Using QPCR for Rapid Quantification of Occupational Exposures to *Brucella abortus*

By

Matthew L. Gruber

A thesis submitted in partial fulfillment of the
requirements for the degree of

Masters of Science in Industrial Hygiene

Montana Tech of The University of Montana

2012

Abstract

Brucella abortus is the causative agent of the zoonotic disease Brucellosis. *B. abortus* is currently found within the Yellowstone ecosystem, which allows for the transmission of the pathogenic agent from wild bison and elk to domestic cattle populations. The transmission between animals has created the possibility of occupational exposures to humans through inhalation and contact with the bacterium.

Current industrial hygiene bacterial monitoring methods consist of air sampling onto a culture plate or air samples collected within various types of filter cassettes, which are then analyzed through culturing methods. Culturing techniques are labor intensive and unreliable in quantifying exposure concentrations, as they are dependent on the ability to maintain cell viability. Quantitative Polymerase Chain Reaction (QPCR) has been used to quantify bacterial exposures through non-culture based methods. The use of a biological air sampling impingement system (SKC BioSampler®) was coupled with QPCR as methodologies for quantifying occupational exposures to *B. abortus*.

The reliability of the sampling system was determined by aerosolizing *B. abortus* DNA at concentrations of 1X, 1.5X and 30X. All concentrations were air sampled with an SCK BioSampler® in triplicate and analyzed by QPCR. The reliability was determined within sample groupings by comparing mean concentrations. Results show that there was no significant difference in the quantification means when sampling with a SKC impingement system and analyzing through QPCR; at concentrations 1X and 30X. Significance was found within the concentration sample 1.5X, due to variance within the grouping sample 1.5X-2. In Addition, the quantified concentrations detected increased as the concentration nebulized increased.

This research project was a feasibility study using QPCR and a SKC BioSampler® to quantify occupational exposures to B. abortus. Methods described within this research could prove to be a valuable tool for quantifying occupational *B. abortus* exposures and may contribute towards the understanding of quantifying bioaerosols.

Keywords: QPCR, *Brucella abortus*, Industrial hygiene, Occupational sampling, SKC BioSampler®.

Acknowledgements

I would like to thank the departments of Industrial Hygiene and Biology at Montana Tech of the University of Montana, Butte, Montana. I would also like to thank the Montana Conservation Genetics Laboratory at the University of Montana, Missoula, Montana. Without the guidance and knowledge of Steve Amish, none of this would have been possible. Thank you for being a great mentor and friend. I significantly appreciate the donations of equipment from SKC Inc. and Anaconda Home Oxygen. I would like to thank all of my family and friends for your love and support, without your encouragement my dreams would not be possible. I would like to thank Danielle for taking this journey with me, your love and support will never be forgotten! To my mother: you have shown me how to perceiver and work hard, the life lessons you taught me, have made me the man I am today.

Table of contents

List of Tables

List of Figures

Introduction

This research project was undertaken from an occupational health prospective to add and establish sampling methodologies for the pathogenic bacterium, *Brucella abortus*. The prevalence of *B. abortus* within the Greater Yellowstone Ecosystem (GYE) has created the possibility of exposures to humans in the workplace (Treanor et al., 2011). Individuals who are exposed to *B. abortus* can become acutely and chronically ill from the pathogen (Young, 1995). Associated occupational monitoring and quantification methods are limited and currently rely on post-exposure assessments and culture-based diagnosis (Navarro et al., 2008). This research investigated the use of Quantitative Polymerase Chain Reactions (QPCR) and an SKC impingement BioSampler® as an alternative to historic culture based analytical methods for quantifying airborne transmission of *B. abortus*.

Statement of Problem

Brucellosis is the most common zoonotic disease in the world, resulting in half a million new cases per year, with a prevalence rate in developing countries reaching 10 cases per 100,000 people (Franco et al., 2007; Queipo-Ortuño et al., 2008). Brucellosis is a significant ailment in regions of Europe, Africa, the Middle East, South America and Asia, which has resulted in increased monitoring in these regions (CDC, 2010). Even though there has been considerable improvement in managing the spread of brucellosis in many countries, there are still areas where the disease is present in wild and domestic animal populations (Treanor et al., 2011). These pockets of infection and potential transmission can represent significant health hazards to human populations.

The spread of brucellosis via *B. abortus* within the Greater Yellowstone Ecosystem (GYE) has been a contentious topic. Infections within wildlife populations can have significant

financial consequences for the United States agricultural industry, which has spent an estimated $3.5 billion on eradication efforts (Newby et al., 2003). The transmission of brucellosis by bison and elk to domestic cattle populations has gained attention from ranchers, scientists, clinicians and state and federal management agencies. The spread of brucellosis to domestic cattle can result in a state losing its ability to export beef on the international market, which is one of the largest economic resources for western states (Treanor et al., 2011).

Brucellosis is transmitted to humans via inhalation of the aerosolized bacterium, oral ingestion and/or direct contact with cuts or mucus membranes (Noviello et al., 2004). Infections have been documented by the Centers for Disease Control (CDC) to be an occupational disease affecting individuals in the agricultural industry, veterinarians, and laboratory personnel. Infections in humans can be incapacitating and immobilizing, while death is rare (CDC, 2010). Brucellosis can systemically infect any organ system within the body and has been show to physiologically present 2-3 weeks following infection (Young, 1995).

Historic detection for brucellosis exposures is limited and does not recognize infection until exposure is suspected or until the manifestation of clinical symptoms is experienced (Navarro et al., 2008). Post-infection detection techniques predominantly consist of blood cultures and serodiagnotics (Young, 1995). There is a need for quantification of exposure prior to the manifestation of symptoms, to inform workers of possible infections and to mitigate negative health effects associated with infection. Historic sampling methods for bioaerosols are time consuming and predominantly rely on culture techniques (An et al., 2006; Newby et al., 2003). Culturing does not allow for a reliable quantification of actual exposures and can result in an underestimation of total organism exposure (An et al., 2006). The use of culturing techniques for semi-quantification or qualification of occupational exposures has low specificity when other

bacteria are present in the sampling setting, as they can be cultured with all viable organisms (An et al., 2006).

Purpose of Research

The purpose of this preliminary study was to establish a method for quantifying airborne occupational exposures to *B. abortus*, utilizing Quantitative Polymerase Chain Reaction (QPCR) and a SKC BioSampler®. The sampling and analysis system was evaluated by comparing inter sample mean concentrations within similar sample groupings. This study was conducted in a laboratory setting. The methodologies established with this research may be adopted for use in an occupational setting.

QPCR Analysis

Quantitative Polymerase Chain Reaction (QPCR) is a sensitive and specific method of quantifying concentrations of DNA within molecular genetics research (Zemanick et al., 2010). Genetics is the study of the physical basis of DNA, with DNA being a large double stranded nucleic acid molecule, carrying the basic code of life. DNA consists of four nucleotides (adenine, guanine, cytosine and thymine) which form long double-helixed strands. The long DNA strands are produced by Polymerase Chain Reaction (PCR), a technique that allows for the amplification of specific DNA segments, in a short amount of time (Newby et al., 2003).

PCR uses three basic principles to accomplish the amplification of DNA. Separation is the initial step used to denature double stranded DNA into single strands suitable for future manipulation. The second step of PCR is annealing, which consists of specifically designed primers binding to the DNA molecules. The extension of DNA is the third process in which the primers build complimentary stands of new DNA (Newby et al., 2003).

QPCR is the molecular technique that amplifies DNA and allows for the quantification of DNA at an initial concentration, prior to any genetic manipulations or techniques used in research (Zemanick et al., 2010). QPCR monitors, in real time, the amount of complementary DNA produced through the repeated steps of cycling. This allows for the quantification of a final concentration by comparing the initial DNA quantity to the final quantity present, following the PCR cycling process (Newby et al, 2003). This research used primers designed to amplify a specific target DNA fragment of interest (156 base pairs), which then allowed for the hybridization of the probe to the DNA fragment (Newby et al., 2003).

The Taqman probe is activated by the 5' exonuclease activity of the hot start taq polymerase, which cleaves the reporter dye from the Quencher dye during the extension of PCR (Newby et al., 2003). As the distance between the newly cleaved dyes becomes greater, the reporter dye gains intensity in florescence, which is measured throughout the repeated cycles of PCR, to quantify a mass to volume ratio of initial DNA concentrations. This QPCR reaction has increased sensitivity and specificity to other general PCR methods (Newby et al., 2003). QPCR compared to Colony Forming Units (CFU) and Most Probable Number (MPN) enumeration, is widely accepted as a superior means of quantification compared to general culturing techniques (Queipo-Ortuño et al., 2008). The Applied BioSystems StepOne™ Plus analysis platform uses a standard curve to quantify a mass to volume ratio, which allows for the accurate quantification of exposures to specific organisms. The quantification of a mass to volume ratio, then allows for a calculation of a total organism exposure (Newby et al., 2003).

SKC BioSampler®

The use of the SCK BioSampler® allows for the collection of bioaerosols into a liquid medium, which eliminates the need to release and harvest cells from conventional air sampling

filters. The SKC BioSampler® provides constant sampling efficiency over longer sampling periods; collection efficiency is reported to be 90% in organisms with an aerodynamic diameter of 0.5 um (*B. abortus* aerodynamic diameter is estimated to be 0.5-0.7 um). A smaller particle size, result in a reduction of sampling efficiency, which continues to decrease as the size of the organism lessens. The device has been shown to create less particle bounce, which allows for the preservation of aggregates of bacterial organisms (SKC Inc., 2002). There is also a decrease of particle re-aerosolization due to the BioSampler® nozzles, which create a swirling airflow, allowing the organisms to be deposited directly into the liquid collection media without re-aerosolizing (SKC Inc., 2002). In addition, the collection media bubbles aggressively, collecting particles that may have been re-aerosolized. The SKC BioSampler® specifically allows for the use of QPCR analysis, by using a liquid medium that maintains cell viability and permits the centrifugation of the collection media to a notably smaller sampling volume of organisms appropriate for quantification (Fabian et al., 2009).

Hypothesis

This study was preliminary research to determine the methodologies and test the reliability of occupational air sampling for *B. abortus*; using QPCR coupled with a SKC BioSampler®. The following hypothesis was tested in this research study.

