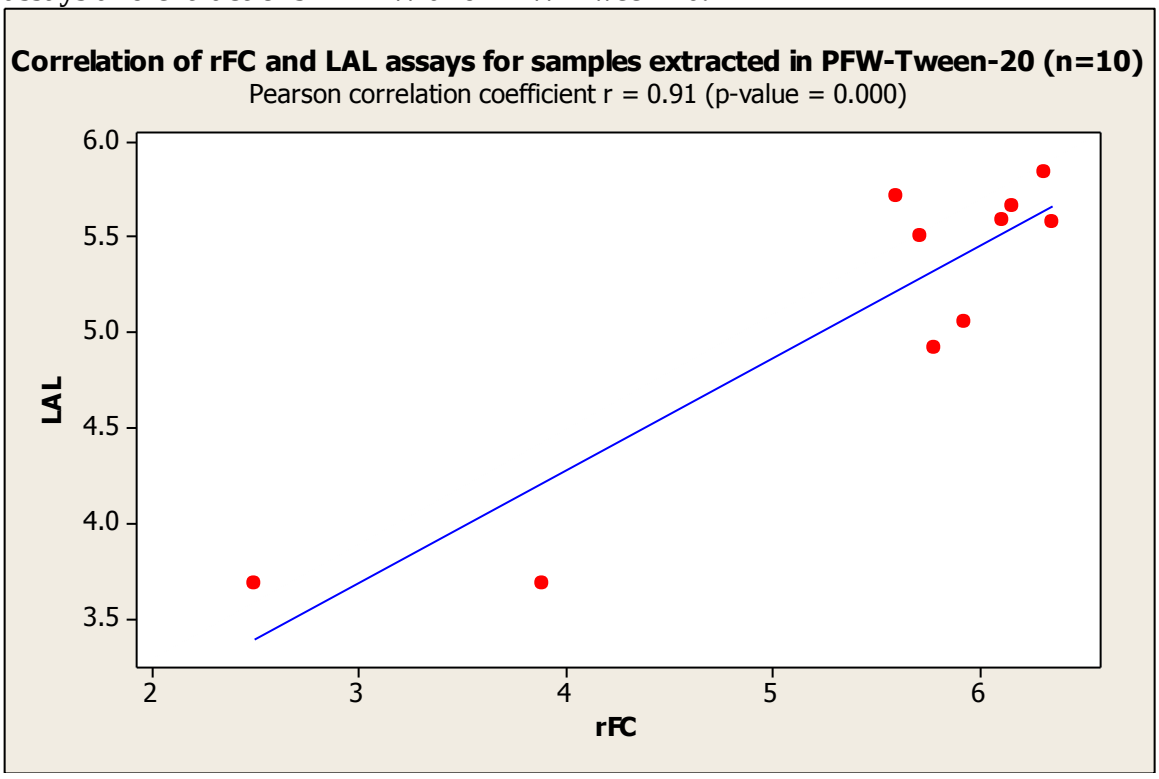
# of pairs	**Paired T-Test (μ1 - μ2)**	**t-value**	**p-value**
16	PFW rFC, PFW LAL	0.85	0.411
10	PFW-Tween 20 rFC, PFW-Tween 20 LAL	1.60	0.144
15	PFW-Tween 20 rFC, PFW rFC	4.51	0.000
10	PFW-Tween 20 LAL, PFW LAL	2.54	0.032