Null Hypothesis: There will be no statistically significant difference in the mean concentrations of *B. abortus* within individual sample groupings 1X, 1.5X and 30X ($p \geq 0.01$).

Alternative Hypothesis: There will be a statistically significant difference in the mean concentrations of *B. abortus* within the same sample groupings 1X, 1.5X and 30X ($p \leq 0.01$).

Background

To provide a foundation of knowledge for this research, the following information will be provided in this section: Bioaerosols, Occupational exposures to *B. abortus*, Brucellosis Toxicology and Brucellosis Epidemiology.

Bioaerosols

An aerosol is a collection of fluid and/or solid particles floating in a gaseous medium for a period long enough to be observed or quantified (Willeke and Baron, 1993). Bioaerosols are suspended particles originating from microorganisms (bacteria, fungi and viruses), typically ranging in size from 20 nm to less than 100 um. Bioaerosols tend to aggregate spontaneously when aerosolized, potentially exposing personnel to large quantities of the bacterium (Willeke and Baron, 1993). Transportation of bioaerosols is effected by many physical and environmental factors, such as size, shape and density of the organism (Willeke and Baron, 1993). The size, shape, and density of the organism are of significance because they are directly related to the aerodynamic diameter and the settling properties of the bioaerosols (Willeke and Baron, 1993).

Bioaerosols can be extremely complicated to recognize, quantify, and associate to occupational diseases (Willeke and Baron, 1993). Bioaerosols such as the Basidiomycete fungus can become sensitizing and can result in severe allergic reactions, while others, such as the influenza virus, can result in severe infections (Chen et al., 2009). There is significant evidence that brucellosis is capable of eliciting acute and chronic effects at extremely low concentrations (Young, 1995). The small aerodynamic diameter of *B. abortus* (0.5-0.7 um) allows the organism to stay suspended for up to 41 hours, resulting in a prolonged period of possible infection (CDC, 2010).

Occupational Exposure

Occupational exposure to Brucellosis occurs from the ingestion or inhalation of Brucella organisms or through organism contact with mucus membranes or cuts in the skin. The CDC states that "brucellosis is the most commonly reported laboratory associated bacterial infection" (CDC, 2010). The most common means of occupational transmission is through the inhalation of the aerosolized bacterium (CDC, 2010). Risk factors contributing to occupational exposures include contact with unknown infected samples, and working with pathogenic organisms with minimal experience. In addition, *B. abortus* has a low infective dose when aerosolized (CDC, 2010). Therefore, it is extremely difficult to monitor occupational exposures to Brucellosis (CDC, 2010). Within the GYE, ranchers, federal and state field workers, veterinarians, and laboratory personnel are of most concern for contracting brucellosis (CDC, 2010).

Toxicology

The fact that biological hazards present at extremely low concentrations along with the lack of established bioaerosol Occupational Exposure Limits (OELs), makes infection difficult to quantify concentrations and relate them to disease (Young, 1995). The Bureau of Emergency and Preparedness and Response estimates the infective dose of *B. abortus* is between 10 and 100 organisms, with an incubation period of 5 days to several months (Young, 1995).

Acute clinical manifestations are highly polymorphic, allowing *B. abortus* to affect any organ or body system, resulting in complications of the following systems: osteoarticular (spondylitis), hepatic, respiratory, genitourinary (orchitits and epididymitis), reproductive (abortion of fetus), cardiovascular (endocarditis) and neurological (neurobrucellosis) (Young, 1995). The physiological symptoms vary from acute febrile illness (fever, sweats, headaches, back pains and weakness) to lasting chronic effects (Young, 1995).

Infections are considered chronic after 12 or more months of acute clinical symptoms from the time of diagnosis. Therefore, chronic infections are categorized into three types, (1) relapse, (2) localized infection and (3) delayed convalescence (Young, 1995). Relapse has been determined with the reappearance of acute symptoms taking place after completion of treatment (Young, 1995). Traditional relapse symptoms mimic initial manifestations such as fever, sweats, headaches, and weakness (Young, 1995).

Clinical diagnostics of relapsed individuals has shown the prevalence of elevated antibody development within their serum (Young, 1995). Localized infections have shown a similarity with relapses, as patients with localized infections have elevated antibodies in the serum, but differ as localized infections can require surgery to drain infections, along with the use of antimicrobial therapy (Young, 1995). Localized infections show a distinctiveness of signs and symptoms associated with the inability to eliminate the foci of infection associated with the previously described organ systems. Delayed convalescence is the perseverance of clinical manifestations without the signs of an infection in individuals who completed traditional treatment, along with the loss of antibodies in the serum. It has been shown that individuals who have delayed convalesce do not benefit from continued antimicrobial treatments. While Brucellosis is rarely deadly, it can be highly devastating and immobilizing to affected individuals (Young, 1995).

Epidemiology

Brucellosis is rare in the United States, but may be underreported as much as 50%, due to medical provider's unfamiliarity with the clinical manifestations and an inability to diagnosis the infection (Franco et al., 2007). Over one thousand fifty- six cases were reported in the US from 1993-2002, with Wyoming having the highest annual incidence rate. Forty-six states had

reported at least one case from 1993-2002, with 26 states reporting one case in 2002 (Pappas et al., 2006).

Sampling and Analysis Literature Review

This section discusses limitations of historic sampling and analytical techniques used to quantify exposures to *B. abortus*. New methods of quantitative analysis that are non-culture based are explored in this literature review section. In addition, research studies regarding air sampling and analysis of bioaerosols are reviewed. Relevant sampling equipment utilized in the monitoring of various bioaerosols and the associated collection media are summarized. Analysis techniques and sampling equipment of bioaerosol quantification are discussed in independent sub-sections.

Limitations of Historic Sampling and Analysis

Bioaerosols of interest for researchers, are those that predominantly result in negative health effects to humans or result in economic losses in industry, such as *Mycobacterium tuberculosis* (TB), *Legionella pneumophila*, Lactococcal bacterial phages, and the H5N1 influenza virus (Chang et al., 2010; Chang and Chou, 2011; Fabian et al., 2009; Wu et al.,2010; Chen and Li, 2005). The historic analytical method for the quantification of bioaerosols in occupational settings has consisted of air sampling, followed by culturing the bacterium (Zemanick et al., 2010). Culturing methods involve collecting organisms into a liquid or onto solid or semi-solid media such as agar gel and growing the cells to achieve observable numbers of colony forming units (CFUs) (Fabian et al, 2009). *B. abortus* organisms are difficult to culture, often taking 1-2 months to incubate (ADHS, 2004). Culturing methods are designed to count CFUs, which may underestimate the total number of organisms sampled. Non-culture based analytical methods have been developed and have been utilized as an alternative analysis method to culturing in quantifying exposures. The use of epifluorescence, light microscopy, flow cytometry, and PCR have commonly been employed to quantify bioaerosols (Chang et al., 2010;

Chang and Chou, 2011; Chen and Li, 2005; Wu et al.,2010; Fabian et al., 2009; Verreault et al., 2011; Zemanick et al., 2010).

Analysis

Microscopy-based quantification methods have been used to quantify total organism concentration within air samples (Baskin et al., 2010). The quantification by epifluorescence for biological organisms is accomplished by combining DNA and fluorescent oligonucleotide probes to a gelatin matrix and incubating, which allows for ligation of the fluorophores and DNA molecules (Baskin et al., 2010). The matrix is then placed in the microscope, a picture is captured and quantification calculations are made according to the fluorescent molecules observed, the microscope magnification setting, and the associated pixel volume (Baskin et al., 2010). The use of epifluorescence has been infamously difficult to perform with the use of microscopes and gelatins, which can increase pipetting errors and decrease the ability to quantify (Baskin et al., 2010). In addition, the use of microscopy and epifluorescence is time and labor consuming and is not species specific. The inability of these methods to define specific species with a high sensitivity and specificity lacks scientific validity in bioaerosol research (An et al., 2006). The use of microscopy is an alternative to culturing based methods, but may not be suitable for the quantification of biological hazard exposures (An et al., 2006).

Flow cytometry has been used as a non-culture based method to quantify bioaerosols (Chen and Li, 2005). The use of fluorochrome with an AGI-30 to quantify levels of *Escherichia coli* and *Bacillus subtilis* was compared with traditional culturing methods. It was determined that the bioaerosol viability during the sampling process and the sequent analysis was dependent on the bioaerosol characteristics and by the physiological characteristics associated with the fluorochrome. The integrity of the cell membrane and the ability to culture was found to be more

linked to the general characteristics of the species being sampled. Flow cytometry can be used as a method for bioaerosol quantification and viability analysis, but contain potential flaws when wishing to quantify distinctive species (Chen and Li, 2005).

Conventional PCR is used to provide a qualitative or semi-quantitative analysis when coupled with electrophoresis, which allows for the visualization of the amplicon (An et al., 2006). Furthermore, PCR has limitations in the ability to quantify bioaerosol exposures with high accuracy, reliability, and reproducibility. The development and implementation of QPCR has increased the validity and usability over conventional PCR and other alternative analysis methods. The use of QPCR has the advantages of quantification for species specific identification over traditional PCR and does not use electrophoresis gels (An et al., 2006). QPCR has become the gold standard in the quantification of biological hazards and specifically bioaerosols. The coupling of QPCR with ethidium monoazide (EMA) has been show to distinguish between viable and non-viable organisms, by gaining entrance into cells that have defects in their cellular membrane (Chang and Chou, 2011). Following the entrance into the cell, EMA then disrupts the covalent links within the DNA, which prohibits the amplification of the DNA during QPCR analysis (Chang and Chou, 2011). The advancement in the ability to detect and quantify organisms that are only viable will provide insight in to overall exposures to bioaerosol. Without EMA treatment prior to QPCR, nonviable cells will be quantified and accounted for in the overall infectious exposure.

Bioaerosol Sampling Research and Associated Equipment

Air sampling research for bioaerosols has included various collection media, including solid and semi-solid filters as well as liquid solutions. The selection of the sampling media has

been dependent on the type of biological hazard being sampled, such as a virus, bacteria, or fungi (Wu et al., 2010). The focus has been on the use of gelatin filters, liquid impingement biosamplers, polytetrafluoroethylene filters, polycarbonate filters, teflon filters and coriolis cyclones, (Chen and Li, 2005; Wu et al.,2010; Verreault et al., 2001; Fabian et al., 2009) .

A study of air sampling methodologies for *L. pneumophila* compared the reliability of five sampling devices; an Institute of Occupational Medicine (IOM) personal inhalable air sampler with a gelatin filter (SKC Inc.); a 37 mm three piece cassette with a polycarbonate filter; an All Glass Impinger (AGI-30); a SKC Biosampler®; and a MAS-100 microbial air monitoring system (Chang and Chou, 2011). The research investigated the differences in filtering and impingement sampling methods and established the sampling equipment most appropriate for the collection and preservation of viable cells. The IOM sampler, AGI-30, and the SKC BioSampler® were determined to be the most appropriate for bioaerosol sampling, with the BioSampler® being the most efficient for preserving culturable cells (Chang and Chou, 2011). The IOM with the gelatin filter was found to outperform the liquid based samplers in total cell collection (Chen and Chou, 2011). This research for *L. Pneumophila* displayed how the appropriateness in selecting the proper sampling devices depends on the needs of the researcher, the organisms of interest, and the availability of the equipment (Chang and Chou, 2011).

The detection of Lactococcal bacteriophages within cheese manufacturing facilities was researched using Polytetrafluoroethylene filters, polycarbonate filters, a SKC BioSampler®, a coriolis cyclone and the NIOSH two stage cyclone bioaerosol personal samplers (Verreault et al., 2011). Samples were collected and then analyzed by QPCR to determine the presence of two Lactococcal latics phage groups (936-like and c2-like) (Verreault et al., 2001). It was found that the NIOSH two stage samplers provided the highest positive rates of phage detection, with 92%

in the first stage and 67% in the second stage (Verreault et al., 2001). The Coriolis sampler detected 72% of positive sample collection, however the second phage of interest (c2-like) was collected in significantly higher concentrations than the NIOSH sampler was able to achieve (Verreault et al., 2001). The polytetrafluoroethylene and polycarbonate filters were found to have equivalent recovery rates, with both filters having a lower recovery rate then the NIOSH sampler and the Coriolis sampler (Verreault et al., 2001). The SKC BioSampler® showed lower detection rates when compared to the other sampling devices and positive samples were found to detect concentrations 10-100 times higher than the other sampling devices were able to achieve (Verreault et al., 2001).

The sampling and quantification of *M. tuberculosis* (TB) in a health care settings has employed a sampling method that uses a 37 mm Nuclepore filter cassette with DNA being extracted from the filter, which is then quantified by PCR (Chen and Li, 2005). The TB research was aimed at validating the filter and QPCR analysis in quantifying airborne exposures in hospitals. The research project determined that the Nuclepore filter 37 mm cassette coupled with QPCR is highly sensitive and resulted in rapid quantification of TB. In addition the NIOSH analytical method (0900) for sampling TB utilizes a polytetrafluoroethylene filter and is a qualitative analysis method that uses the basic PCR analysis for detecting airborne TB (NMAM, 2003).

Conclusion

In this research project it was determined to be advantageous to utilize a SKC BioSampler®, as it was generously donated to the project, has been demonstrated to be appropriate for use with bacteria, and is compatible with QPCR analysis. The ability to centrifuge and isolate DNA (Nucleotides) from a liquid media also played a role in the decision

making process of using a SKC BioSampler®. QPCR analysis was chosen because of its accuracy, precision and high specificity for species specific detection and quantification of biologicals (An et al., 2006). QPCR has been previously validated and optimized for *B. abortus* and is an advantageous analytical method compared to other non-culture quantification methods for *B. abortus*; as they are not widely used for the particular biological agent. In addition, QPCR has increased the speed of analysis and can act as a high throughput system for sample analysis.

Preliminary Methods

Preliminary research methods that were tested and optimized for this research, such as the collection of *B. abortus* through field collection, QPCR optimization and a Pilot study are detailed in this section.

Sample Collection

B. abortus samples were collected in the fall of 2010 for a National Institute of Health Research project from the Greater Yellowstone Ecosystem (GYE). Field crews collected samples from bison and elk in Jackson, Dubois, and Cody, Wyoming. Field crews opportunistically sampled from hunter-killed animals, taking inguinal and super mammary lymph nodes. Samples were sent to the University of Wyoming Wildlife Veterinary Institute to be isolated from the lymph. All samples were cultured to produce a working stock of whole killed organisms. These samples were used in this study as a working stock of DNA, to simulate exposures to *B. abortus*.

QPCR Optimization

Initial experiments were optimized for Brucella Q-PCR detection of the 5'-exonuclease Taqman probe IS711. Brucella-positive controls were obtained from colleagues in Portugal. An Applied BioSystems StepOne™ real-time PCR system was utilized in all experiments. Optimization began with 15 ul reactions, using two replicates on a six point standard dilution series with a dilution factor of 1:10, starting with a concentration of 1 ng/ul. Amplification for the first four experiments conducted showed little to no amplification and resulted with incorrect efficiency values well over 100%. Specifically, no amplification occurred in 16 of 28 standard dilution samples. Errors in the initial experiments were due to high standard deviations in the dilution replicate groups, along with the failure in the exponential and threshold algorithms associated with the ABI StepOne™ analysis platform. The initial R^2 value of 0.877, revealed a

variance in the fit between the standard curve regression line and the threshold amplification in this study (Applied BioSystems, 2010).

The possibility of low DNA template quantity was hypothesized to be affecting the reactions. Therefore, 0.5 ul of Bovine Serum Albumin (BSA) was added to each reaction containing Taqman Genotyping master mix. In addition, the elongation/annealing time was increased from 60 seconds to 90 seconds and the reaction volume was increased to 25 ul. The primers and probe were re-diluted at larger volumes to reduce pipetting errors. To increase amplification efficiency, a new dilution series using electronic pipettes was created for PCR reaction setup, as they were newly calibrated. DNA concentrations were adjusted to provide a 10^6 fold dilution in 5 ul DNA volumes. A standard dilution series of six points with four replicates at a dilution factor of 1:10 was used.

An amplification efficiency of 99.6% with the Taqman Genotyping Master Mix and a slope of -3.32 with a corresponding R^2 value of 0.995 were produced within the standard curve. The improved reactions provided amplification in 7 of 12 (58%) samples conducted in 3 independent optimized experiments at 10 fg, which is equated to successful amplification with just a few copies of DNA present (7.5fg=1 Brucella organism) (Newby et al., 2003). Optimized results for the next dilution series up (100 fg) provided a 100% amplification success in 12 of 12 reactions. Taqman Environmental Master Mix (EMM) is intended for use in research samples that have high levels of PCR inhibitors. The QPCR optimization utilizing the EMM produced results of 99.9% amplification efficiency, a slope of -3.322 with an R^2 of 0.98. Amplification success was determined to be 63% at 10 fg, and 100% at 100 fg, as illustrated in Table 1.

	1 ng/ul	100 pg/ul	10 pg/ul	1 pg/ul	100 fg/ul	10 fg/ul
Genotyping Master Mix Amp	**12**	**12**	**12**	**12**	**12**	**7**

Genotyping Failure	**0**	**0**	**0**	**0**	**0**	**5**
Environmental Amp	**8**	**8**	**8**	**8**	**8**	**5**
Environmental Failure	**0**	**0**	**0**	**0**	**0**	**3**
Genotyping Success	**100%**	**100%**	**100%**	**100%**	**100%**	**58%**
Environmental Success	**100%**	**100%**	**100%**	**100%**	**100%**	**63%**
Total Amplification Success	**100%**	**100%**	**100%**	**100%**	**100%**	**60%**

Table : QPCR Optimization Results for quantifiing *B. abortus* concentrations. A 1:10 dilution series explored the amplification efficeiency between two potential reagents (genotyping master mix and environmental master mix) and spiked *B. abortus* samples. The total amplification success is shown to be 100% down to 100 fg/ul and 10 fg/ul provided 60% total amplification success. Both respective master mix's showed 100% amplification down to 100 fg/ul, while genotyping provided 58% and enviromental 60% amplification at 10 fg/ul.

Pilot study

A pilot study was conducted to refine the methodology for this research. The quantification of primary stock concentrations was conducted in the lab of Kuenzi and Pedulla, room 205 in the Chemistry and Biology Building (CBB) located on the campus of Montana Tech of the University of Montana in Butte, Montana. Three primary stocks of DNA samples were Nano dropped to determine nucleic acid concentrations prior to dilution to working concentrations. Six DNA samples were spiked in a Relimed® Compressor Nebulizer for aerosolization. A standard dilution series was used to obtain 1 ml of DNA in sample concentrations of 30X (15.23 ng/ul), 1.5X (750 pg/µl), 1X (500 pg/µl), with all samples aerosolized at a loaded volume of 10 ul. The same base concentrations samples were also aerosolized at a loaded volume of 20 ul.

A Gast model 1532 high flow air sampling pump was calibrated while connected to the SKC BioSampler® at 12.5 liters per minute (LPM) with a Gilian high flow Gilibrator. The air sampling duration was 30 minutes. The BioSampler® was placed ten inches from the nebulizer at a height of eight inches.

Two different sample preparations were conducted to determine the specific methodologies needed to pellet DNA from the liquid sampling media. The 10 ul loaded samples were centrifuged at 3800 rpm for 20 min, while the 20 ul loaded samples were centrifuged at 10,000 rpm for 10 min. Both samples were then adjusted to 1.5 ml volumes and centrifuged at 13,000 rpm for 5 min. The samples were then adjusted to a final sample volume of 100 ul.

Pilot samples were then analyzed by QPCR to determine the optimum sampling variables to be utilized in the research (Table 2). The Data provided from this pilot revealed that to gain the highest degree of sample amplification, DNA at a 20 ul volume would be introduced and the samples would be centrifuged at 10,000 rpm for 10 min. Samples tested under these parameters provided the greatest amplification percentages, which guided the research forward.

Table : Pilot study amplification results for airborne *B. abortus* exposures. To determine the parameters in conducting research, two groups of spiked sample volumes were tested to determine the variable necessary to optimize experimentation. Sample volumes of 10 ul and 20 ul were spiked in air samples and analyzed by QPCR. Results showed that 20 ul spiked samples produced greater overall total amplification efficiency, when compared to the 10 ul spiked samples.