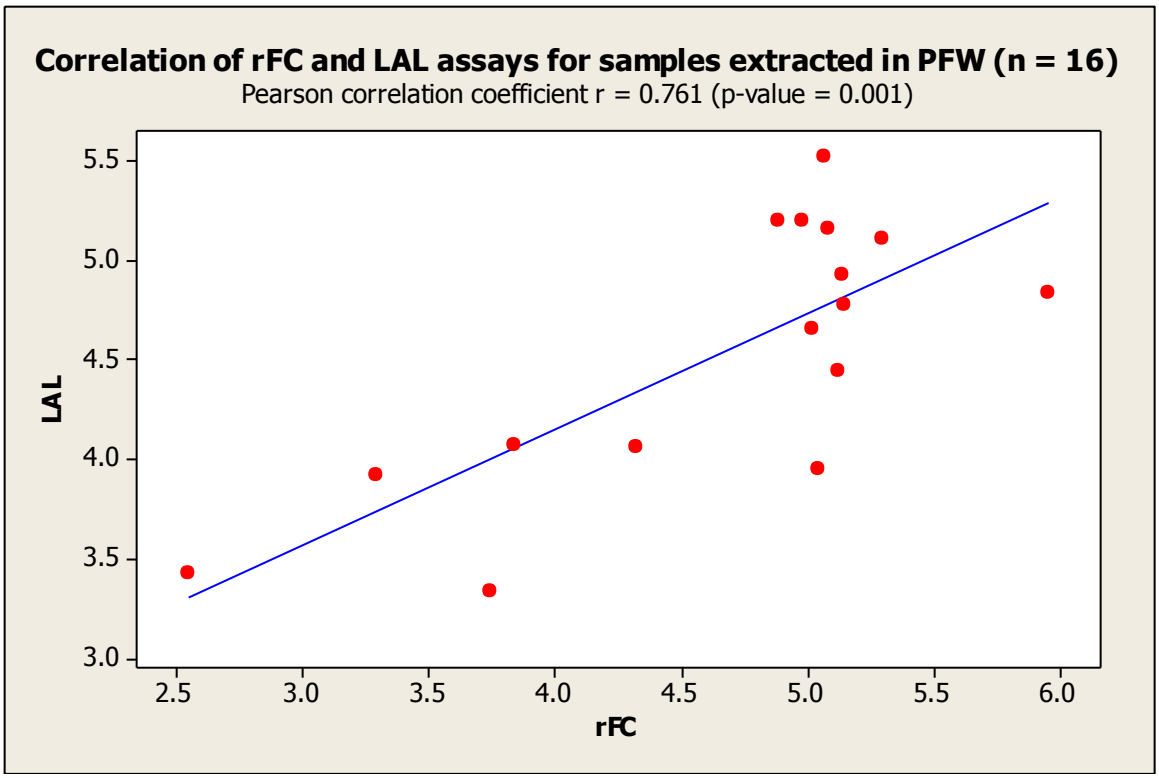
Figure 5.2. Box-plot of endotoxin concentrations for IOM (inhalable) samples.