Samples	No amp	Amp	Total	total amp %
30x-10ul	1	5	6	83%
1.5x-10ul	4	2	6	33%
1x-10ul	2	4	6	67%
30x-20ul	0	8	8	100%
1.5x-20ul	2	6	8	75%
1x-20ul	4	5	8	0.63%

Methods and Materials

The techniques, equipment and method of statistical analysis used in this research are defined. The use of a SCK BioSampler® for air sampling, QPCR for quantitative analysis and Minitab ANOVA were employed in this study.

Sampling Location and Set Up

Sampling was conducted in the Science and Engineering, room 216D, on the campus of Montana Tech of the University of Montana in Butte, Montana. The sampling site was selected to decrease the effects of air current disturbances on the sampling site, such as activity by other lab personnel and the ventilation systems. The sampling room dimensions were 3.6 m x 1.8 m x 2.7 m, giving a total volume of 17.5 m^3. The sampling room had one door, no windows, two tables, three chairs and sampling laboratory equipment present. The laboratory table dimensions were 1.8 m x 0.762 m and 1.25 m x 0.628 m x 0.628 m, respectively. The first table contained the sampling pump, BioSampler® and nebulizer; the second table was used to support all associated reagents and a centrifuge. The ventilation inlet was restricted by stuffing intake vents with rags and sealing off with plastic to eliminate air current disturbances. The sampling room was disinfected prior to sampling by wiping all walls and mopping the floor with a 10% bleach solution.

DNA Extraction

All DNA extraction processes took place at the Montana Conservation Genetics Laboratory in the Biological Research Building, room 011, The University of Montana in Missoula, Montana. Genomic DNA was extracted using a Qiagen DNeasy blood and tissue kit and the cultured cells procedure. The protocol consisted of the adding 1 ml of cultured cells to 200 ul PBS, with the addition of 20 ul proteinase K and 200 ul buffer AL, then mixed

thoroughly by vortexing and incubating at 56˚C for 10 min. Add 200 ul ethanol (96%-100%) and mixed by vortexing. A DNeasy mini spin column was utilized to collect DNA from the prepared lysed cell extraction mixture, which was pipetted into a 2 ml collection tube holding the min spin column and centrifuged at 6000 x g for 1 minute, with the flow-through and 2 ml collection tube being discarded after centrifugation. The mini spin column was then placed in a new 2 ml collection tube and 500 ul of buffer AW1 was added and centrifuged at 6000 x g for 1 minute, with the flow-through and collection tube being discarded following centrifugation. The mini column was placed in new 2 ml collection tube and 500 ul AW2 buffer was added to the column, which was centrifuged at 20,000 x g for 3 minutes and the flow through and the collection tube were discarded following centrifugation.

The mini column was removed from the 2 ml collection tube and placed in a clean 1.5 ml microcentrifuge tube and 200 ul buffer AE was directly pipetted onto the column membrane. The tube was incubated at room temperature for 1 minute, and then centrifuged at 6000 x g for 1 minute to elute the DNA. The column was placed in a new 1.5 ml microcentrifuge and the DNA elution was directly pipetted onto the column membrane, and then centrifuged at 6000 x g for 1 minute (this increases DNA yield by releasing any DNA remaining on column membrane).

Air Sampling

The air sampling section of methods and materials describes the equipment utilized, such as a SKC BioSampler®, Gast high flow pump and a Relimed® medical nebulizer for bacterial aerosolization. The method of sample collection following the air sampling experimentation is described.

SKC BioSampler® and Gast High Flow Pump

Ten air samples were collected, with three independent sampling sessions at each concentration, denoted as 1X, 1.5X and 30X. In addition, a negative control sample was collected at the conclusion of all sampling experimentation; the same protocol was used to sample the negative control that was employed in the spiked concentration air sampling. Air samples were collected using an SKC BioSampler® impingement system connected to a Gast model 1532 high flow pump. The Gast pump was pre and post calibrated using a Gilian high flow Gilibrator primary flow meter. Each sampling session was calibrated by running the pump for 5 min, adding 15 ml TE buffer to the BioSampler® collection base and assembling the BioSampler®. The BioSampler® was then connected to the Gilibrator and adjusted to an average flow rate of 12.5 LPM (see Appendix C, Table 4 for calibration sheet). The BioSampler® was then disconnected from the Gilibrator.

Organism Aerosolization

A Relimed® compressor nebulizer was used to suspend *B. abortus* DNA. The nebulizer suspends a mist at 0.5 to 10 micrometers. The aerosolization was completed by adding 20ul of *B. abortus* DNA to 5 ml TE buffer in the nebulizing unit. A 75 degree mouth piece was attached, the nebulizer was connected to a base stand and the nebulizer pump was turned on. The nebulizer was placed at a height of 8 inches from the working surface and set approximately 10 inches from the BioSampler® (see Figure 1). Following the setup of the nebulizer, the sample start time was recorded to track a 30 minute sampling session and the door was closed.

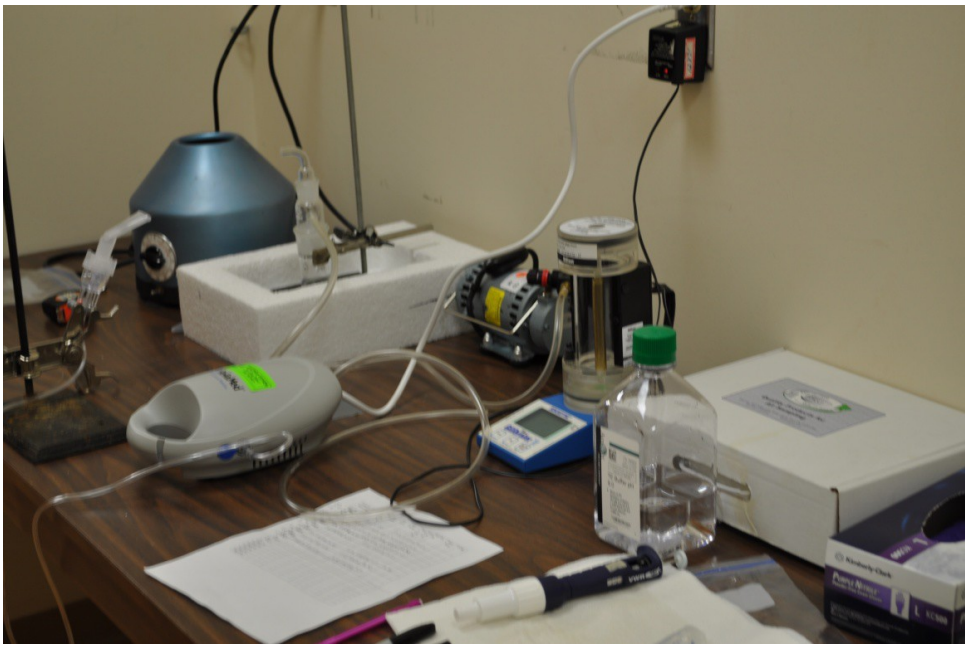

Figure : Sampling set up used in conducting experimental research. Displayed is the SKC BioSampler®, nebulizing unit, sampling pump, calibration equipment, reagents and centrifuge.

Sample Collection

Following the sampling session, The BioSampler® was disassembled to extract the sampling medium. The liquid sampling media was pipetted into a 15 ml falcon tube, labeled with a sample number, time, date, and the researcher's initials. All samples were directly centrifuged at 10,000 rpm for 10 minutes to concentrate and pellet the DNA in the bottom of the falcon tube. The supernate was drawn off by carefully pipetting to a volume of 1 ml. The 1 ml sample was then vortexed to re-suspend the DNA. The 1 ml falcon tube sample was transferred to a 1.5ml microcentrifuge tube and centrifuged at 13000 rpm for 5 minutes to pellet the DNA; the supernate was then drawn off to obtain a final volume of 100ul. Samples were then ready for QPCR analysis.

QPCR

All QPCR analysis was conducted at the Montana Conservation Genetics Laboratory in the Biological Research Building, room 011, The University of Montana in Missoula, Montana. Amplification and detection of DNA by QPCR was performed on an Applied BioSystems StepOne™ real-time PCR system, using Taqman genotyping master mix, primers, and probe. The prepared DNA samples were used as sample templates in the PCR reaction. Reaction mixtures consisted of 12.5 ul of Taqman master mix, 0.25 ul 5 um Taqman Probe, 0.25 ul 10 um forward primer, 0.25 ul 10 um reverse primer, and 11.75 ul DNA, for a total reaction volume of 25 ul.

The set up consisted of four replicates of a five point dilution series, two negative controls and two positive controls. A thin, 96 well reaction plate with snap caps was used to seal all reactions. Reaction plates were centrifuged to allow for all reagents to settle on the bottom of the plates. The Primers described by Newby et al. (2003); including the forward primer 5'-CCATTGAAGTCTG GCGAGC-3' and reverse primer5'CGATGCGAGAAAACATTGACCG-3', along with the 5'exonuclease probe 5'-FAM-GCATGCGCTATGATCTGGTTACGTT-(TAMRA)-3' were used. The primers produced a 156bp DNA fragment from the Brucella genome that includes portions of lab gene and the insertion element IS711. The thermal profile was initiated by stimulating the hot start taq polymerase at 95°C for 10 minutes, followed by 60 cycles of 15 seconds at 95°C and 90 seconds at 60°C.

Statistical Analyses

Statistical analysis was conducted to determine the significance between mean concentrations within sampling groups. Analysis of variance was then used to determine significance of the quantified means within each concentration grouping (1X, 1.5X and 30X).

The decision rule is that if the average means between inter concentration samples provided a P-value ≤ 0.01; the Null hypothesis would be rejected. The statistical analysis was completed using the computer program Minitab Version 16.

Results

The aim of this research was to validate the quantification of aerosolized *B. abortus* DNA in a simulated occupational setting. Results of air sampling for *B. abortus* were conducted thought QPCR analysis. Each concentration grouping was analyzed with the ABI StepOne™ Plus QPCR platform. Spiked samples were analyzed though a standard curve experiment, while the negative control sample was analyzed though a presence/absence experiment. Analysis of variance (ANOVA) within Minitab determined any significance of variance in the quantification of mean concentrations for each independent air sampled concentration group.