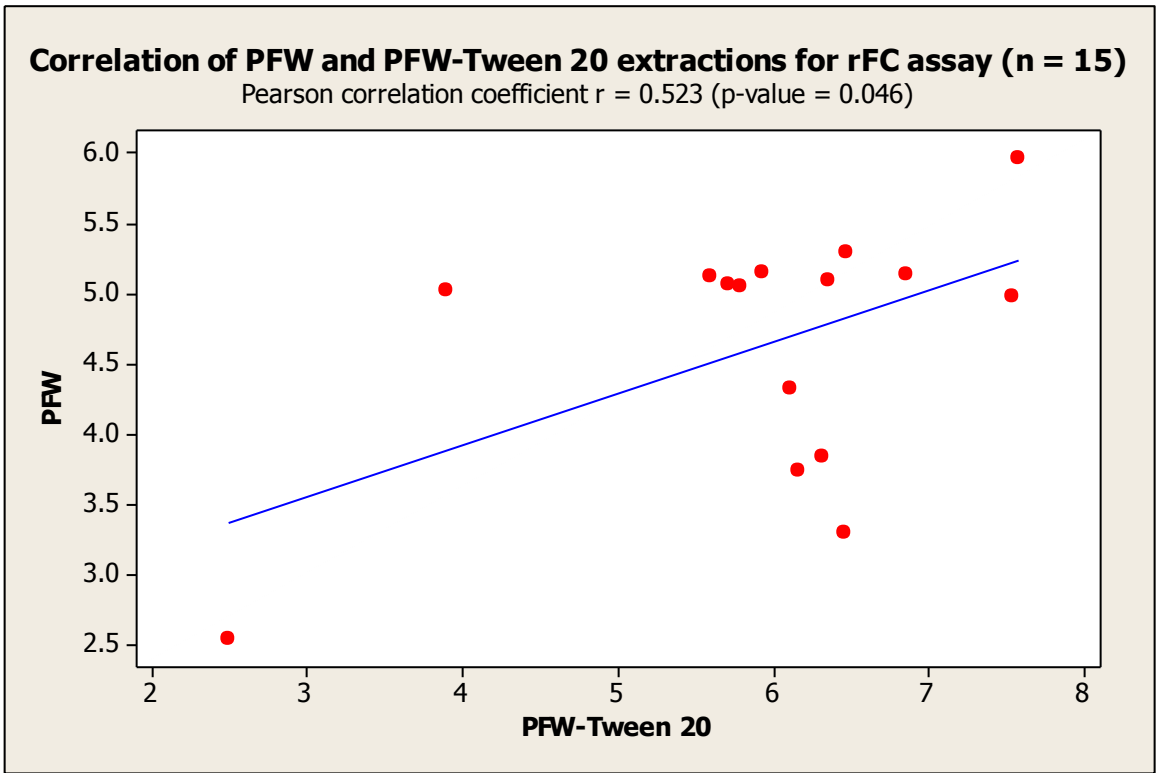


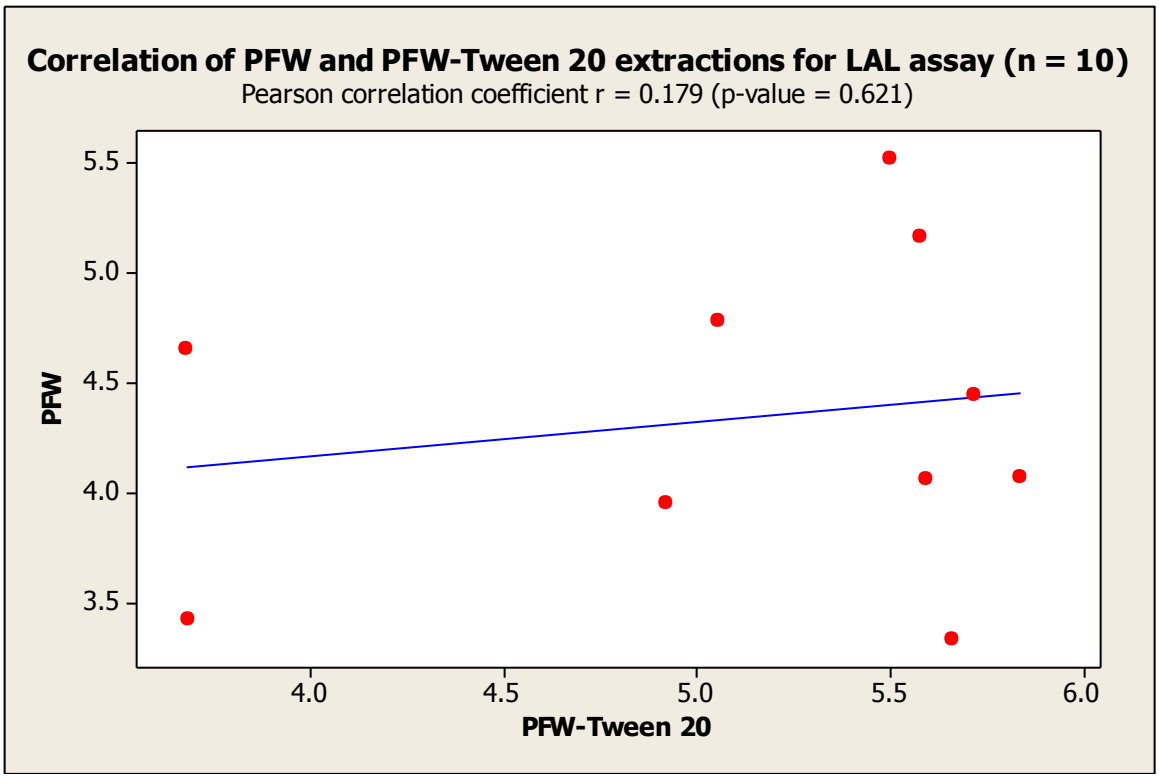
*Ext = extraction media; Assay = type of bioassay for endotoxin.

Figure 5.3. Correlations scatterplots for each comparison between rFC and LAL assays and extractions in PFW and PFW-Tween 20.




Correlation of rFC and LAL assays for samples extracted in PFW (n = 16)
Pearson correlation coefficient r = 0.761 (p-value = 0.001)
LAL
rFC
5.5
5.0
4.5
4.0
3.5
3.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0


Correlation of PFW and PFW-Tween 20 extractions for rFC assay (n = 15)
Pearson correlation coefficient r = 0.523 (p-value = 0.046)
PFW
PFW-Tween 20
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2
3
4
5
6
7
8


Correlation of PFW and PFW-Tween 20 extractions for LAL assay (n = 10)
Pearson correlation coefficient r = 0.179 (p-value = 0.621)
PFW
5.5
5.0
4.5
4.0
3.5
4.0
4.5
5.0
5.5
6.0
PFW-Tween 20