QPCR

The aerosolization and subsequent air sampling of *B. abortus* DNA was performed for each concentration grouping (1X, 1.5X, and 30X) in triplicate. The three concentrations grouping were each analyzed in four independent QPCR reactions. Each reaction provided an independent quantification of the concentration associated to the sample. Quantified concentrations are expressed as a mass, which is referenced to standard curve concentration. The ABI StepOne™ Plus analysis platform provided a QPCR efficiency of 92.04%, R^2= 0.975 and a standard curve slope of -3.529(Appendix A-Figure 4). Efficiencies between 90% and 110% are acceptable, with an R^2= 0.99 being desirable and a slope of -3.3 correlating to 100% reliability (Applied BioSystems, 2010). QPCR reported a total amplification of unknown concentration samples at 97% (35 of 36), with the non-amplifying sample being 30x-2 (found in table 3, row 2, column B)

The reported mean concentrations for sample groupings are reported in Table 3, column G. Sample concentration 30x is reported in rows 1-3, with each independent quantification reaction being reported in columns B-E (Table 3).The mean concentration of the four independent QPCR quantifications are reported in column F, along with the associated standard

deviation. Mean concentrations for Sample grouping 30X reported samples 30X-1 at 3.06 pg/ul, 30X-2 at 3.73 pg/ul and 30X-3 at 4.504 pg/ul (Table 3). Samples within concentration grouping 1.5X are reported in rows 4-6 and report the mean concentrations in column F (Table 3). Quantified mean concentrations for sample group 1.5X are 1.5X-1 at 0.451 pg/ul, 1.5X-2 at 1.022 pg/ul and 1.5X-3 at 0.1668 pg/ul (Table 3). Reported concentrations for group 1X are reported in rows 7-9, with mean concentration shown in column F. Concentration group 1X reported mean concentrations for samples; 1X-1 at 0.415, 1X-2 at 0.577 and 1X-3 at 0.361 pg/ul (Table 3). Sample grouping 30X had a PCR total mean of 3.76 pg/ul, sample 1.5X had a PCR total mean of 0.55pg/ul and sample 1X reported a total mean PCR concentration of 0.362 pg/ul Table 3, Column G (Figure 2, page 29). All sample concentrations are measured in pictograms per microliter (pg/ul).

Table : QPCR Results (pg/ul) of *B. abortus.* Experimental air sampling results analyzed though QPCR for sample groupings 1X, 2X and 3X. Each sample grouping was conducted in triplicate, with respective samples being independently aerosolized and analyzed via QPCR. Individual samples were summed and averaged (column F) across independent QPCR reaction to quantify a total mean QPCR concentration (column G) within sample groupings. Standard deviation was calculated across individual QPCR samples (column F), along with the associated p-value for each concentration grouping (H).

	A	B	C	D	E	F	G	H
	Sample	**PCR-1**	**PCR-2**	**PCR-3**	**PCR-4**	**PCR**	**PCR**	**P-Value**
1	30x-1	1.76266	2.96849	4.89729	2.61028	3.06	3.76	0.453
2	30x-2	0	4.85628	2.78759	3.54481	3.73		
3	30x-3	4.27133	5.26018	6.56979	1.91296	4.504		
4	1.5x_1	0.56338	0.55212	0.53843	0.15124	0.451	0.55	0.001
5	1.5x_2	0.79768	1.39285	0.90782	0.99157	1.022		
6	1.5x_3	0.29896	0.14064	0.12879	0.09874	0.1668		
7	1x-1	0.35514	0.43124	0.38403	0.48963	0.415	0.362	0.061
8	1x-2	0.46985	0.45680	0.69719	0.68199	0.5765		
9	1x-3	0.17529	0.49479	0.37236	0.40343	0.3615		
1	Neg	No AMP	No AMP					

Negative Control

Negative controls were analyzed through a presence/absence detection method; because negative control samples were not conducted until after spiked air sampling experimentation and

the quantitative analysis was performed. Negative control samples were analyzed by the ABI StepOne™ Plus analysis platform. Analysis was performed by comparing the negative air samples to two different known spiked concentrations. The presence of no amplification shown in the two independent QPCR samples analyzed can be seen as absent when compared to the four positive amplified presence samples (Appendix A, Figure 5).

Statistical Analysis

Analysis of variance (ANOVA) and the Tukey method within Minitab reported p-values for significant differences in the mean concentrations within sample groupings. Associated p-values are 0.453 for sample 30X, 0.001 for sample 1.5X, and 0.061 for sample 1X (Table 3, column H). Samples 1X and 30X were found to have no significant difference (P-Value $\geq$ 0.01) within the mean quantified concentrations, while sample concentration group 1.5X reported inconsistencies within the mean quantified concentrations. Particularly in concentration 1.5X, samples 1 and 3 showed no significant difference between samples, but did show significant differences when compared to sample 1.5X-2. To further investigate the overall sampling of QPCR and the SKC BioSampler®, the concentration groupings 1X, 1.5X, and 30X were analyzed to determine the actual sampling difference between concentration groupings. The data showed the total mean concentrations following a concentration variance of 1X, 1.2X, and 8.5X instead of the theoretical 1X, 1.5X, and 30X format (Figure 2).

Figure : Spiked sample mean concentration relationships for experimental airborne *B. abortus* exposures. Minitab calculated the concentration variance among sample grouping of 1X, 2X and 30X. This graph displays the variance to be 1X, 1.2X and 8.5X, which deviates from the theoretical variance of 1X, 2X and 30X

Discussion and Conclusion

This study was essentially the first of its type in coupling QPCR and the SKC BioSampler® to quantify occupational exposures to *B. abortus*. Therefore, numerous unknowns were present in determining the preliminary methodologies for this research. The main question was if *B. abortus* could be aerosolized and quantified from air samples in the simulated laboratory setting and reliably quantified.

Historic methods for quantifying exposures to biological hazards rely on air sampling and analysis though culture based methods (Willeke and Baron, 1993). Advanced research methods are utilizing solid and liquid collection mechanisms to capture a wide variety of viable biological organisms. The quantification abilities for bioaerosols, which that rely on non-culture based techniques, such as QPCR, have become considered the gold standard in research. This research project determined that the use of QPCR coupled with a liquid impingement sampling device provided acceptable methods of quantification for aerosolized *B. abortus*.

Statistical software was used to determine if there were significant differences in mean concentrations of similar sampling groupings quantified through QPCR. The data showed that as the sample concentrations aerosolized increased, associated concentrations also increased. The failure to reject the null hypothesis occurred in samples 1X and 30X, while sample 1.5X rejected the null hypothesis, according to the data (Appendix D, Descriptive statistics, One-Way ANOVA and Graphical plots). The rejection of the null hypothesis for sample 1.5X was due to the variation seen between samples 1.5X-1, 3 and sample 1.5X-2 (Appendix D, Figure 13). The Tukey method of analysis within the ANOVA programs provided us with ability to differentiate between significant differences with grouping concentrations (Appendix D, Table 8).

The results from this study provided contradictory results as two of the three sampling trials supported the research during air sampling experimentation. The research demonstrated that aerosolized *B. abortus* DNA can be collected through a SCK BioSampler® and quantified with QPCR. We found the relationship that as spiked sample concentrations were increased and aerosolized, the quantification of their mean concentrations also increased when analyzed with QPCR (as illustrated in Figure 2). It should be noted that the quantified exposure level conducted in this research has been found to fall within or exceed the infective dose of 10-100 organism. In this research project, it is estimated that one organism relates to 7.5 fg. The lowest exposure level detected is 0.0987 pg/ul, while the greatest exposure reported is 6.5697 pg/ul. The adjusted exposure levels relate to 13 and 876 organisms respectively (adjustments made by converting exposure units from pg to fg and dividing by 7.5fg).

By utilizing a liquid impingement BioSampler® and QPCR, there were many advantages over conventional filters and culturing techniques used in qualifying biological hazards. The ability to yield purified DNA from a liquid media within the BioSampler® allowed for direct use in the QPCR assay and permitted the research to be performed under limited biosafety precautions. The use of DNA to simulate a laboratory exposure has allowed the development of preliminary sampling methodologies and analytical techniques using the actual equipment required to complete such research. This research demonstrated the potential of these methods and techniques being employed throughout the GYE to occupationally sample for *B. abortus*.

This research is the preliminary step to establishing methodologies that allow for rapid quantification of occupational exposures to aerosolized *B. abortus*. There is a significant amount of research required to empirically validate the sampling and analysis techniques to accurately and reliably quantify exposures. Funding would be a significant contribution to this project as the

use of QPCR reagents are extremely expensive and the production of a large sampling set would provide a more accurate and robust data set.

Limitations and recommendations

Limitations

Various limitations could have influenced the results of this research. Variables that influence the accuracy and precision in the quantification of bioaerosols, such as bioaerosol sampling and collection efficiency, PCR inhibitors, the use of simulated organism exposures (DNA) and the variability in low DNA template concentrations that were used in the research may have decreased the ability to sample and quantify aerosolized particles. Not conducting negative control air samples between each spiked sampling session could have led to residual DNA template within the sampling equipment, resulting in elevated quantification within sampling groups or providing quantification when no DNA was actually sampled. However, a negative control was conducted following sampling experimentation, which provided no evidence of contamination.

Recommendations for Future Research

The following recommendations are to direct future research to build upon the work performed in this study. The challenge in working with organisms that carry a Biosafety Level is to establish methods that are as real world as possible. 1. Creating a containment box that act as a closed system would allow for the use of whole killed *B. abortus* organisms in aerosolization and subsequent DNA extraction and QPCR analysis. This could reduce the amount of inhibitors encountered and yield more transparent results between sample groupings. 2. Procure a larger sample size and data set to strengthen the results of this research. 3. Adjust the sampling design to add a negative control air sample in-between each spiked air sampling trial, to ensure a clean system and that no residual organisms are left in the system and sampled in the air sampling sessions. 4. Use EMA to quantify only infective cells.