CHAPTER 6: DISCUSSION AND CONCLUSIONS

Endotoxin concentrations were highest within the rFC/ PFW-Tween 20 group and the rFC and LAL assays with PFW and PFW-Tween 20 extraction were found to have a strong, positive correlation. These results are consistent with many other similar studies [25, 32, 36, 45, 69, 70, 72, 73]. For PFW-Tween 20 extraction, the mean endotoxin concentration for rFC was found to be about 3 times higher than LAL, and the Paired T test (Mini-Tab Student Version) showed a significant difference between these two groups. It is important to note that there were 5 LAL samples that were greater than the limit of detection, due to a much narrower LAL standard curve (0.1 EU/ml to 1.0 EU/ml for LAL as opposed to 0.01 to 10 EU.ml for rFC). Therefore, the 5 pairs with the highest endotoxin concentrations were left out of the Paired T test. Contradictory to these results, Alwis and Milton (2006) found that the LAL (kinetic) gave higher results than the rFC for house dust and found no significant glucan interference [32]. Saito et al. (2009) showed consistent results with this study, indicating the rFC gave higher results than the LAL. This may be due to inherent differences in dust, LAL reagents, or presence of Actinobacteria [25]. Glucan activity did not appear to be a factor in this study since rFC estimates were higher than LAL. More research needs to be done to determine if Actinobacteria is present in sheep dust.

The highest levels of endotoxin were associated with the airborne samples collected in June of 2010. The activity on that date of collection was different from the other two dates because workers were sheering the sheep. This is most likely the cause

of the 5 samples extracted in PFW-Tween 20 that were beyond the limit of quantification of the LAL assay. This type of activity could result in higher endotoxin exposure to the worker and more severe health effects. More research into the health effects after or during sheering activity is needed.

Quality control is necessary for obtaining valid results from endotoxin. [24] In this study, the spike recoveries gave minimal information on possible inhibition or enhancement. The data available indicate that there is inhibition in the LAL assay with PFW extraction and slight enhancement in the rFC assay with PFW-Tween 20 extraction. There is also a possibility of enhancement with PS tubes as indicated in the bulk dust study. More data are needed to make any determination of effects. Due to the limited sample size and limited number of dilutions, it is difficult to draw any conclusions.

Differences in origin of dust, laboratory procedures, extraction methods, storage of samples and type of bioassay may affect endotxin results significantly. [36, 45, 41, 25, 32, 69, 70, 71, 72, 73] Extraction with PFW-Tween 20 gives consistently higher results than extraction with other media. [41, 70] Noss et al. (2010) hypothesizes that the addition of Tween 20 may improve endotoxin release from hydrophobic filters used in collection of airborne dust samples. [73] Spaan et al. (2008) showed samples extracted in PFW-Tween 20 have higher concentrations, however, standard reconstituted in PFW-Tween 20 showed inhibition that resulted in a calibration curve shifted toward higher values. In order to overcome this inhibition, Spaan et al. recommends using PFW-Tween 20 for extractions with serial dilutions and LPS standards in PFW. [69] Liebers et al. (2007) found lower endotoxin concentrations when extractions were frozen and extraction volume was increased from 5 ml to 10 ml and Reynolds et al. (2005) found centrifugation

to give higher endotoxin concentrations, possibly due to the release of LPS from the cell. [41, 45] Other studies have shown no differences due to freezing. [69,72] As indicated previously, this study on the bulk dust from a sheep farm showed no effect of centrifugation. These different results among different agricultural environments may suggest the need to investigate each one individually. The hazards of sheep dust may differ from that of cow, horse, chicken, etc since they differ in microbial and chemical composition. [25, 36, 41, 45]

Conclusions and Research Needs

While the LAL is the most widely used and accepted assay for endotoxin [41], it is more expensive and has more laboratory steps which may introduce error. [74] The rFC is more specific to endotoxin detection and has less variability. Fluorescence spectrometry is more selective than UV/ Vis absorption because many different molecules absorb strongly in this spectrum but do not exhibit fluorescence. Also, two wavelengths, excitation and emission, are used in fluorometry, and only one is used in absorption.[75] Although the LAL is well established, the rFC may have more advantages overall. Therefore it is recommended that the rFC assay be used in more research on airborne dust samples from agricultural environments. Also, more research must be done to characterize the exposure and health effects from working in a sheep farm environment. Endotoxin concentrations are variable, but may be very high depending on environmental conditions and work activity. In order to establish a dose-response relationship between sheep dust and health effects, more research must be done to better define the dust components and the effects that arise from exposure.

Study Limitations

The small sample size is a major limitation of this study. A larger sample size would provide more statistical power of the observations. Also, the samples collected in September of 2009 were frozen for a much longer period of time (one year as opposed to one or two months) which may affect the levels of detection. Another limitation was that the dilutions, 1:10 and 1:25, were not enough to bring some of the samples into range of the assays. Seven samples analyzed by LAL and one by rFC were not detectable.

REFERENCES

1. Castellan RM, Olenchock SA, Hankinsen JL. Acute bronchoconstriction induced by cotton dust: Dose-related responses to endotoxin and other dust factors. Ann Internal Med 1984; 101: 157-163.
2. Castellan RM, Olenchock SA, Kinsley KB, Hankinsen JL. Inhaled endotoxin and decreased spirometric values, an exposure-response relation for cotton dust. N Engl J Med 1987; 317: 605-610.
3. Reynolds SJ, Parker D, Vesley D, Smith D, Woellner R. Cross-sectional epidemiological study of respiratory disease in turkey farmers. Amer J Ind Med 1993; 24: 713-722.
4. Reynolds SJ, Donham KJ, Whitten P, Merchant JA, Burmeister LE, Popendorf WJ. Longitudinal evaluation of dose response relationships for environmental exposure and pulmonary function in swine production workers. Am J Ind Med 1996; 29: 33-40.
5. Donham K, Cumro D, Reynolds SJ, Merchant JA. Dose-response relationships between occupational aerosol exposures and cross-shift declines of lung function in poultry workers: recommendations for exposure limits. J Occup Environ Med 2000; Mar; 42(3):260-9.
6. Rylander R, Haglind P, Lundholm M. Endotoxin in cotton dust and respiratory function decrement among cotton workers in an experimental cardroom. Am Rev Respir Dis 1985; 131: 209-213

7. Schwartz DA, Thorne PS, Yagla SJ, Burmeister LF, Olenchock SA. The role of endotoxin in grain dust-induced lung disease. Am J Respir Crit Care Med 1995; 152: 603-608.
8. Vogelzang PF, van der Gulden JW, Folgering H, Heederik D, Tielen MJ, van Schayck CP. Longitudinal changes in bronchial responsiveness associated with swine confinement exposure. 2000; 117; 1488-1495.
9. Zhiping W, Malmberg P, Larsson B-M, Larsson K, Larsson L, Saraf A. Exposure to bacteria in swine-house dust and acute inflammatory reactions in humans. Am J Respir Crit Care Med 1996; 154:1261-1266.
10. Heederik D, Brouwer R, Biersteker K, Boleij JSM. Relationship of airborne endotoxin and bacteria levels in pig farms with the lung function and respiratory symptoms of farmers. Int Archives Occ Environ Health. 1991; 62: 595-601.
11. Zejda JE, Barber E, Dosman JA. 1994. Respiratory health status in swine producers relates to endotoxin exposure in the presence of low dust levels. J Occ Med 1994; 36: 49-56.
12. Michel O, Kips J, Duchateau J, Vertongen F, Robert L, Collet H, et al. 1996. Severity of asthma is related to endotoxin in house dust. Am J Respir Crit Care Med 1996; 154: 1641-1646.
13. Rizzo MC, Naspitz CK, Fernandez-Caldas E, Lockey RF, Mimica I, Sole D. Endotoxin exposure and symptoms in asthmatic children. Pediatr Allergy Immunol 1997; 8:121-6.

14. Gehring U, Bolte G, Borte M, Bischof W, Fahlbusch B, Wichmann HE, et al. Exposure to endotoxin decreases the risk of atopic eczema in infancy: A cohort study. J Allergy Clin Immunol 2001; 108: 847-54.
15. Park JH, Gold DR, Spiegelman DL, Burge HA, Milton DK. House dust endotoxin and wheeze in the first year of life. Am J Respir Crit Care Med 2001; 163: 322-328.
16. Gereda JE, Leung DYM, Thatayatikom A, Streib JE, Price MR, Klinnert MD, et al. Relation between house-dust endotoxin exposure, type 1 t-cell development, and allergen sensitization in infants at high risk of asthma. Lancet 2000; 355: 1680-1683.
17. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, Maisch S, Carr D, Gerlach F, Bufe A, Lauener RP, Schierl R, Renz H, Nowak D, von Mutius E. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002; 347: 869-877.
18. von Mutius E, Braun-Fahrlander C, Schierl R, Riedler J, Ehlermann S, Maisch S, Waser M, Nowak D. Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin Exp Allergy 2000; 30: 1230-1234.
19. Eduard W, Douwes J, Omenaas E, Heederik D. Do farming exposures cause or prevent asthma? Results from a study of adult Norwegian farmers. Thorax 2004; 59: 381-386.
20. George C, Jin H, Wohlford-Lenane C, O'Neill M, Phipps J, O'Shaughnessy P, Kline J, Thorne P, Schwartz D. Endotoxin responsiveness and subchronic

grain dust-induced airway disease. Am J Physiol (Lung Cell Mol Physiol) 2001; 280: L203-L213.