References

An, H. R., Mainelis, G., & White, L. (2006). Development and calibration of real-time PCR for quantification of airborne microorganisms in air samples. *Atmospheric Environment, 40*(40), 7924-7939. doi:10.1016/j.atmosenv.2006.07.020

Applied BioSystems. (2010). *Applied BioSystems StepOne™ and StepOnePlus™ real-time PCR systems.* Retrieved 05/23/1012, 2012, from http://tools.invitrogen.com/content/sfs/manuals/cms_046737.pdf

ADHS-Arizona Department of Health Services. (2004). *BRUCELLOSIS: Bioterrorism agent profiles for health care workers.* Retrieved 10/28, 2011, from http://www.azdhs.gov/phs/edc/edrp/pdf/brucellosisset.pdf

Baskin, D. S., Widmayer, M. A., & Sharpe, M. A. (2010). Quantification and calibration of images in fluorescence microscopy. *Analytical Biochemistry, 404*(2), 118-126. doi:10.1016/j.ab.2010.05.029

CDC-Center for Disease Control, National Center for Emerging and Zoonotic Infectious Diseases. (2010). *Brucellosis: General information.* Retrieved 05/01, 2012, from www.cdc.gov/nczved/divisions/dfbmd/diseases/brucellosis/

Chang, C. -. & Chou, F. -. (2011). Methodologies for quantifying culturable, viable, and total legionella pneumophila in indoor air. *Indoor Air, 21*(4), 291-299. doi:10.1111/j.1600-0668.2010.00701.x

Chang, C., Chou, F., & Hung, P. (2010). Evaluation of bioaerosol sampling techniques for legionella pneumophila coupled with culture assay and quantitative PCR. *Journal of Aerosol Science, 41*(12), 1055-1065. doi:10.1016/j.jaerosci.2010.09.002

Chen, P., & Li, C. (2005). Quantification of airborne mycobacterium tuberculosis in health care setting using real-time qPCR coupled to an air-sampling filter method. *Aerosol Science and Technology, 39*(4), 371-376. Doi: 10.1080/027868290945767

Chen, P., & Li, C. (2005). Sampling performance for bioaerosols by flow cytometry with fluorochrome. *Aerosol Science and Technology, 39*(3), 231-237. Doi: 10.1080/027868290925534

Chen, P., Lin, C. K., Tsai, F. T., Yang, C., Lee, C., Liao, Y., Lin, K. (2009). Quantification of airborne influenza and avian influenza virus in a wet poultry market using a Filter/Real-time qPCR method. *Aerosol Science and Technology, 43*(4), 290-297. Doi: 10.1080/02786820802621232

Fabian, P., McDevitt, J. J., Houseman, E. A., & Milton, D. K. (2009). Airborne influenza virus detection with four aerosol samplers using molecular and infectivity assays: Considerations for a new infectious virus aerosol sampler. *Indoor Air, 19*(5), 433-441. doi:10.1111/j.1600-0668.2009.00609.x

Franco, M. P., Mulder, M., Gilman, R. H., & Smits, H. L. (2007). Human brucellosis. *The Lancet Infectious Diseases, 7*(12), 775-786. Doi: 10.1016/S1473-3099(07)70286-4

Hospodsky, D., Yamamoto, N., & Peccia, J. (2010). Accuracy, precision, and method detection limits of quantitative PCR for airborne bacteria and fungi. *Applied and Environmental Microbiology, 10*(0099-2040), 7004-7012.

NMAM- NIOSH Manual of Analytical Methods, Third Supplement: DHHS (NIOSH) Publication No. 2003-154

Navarro-Martinez, A., Navarro, E., Castano, M., & Solera, J. (2008). Rapid diagnosis of human brucellosis by quantitative real-time PCR: A case report of brucellar spondylitis. *J Clin Microbiol, 46*(1), 385-387.

Newby, D. T., Hadfield, T. L., & Roberto, F. F. (2003). Real-time PCR detection of Brucella abortus: A comparative study of SYBR green I, 5′-exonuclease, and hybridization probe assays. *American Society for Microbiology, 69*(8)

Noviello, S., Gallo, R., Kelly, M., Limberger, R., DeAngelis, K., Cain, Dumas, N. (2004). Laboratory-acquired brucellosis. *Emerging Infectious Diseases, 10*(10), 1848-1850.

Pappas, G., Papadimitriou, P., Akritidis, N., Christou, L., & Tsianos, E. V. (2006). The new global map of human brucellosis. *The Lancet Infectious Diseases, 6*(2), 91-99. Doi: 10.1016/S1473-3099(06)70382-6

Queipo-Ortuño, M. I., Colmenero, J. D., Bravo, M. J., García-Ordoñez, M. Á., & Morata, P. (2008). Usefulness of a quantitative real-time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis. *Clinical Microbiology and Infection, 14*(12), 1128-1134. doi:10.1111/j.1469-0691.2008.02095.x

SKC Inc. (2002). *Biosampler operating instructions®.* (Operating manual No. 37084). PA: SKC Inc.

Treanor, J. J., Geremia, C., Crowley, P. H., Cox, J. J., White, P. J., Wallen, R. L., & Blanton, D. W. (2011). Estimating probabilities of active brucellosis infection in Yellowstone bison through quantitative serology and tissue culture. *Journal of Applied Ecology, 48*, 1324-1332.

Verreault, D., Gendron, L., Rousseau, G., Veillette, M., & Masse, D. (2011). Detection of airborne lactococcal bacteriophages in cheese manufacturing plants. *Applied and Environmental Microbiology, 2*(0099-2240), 491-497.

Willeke, K., & Baron, P. (1993). In Van Norstrand R. (Ed.), *Aerosol measurement: Principles, techniques and applications*. (1st Ed.). United States of America: Van Norstrand Reinhold.

Wu, Y., Shen, F., & Yao, M. (2010). Use of gelatin filter and BioSampler in detecting airborne H5N1 nucleotides, bacteria and allergens. *Journal of Aerosol Science, 41*(9), 869-879. doi:10.1016/j.jaerosci.2010.05.006

Young, E. (1995). An overview of human brucellosis. Clin infect dis. 1995 Aug; 21(2):283–290. *Clinical Infectious Diseases, 21*(2), 283-289.

Zemanick, E. T., Wagner, B. D., Sagel, S. D., Stevens, M. J., Accurso, F. J., Harris, J. K., & Gaggar, A. (2010). Reliability of quantitative real-time PCR for bacterial detection in cystic fibrosis airway specimens. PLoS ONE, 5(11), 15101.

Appendix A: QPCR Figures

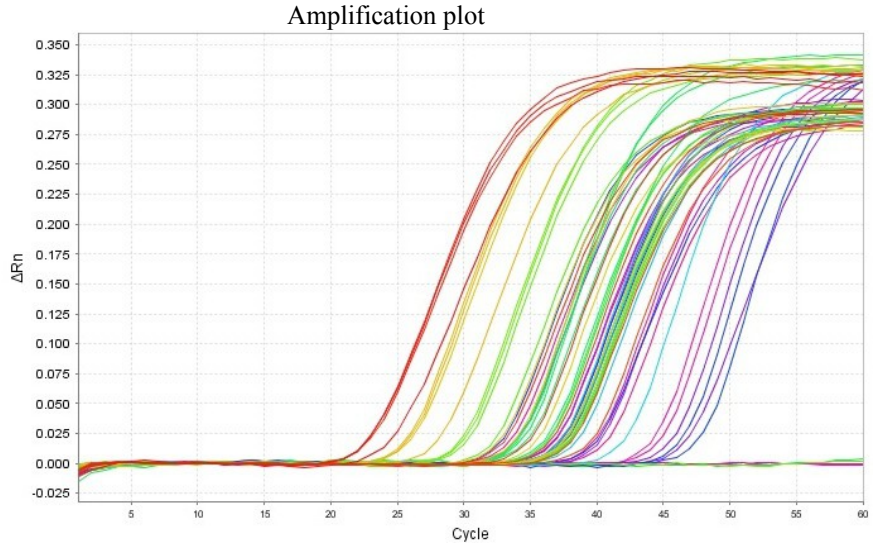

Figure : Representative amplification plot for all air sampling reactions. The amplification plot is used in QPCR experiments to express *B. abortus* concentration. The pathogen concentration is quantified during real-time PCR analysis based on the cycle in which fluorescence change is determined to be statistically increased above background levels. The amplification plot displays the change of fluorescence (y-axis) during PCR cycling (x-axis). Individual curves are grouped into similar quantified concentrations by color. Non curved samples that lack amplification, represent no amplification.

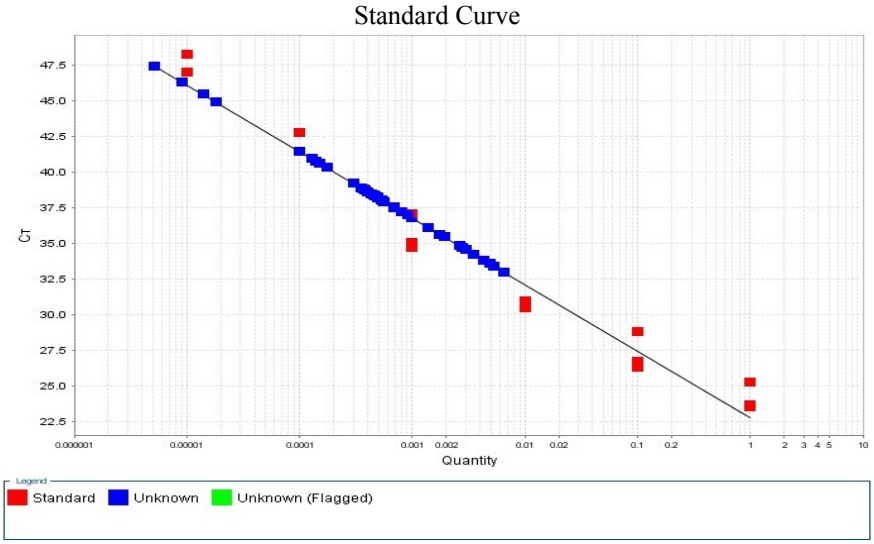

Figure : ABI StepOne™ standard curve graph for all air sample reactions. Standard curves are used to determine the absolute quantity of *B. abortus* in an air sample. Each red bar represents a known standard dilution concentration (1:10 dilution), while each blue bar represents an unknown experimental air sample concentration. Curves are generated by plotting fluoresce threshold cycle (y-axis) to quantified concentrations (x-axis).