21. Savov J, Gavett S, Brass D, Costa D, Schwartz D. 2003. Neutrophils play a critical role in the development of LPS-induced airway disease. Am J Physiol: Lung Cell Mol Physiol 2003; 283: L952-L962.
22. Lorenz E, Jones M, Wohlford-Lenane C, Meyer N, Frees K, Arbour N, Schwartz D. Genes, other than TLR4, are involved in the response to inhaled LPS. Am J Physiol: Lung Cell Mol Physiol 2001; 281: L1106-1114.
23. Poole J, Dooley G, Saito R, Burrell A, Bailey K, Romberger D, Mehaffy J, Reynolds S. Muramic acid, endotoxin, 3-hydroxy fatty acids, and ergosterol content explain monocyte and epithelial cell inflammatory responses to agricultural dust. J of Toxicol Environ Health 2010; Part A: 73: 684-700.
24. Hollander A, Heedrick D, Versloot P, Douwes J. Inhibition and enhancement in the analysis of airborne endotoxin levels in various occupational environments. Am Ind Hyg Assoc 1993; J 54: 647-653.
25. Saito R, Cranmer B, Tessari J, Larsson L, Mehaffy J, Keefe T, Reynolds S. Recombinant Factor C (rFC) assay and gas chromatography/ mass spectroscopy (GC/MS) analysis of endotoxin variability in four agricultural dusts. Ann Occup Hyg 2009; 53(7): 713-22.
26. Occupational Safety and Health Administration (OSHA) [ONLINE] Table Z-1 Limits for air contaminants. 1910.1000. http://www.osha.gov/pls/oshaweb/owadisp.show_document?p_table=STANDARDS&p_id=9992

27. Smit LA, Wouters IM, Hobo M, Eduard W, Doekes G, Heederik D. Agricultural seed dust as a potential cause of Organic Dust Toxic Syndrome. Occup Environ Med 2006; 63: 59-67.
28. Liebers V, Bruning T, Raulf-Heimsoth, M. Occupational Endotoxin-Exposure and Possible Health Effects on Humans. Am J Ind Med 2006; 49: 474 – 491.
29. Bang FB. A bacterial disease of Limulus polyphemus. Bull Johns Hopkins Hosp 1956; 98: 325.
30. Levin J, Bang FB. Clottable protein in Limulus: its localization and kinetics of its coagulation by endotoxins. Thromb. Diath.Haemorrh. 1968; 19: 186.
31. Reynolds SJ, Milton DK. Comparison of methods of analysis of airborne endotoxin. Appl Occup Environ Hyg 1993; 8: 761-767.
32. Alwis KU, Milton DK. Recombinant Factor C assay for measuring endotoxin in house dust: Comparison with LAL, and (1-3)-beta-D-Glucans. Am J Ind Med 2006; 49: 296-300.
33. Helander I. Pulmonary toxicity of endotoxins: Comparison of lipopolysaccharides from various bacterial species. Infect Immun 1982; 35: 528-532.
34. Roslansky PF, Novitsky TJ. Sensitivity of Limulus amebocyte lysate (LAL) to LAL-reactive glucans. J Clin Microbiol 1991; 29: 2477-2483.
35. Douwes J, Zuidhof A, Doekes G, van Der ZEE, Wouters I, Marike Boezen H, Brunekreef B. (1--> 3)-beta-D-glucan and endotoxin in house dust and peak flow variability in children. Am J Respir Crit Care Med 2001; 162: 1348-1354.

36. Thorne PS, Perry SS, Saito, R, O'Shaughnessy PT, Mehaffy J, Metwalli N, Keefe T, Donham K, Reynolds, SJ. Evaluation of the Limulus amebocyte lysate and recombinant factor C assays for assessment of airborne endotoxin. Appl Environ Micro 2010; Aug: 4988-4995.
37. Nakamura T, Furunaka H, Miyata T, Tokunaga F, Muta T, Iwanaga S, Niwa M, Takao T, Shimonishi Y. Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (Tachypleus tridentatus). Isolation and chemical structure. J Biol Chem 1988; 263: 16709-16713.
38. Ding JL, Navas MA, 3rd, Ho B. Two forms of factor C from the amoebocytes of Carcinoscorpius rotundicauda: purification and characterisation. Biochim Biophys Acta 1993; 1202: 149-156.
39. Ding JL, Navas MA, 3rd, Ho B. Molecular cloning and sequence analysis of factor C cDNA from the Singapore horseshoe crab, Carcinoscorpius rotundicauda. Mol Mar Biol Biotechnol 1995; 4: 90-103.
40. Chen L. 2003. Pyrogene - a new endotoxin detection method using recombinant Factor C. Presented for the 5th International Symposium on Future of Rural Peoples: Rural economy, Healthy people, Environment, Rural communities (Exposure to Endotoxin and Lung), Saskatoon, Saskatchewan, Canada, Oct. 19-23.
41. Liebers V, Raulf-Heimsoth M, Linsel G, Goldscheid N, Duser M, Stubel H, Bruning Th. Evaluation of Quantification Methods of Occupational Endotoxin Exposure. J of Toxicol Environ Health 2007; Part A; 70: 1798-1805.
42. American Sheep Industry (ASI) [ONLINE] http://www.sheepusa.org