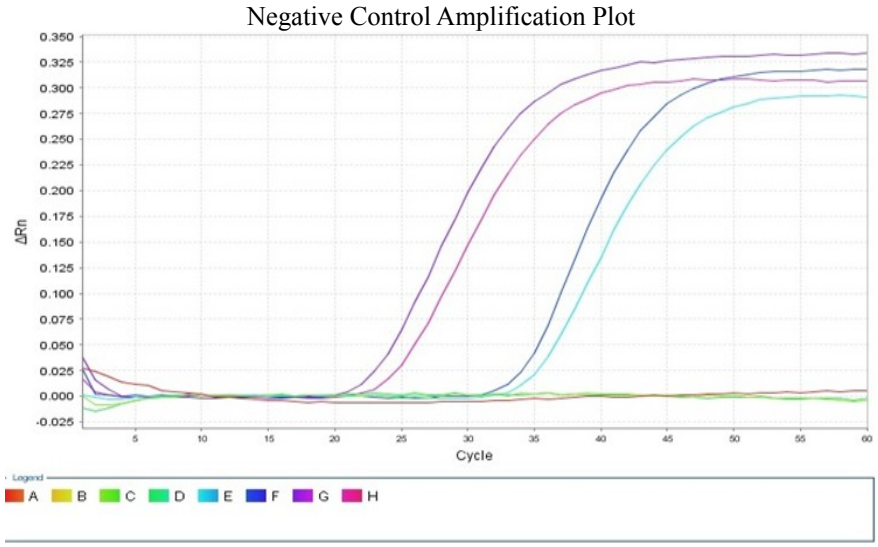

Figure : Representative negative control amplification plot. The amplification plot is used in QPCR experiments to express *B. abortus* concentration. The pathogen concentration is quantified during real-time PCR analysis based on the cycle in which fluorescence change is determined to be statistically increased above background levels. The amplification plot displays the change of fluorescence (y-axis) during PCR cycling (x-axis). Individual curves are grouped into similar quantified concentrations by color. Non curved samples represent negative controls (no amplification) and curved samples represent positive controls.

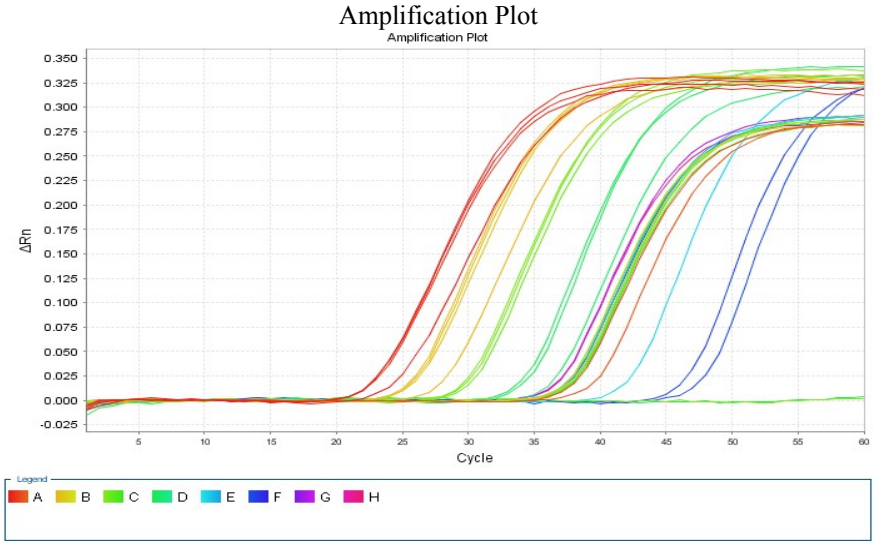

Figure : Amplification Plot for samples in concentration grouping 1X. The amplification plot is used in QPCR experiments to express *B. abortus* concentration. The pathogen concentration is quantified during real-time PCR analysis based on the cycle in which fluorescence change is determined to be statistically increased above background levels. The amplification plot displays the change of fluorescence (y-axis) during PCR cycling (x-axis). Individual curves are grouped into similar quantified concentrations by color. Non curved samples that lack amplification, represent no amplification.

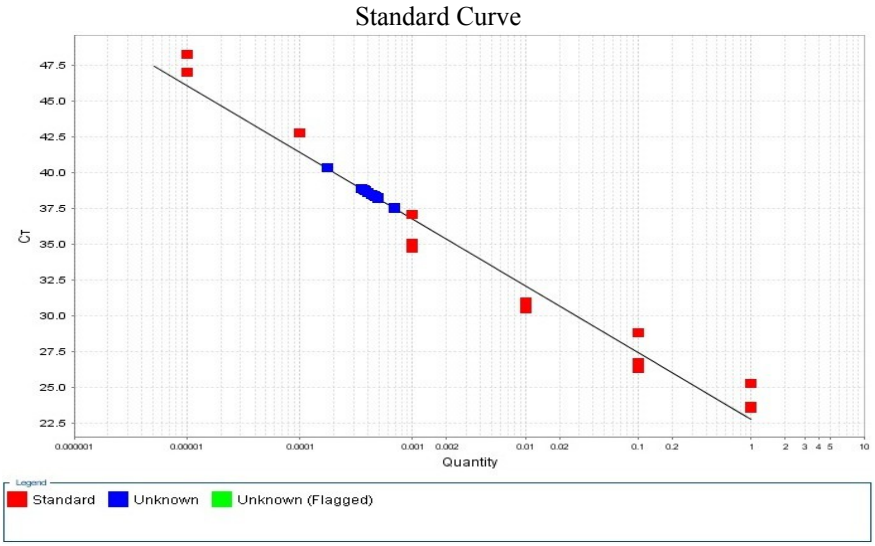

Figure : Standard Curve for samples of concentration grouping 1X. Standard curves are used to determine the absolute quantity of *B. abortus* in an air sample. Each red bar represents a known standard dilution concentration (1:10 dilution), while each blue bar represents an unknown experimental air sample concentration. Curves are generated by plotting fluoresce threshold cycle (y-axis) to quantified concentrations (x-axis).

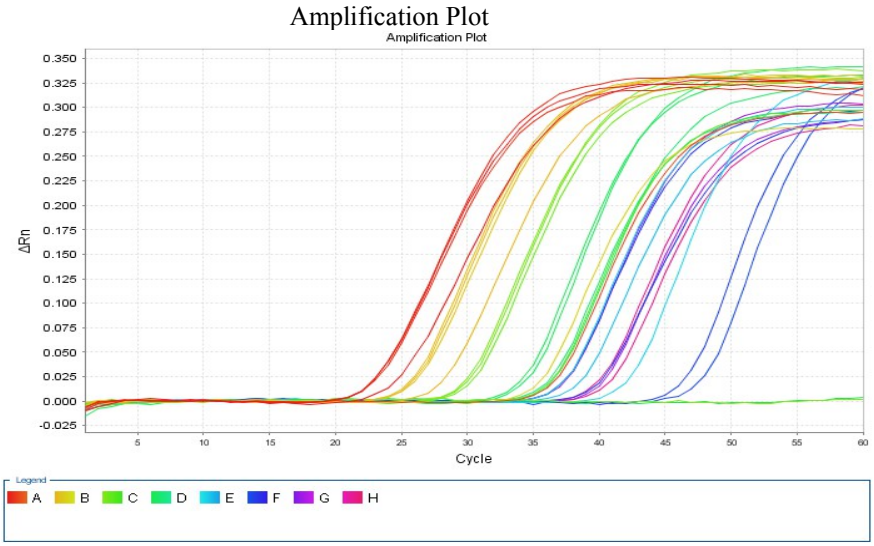

Figure : Amplification Plot for samples of concentration grouping 1.5X. The amplification plot is used in QPCR experiments to express *B. abortus* concentration. The pathogen concentration is quantified during real-time PCR analysis based on the cycle in which fluorescence change is determined to be statistically increased above background levels. The amplification plot displays the change of fluorescence (y-axis) during PCR cycling (x-axis). Individual curves are grouped into similar quantified concentrations by color. Non curved samples that lack amplification, represent no amplification.

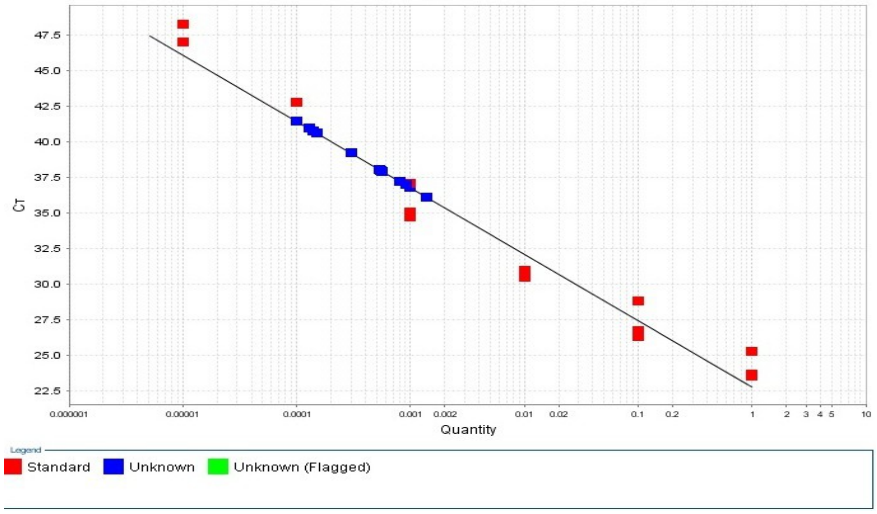

Figure : Standard Curve for samples of concentration grouping 1.5X. Standard curves are used to determine the absolute quantity of *B. abortus* in an air sample. Each red bar represents a known standard dilution concentration (1:10 dilution), while each blue bar represents an unknown experimental air sample concentration. Curves are generated by plotting fluoresce threshold cycle (y-axis) to quantified concentrations (x-axis).