43. United States Department of Labor, Bureau of Labor Statistics (BLS) [ONLINE] http://bls.gov/

44. Martinez KF, Rao CY, Burton NC. Exposure assessment and analysis for biological agents. Grana. 2004; 43: 193-208.

45. Reynolds S.J., Milton D.K., Heederik D., Thorne P.S., Donham K.J., Croteau E.A., Kelly K.M., Douwes J., Lewis D., Whitmer M., Connoughton I., Koch S., Malmberg P., Larsson B., Deddens J, Saraf, A., Larsson, L. Interlaboratory evaluation of endotoxin analyses in agricultural dusts – comparison of LAL assay and mass spectrometry. J. Environ. Monit 2005; 7: 1371-1377.

46. Rylander R. and Fernanda M. Airways inflammation among workers in poultry houses. Int. Arch. Occup. Environ. Health 2006; 79: 487-490.

47. Smid T, Heederik D, Houba R, Quanjer PH. Dust and endotoxin-related respiratory effects in the animal feed industry. Am Rev Respir Dis 1992; 146: 1474-1479.

48. Liebers V, Raulf-Heimsoth M, Bruning T. Health effects due to endotoxin inhalation (review). Arch Toxicol 2008; 82: 201-210.

49. Smit L, Wouters I, Hobo M, Eduard W, Doekes G, Heederik D. Agricultural seed dust as a potential cause of Organic Dust Toxic Syndrome. Occup Environ Med 2006; 63: 59-67.

50. Smit L, Heederik D, Doekes G, Lammers J, Wouters I. Occupational endotoxin exposure reduces the risk of atopic sensitization but increases the

risk of bronchial hyperresponsiveness. Int Arch Allergy Immunol 2009; 152:151-58.

51. Wilson PJ, Khozani TT, Juurlink BH, Senthilsven A, Rennie DC, Gerdtz V, Gawaziuk J, Schneberger D, Burch LH, Dosman JA. In vitro production of tumor necrosis factor-alpha by human monocytes stimulated with lipopolysaccharide is positively correlated with increased blood monocytes after exposure to a swine barn. J Toxicol Environ Health 2008; Part A; 71: 1401-1406.
52. Senthilselvan A, Cockcroft D. Assessment of endotoxin levels in the home and current asthma and wheeze in school age children. Indoor Air 2007; 18: 447-453.
53. Vogelzang P, van der Gulden J, Folgering H, Kolk J, Heederik D, Preller L, Tielen M, van Schayck C. Endotoxin exposure as a major determinant of lung function decline in pig farmers. Am J Respir Crit Care Med 1998; 157: 15-18.
54. Christiani D, Wang X, Pan L, Zhang H, Sun B, Dai H, Eisen E, Wegman D, Olenchock S. Longitudinal changes in pulmonary function and respiratory symptoms in cotton textile workers. Am J Respir Crit Care Med 2001; 163: 847-853.
55. Burch J, Svendsen E, Siegel P, Wagner S, von Essen S, Keefe T, Mehaffy J, Martinez A, Bradford M, Baker L, Cranmer B, Saito R, Tessari J, Prinz L, Anderson C, Christiansen O, Koehncke N, Reynolds S. Endotoxin exposure and inflammation markers among agricultural workers in Colorado and Nebraska. J of Toxicol Env Health 2010; Part A; 73: 5-22.

56. Spurzem JR, Romberger DJ, Von Essen SG. Agricultural Lung Disease. Clin Chest Med 2002; Dec; 23(4):795-810.

57. LeVan T, Von Essen S, Romberger D, Lambert G., Martinez F, Vasquez M, Merchant J. Polymorphisms in the CD14 gene associated with pulmonary function in farmers. Am J Respir Crit Care Med 2005; 171: 773-779.

58. InvivoGen [ONLINE] Agonositic and antagonistic effects of LPS on TLR4; Available at www.invivogen.com

59. Mueller M, Lindner B, Kusumoto S, Fukase K, Schromm A, Seydel U. 2004. Aggregates are the biologically active units of endotoxin. J of Biol Chem 2004; 279: 26307-26313.

60. Brass D, Hollingsworth J, Fessler M, Savov J, Maxwell A, Whitehead G, Burch L, Schwartz D. The IL-1 type 1 receptor is required for the development of LPS-induced airways disease. J Allergy Clin Immunol 2007; 120: 121-7.

61. Alexis N, Lay J, Almond M, Bromberg P, Patel D, Peden D. Acute LPS inhalation in healthy volunteers induces dendritic cell maturation *in vivo*. J Allergy Clin Immunol 2005; 115: 345-50.

62. Ekman A, Fransson M, Rydberg C, Adner M, Cardell L. Nasal Challenge with LPS stimulates the release of Macrophage Inflammatory Protein 1aplha. Int Arch Allergy Immunol 2009; 149: 154-160.

63. Eder W, Klimecki W, Yu L, von Mutius E, Reidler J, Braun-Fahrlander C, Nowack D, Martinez F. Opposite effect of CD14/-260 on serum IgE levels in

children raised from different environments. J Allergy Clin Immunol 2005; 116: 601-7.

64. Rennie D, Lawson J, Kirychuk S, Paterson C, Wilson P, Senthilselvan A, Cockcroft D. Assessment of endotoxin levels in the home and current asthma and wheeze in school age children. Indoor Air 2008; 18: 447-453.
65. Mastrangelo G, Grange J, Fadda E, Fedeli U, Buja A, Lange J. Lung cancer risk: Effect of dairy farming and the consequence of removing that occupational exposure. Am J Epidemiol 2005; 161(11): 1037 – 1046.
66. Janke, M. [ONLINE] Innovation in endotoxin detection – Moving out of the stone age, Manufacturing; International Pharmaceutical Industry; Available at http://www.lonza.com/group/en/products_services/products/catalog_new.ParSys.0007.File0.tmp?path=eshop\endotoxin\IPI_Moving_Out_of_Stone_Age_Autumn_2009.pdf
67. Chen, L. [ONLINE] In the lab: Reckoning recombinant factor, Pharmaceutical Formulation & Quality; Dec/Jan 2006; Available at http://www.pharmaquality.com
68. Lonza. Assay Kit Insert. 8830 Biggs Ford Road. Walkersville, MD 21793
69. Spaan S, Heederik D, Thorne P, Wouters I. Optimization of airborne endotoxin exposure assessment: Effects of filter type, transport conditions, extraction solutions, and storage of samples and extracts. Appl Environ Micro 2007; Ocotber; 6134 – 6143.

70. Spaan S, Doekes G, Heederik D, Thorne P, Wouters I. Effects of Extraction and Assay Method on Analysis of Airborne Endotoxin. Applied Environ Micro 2008; June: 3804 – 3811.
71. Reynolds S, Thorne P, Donham K, Croteau E, Kelly K, Lewis D, Whitmer M, Heederik D, Douwes J, Connaughton I, Kock S, Malmberg P, Larsson B, Milton D. Comparison of Endotoxin Assays Using Agricultural Dusts. AIHA Journal 2002; 63: 430-438.
72. Douwes J., Versloot P., Hollander A., Heederik D., DoekesG. Influence of various dust sampling methods on the measurement of airborne endotoxin. Appl Environ Micro 1995; May; 1763-1769.
73. Noss I, Doekes G, Sander I, Heederik D, Thorne P, Wouters I. Passive Airborne Dust Sampling with the Electrostatic Dustfall Collector: Optimization of Storage and Extraction Procedures for Endotoxin and Glucan Measurement. Ann Occup Hyg 2010; 54(6): 651-58.
74. Luna Bevin. Endotoxin air sampling: A comparison between the Recombinant Factor C (rFC) and Limulus Amebocyte Lysate (LAL) endtoxin assays. Master's Thesis. Department of Environmental and Radiological Health Sciences – Colorado State University Fort Collins, CO Fall 2009.
75. Whery, E.L. (1997) Chapter 26. Molecular Fluorescence and Phosphorescence Spectrometry. In Settle, F. (Ed.), Handbook of Instrumental Techniques for Analytical Chemistry (pp. 507-539). Retrieved from http://www.prenhall.com/settle/chapters/ch26.pdf