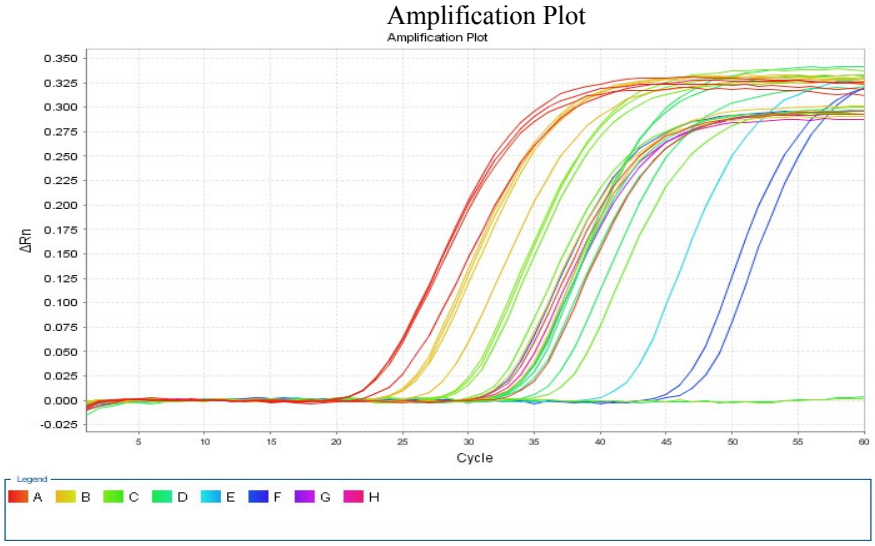

Figure : Amplification Plot for samples of concentration grouping 30X. The amplification plot is used in QPCR experiments to express *B. abortus* concentration. The pathogen concentration is quantified during real-time PCR analysis based on the cycle in which fluorescence change is determined to be statistically increased above background levels. The amplification plot displays the change of fluorescence (y-axis) during PCR cycling (x-axis). Individual curves are grouped into similar quantified concentrations by color. Non curved samples that lack amplification, represent no amplification.

Standard Curve

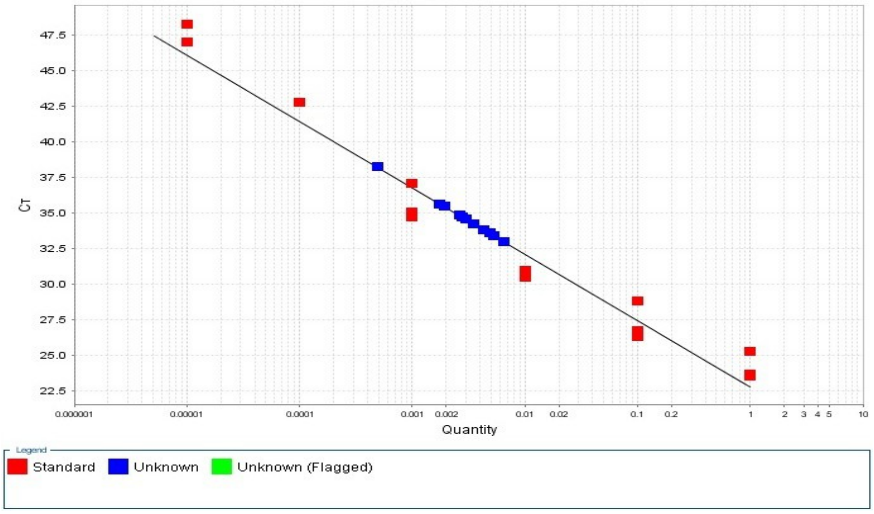

Figure : Standard Curve for samples of concentration grouping 30X. Standard curves are used to determine the absolute quantity of *B. abortus* in an air sample. Each red bar represents a known standard dilution concentration (1:10 dilution), while each blue bar represents an unknown experimental air sample concentration. Curves are generated by plotting fluoresce threshold cycle (y-axis) to quantified concentrations (x-axis).

Appendix B: Sampling Protocol

1. Turn on Gast sampling Pump
    a. Allow for a five min warm up
2. SKC BioSampler®
    a. Add 15 ml TE buffer sampler reservoir
    b. Assemble glass
    c. Attach Pump Tubing
3. Calibrate
    a. Turn on Gilibrator
    b. Attach to sample inlet of BioSampler®
    c. Adjust to 12.5 LPM
    d. Take 4 calibration readings
    e. Disconnect
4. Nebulizer
    a. Turn on pump
    b. Assemble tubing
    c. Add 5 ML TE and 20ul DNA sample
    d. Connect nebulizing unit to nebulizer pump
5. Sample time
    a. 30 min
6. Shut door
7. After 30 min shut down sampling Train
8. Disassemble BioSampler®
    a. Pipette TE sampling medium to a labeled 15 ml falcon tube
9. Sample preparation for analysis
    a. Centrifuge 15 ml sample
        a.i. 10 min at 10,000 rpm
    b. Pour off supernate
        b.i. Obtain 1.5 ml of air sample media
    c. Transfer to 1.5 ml microcentrifuge tube
        c.i. Centrifuge 5 min at 13,000 rpm
10. Pipette to final volume of 100ul

Appendix C: Calibration Data

Table : Pre and post calibration data for *B. abortus* air sampling experimentation. Individual samples within concentration grouping 1X, 1.5X, 30X and negative controls are reported. Each individual sample was pre and post calibrated four times, pre and post calibrations were averaged for a final calibration flow rate. Experimentation sessions were conducted over a 30 minute time frame. Calibration data is used as a control to monitor experimental flow rates and ensure sampling collection is preformed according to specification (12.5 lpm)

Date	Concentra	PRE-Cal#1	PRE-Cal#2	PRE-Cal#3	PRE-Cal#4	Pre-Total	Post Cal #	Post Cal #	Post Cal #	Post Cal #	Post-Total	Q Avg	Sample Duration
4/10/2012	1x-1	12.52	12.42	12.44	12.43	12.45	12.6	12.56	12.55	12.51	12.55	12.5	30 minutes
4/10/2012	1x-2	12.53	12.48	12.47	12.48	12.49	12.58	12.47	12.49	12.5	12.51	12.5	30 minutes
4/10/2012	1x-3	12.58	12.52	12.58	12.54	12.51	12.69	12.62	12.59	12.55	12.6125	12.56125	30 minutes
4/10/2012	1.5x-1	12.42	12.48	12.39	12.48	12.44	12.48	12.53	12.43	12.6	12.51	12.475	30 minutes
4/10/2012	1.5x-2	12.43	12.56	12.51	12.47	12.4925	12.6	12.51	12.52	12.53	12.54	12.51625	30 minutes
4/10/2012	1.5x-3	12.6	12.5	12.5	12.47	12.51	12.55	12.55	12.6	12.56	12.56	12.535	30 minutes
4/10/2012	30x-1	12.54	12.48	12.52	12.5	12.51	12.44	12.5	12.47	12.44	12.4625	12.48625	30 minutes
4/10/2012	30x-2	12.58	12.54	12.52	12.49	12.5325	12.58	12.45	12.4	12.5	12.4825	12.5075	30 minutes
4/10/2012	30x-3	12.52	12.44	12.52	12.44	12.48	12.62	12.68	12.44	12.59	12.5825	12.53125	30 minutes
4/19/2012	Neg Con	12.5	12.46	12.47	12.5	12.4825	12.46	12.54	12.61	12.47	12.52	12.50125	30 minutes

Appendix D: Minitab Data

Sample 30X

Table : Minitab descriptive statistics for concentration grouping 30X. Descriptive statistics quantitatively report the fundamental structures used in statistical analyses. The summaries of sample size, mean concentrations and standard deviations were used in our research.

Variable	N	N*	Mean	SE Mean	StDev	CoefVar	Minimum	Median	Q3
30x-1	4	0	3.060	0.663	1.325	43.32	1.763	2.789	4.415
30x-2	3	0	3.730	0.604	1.047	28.06	2.788	3.545	4.856
30x-3	4	0	4.504	0.983	1.967	43.68	1.913	4.766	6.242

Variable	Maximum
30x-1	4.897
30x-2	4.856
30x-3	6.570

Figure : Mean concentration quantification for sample grouping 30X. The group was sampled in triplicate, with four independent QPCR analysis for each independent sample. Each bar represents a sample within the 30X grouping that was analyzed via QPCR. The horizontal line within each bar represents the mean concentration among the four independent samples analyzed by QPCR.

Table : One-Way Analysis Of Variance (ANOVA) for concentration grouping 30X. ANOVA is a statistical test, which determines if the observed variance of mean concentrations within independent samples is significantly different. Statistical significance was determined by evaluating the associated p value to the hypothesis.

```
Source  DF     SS    MS     F      P
Factor   2   4.18  2.09  0.88  0.453
Error    8  19.07  2.38
Total   10  23.24

S = 1.544   R-Sq = 17.96%   R-Sq(adj) = 0.00%


                        Individual 95% CIs For Mean Based on
                        Pooled StDev
Level  N   Mean  StDev   -+---------+---------+---------+--------
30x-1  4  3.060  1.325   (----------*-----------)
30x-2  3  3.730  1.047     (-------------*-------------)
30x-3  4  4.504  1.967              (-----------*-----------)
                         -+---------+---------+---------+--------
                        1.5       3.0       4.5       6.0

Pooled StDev = 1.544


Grouping Information Using Tukey Method

       N   Mean  Grouping
30x-3  4  4.504  A
30x-2  3  3.730  A
30x-1  4  3.060  A

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 97.87%


30x-1 subtracted from:

        Lower  Center  Upper  -------+---------+---------+---------+--
30x-2  -2.699   0.670  4.038         (-------------*------------)
30x-3  -1.675   1.444  4.562             (------------*-----------)
                              -------+---------+---------+---------+--
                                  -2.5       0.0       2.5       5.0


30x-2 subtracted from:

        Lower  Center  Upper  -------+---------+---------+---------+--
30x-3  -2.594   0.774  4.142          (------------*-------------)
                              -------+---------+---------+---------+--
                                  -2.5       0.0       2.5       5.0
```

Sample 1.5x

Table : Minitab descriptive statistics for concentration grouping 1.5X. Descriptive statistics quantitatively report the fundamental structures used in statistical analyses. The summaries of sample size, mean concentrations and standard deviations were used in our research.

Variable	N	N*	Mean	SE Mean	StDev	CoefVar	Minimum	Median	Q3
1.5x-1	4	0	0.451	0.100	0.200	44.38	0.151	0.545	0.561
1.5x-2	4	0	1.022	0.130	0.259	25.37	0.798	0.950	1.293
1.5x-3	4	0	0.1668	0.0449	0.0899	53.88	0.0987	0.1347	0.2594

Variable	Maximum
1.5x-1	0.563
1.5x-2	1.393
1.5x-3	0.2990

Figure : Mean concentration quantification for sample grouping 1.5X. The group was sampled in triplicate, with four independent QPCR analysis for each independent sample. Each bar represents a sample within the 1.5X grouping that was analyzed via QPCR. The horizontal line within each bar represents the mean concentration among the four independent samples analyzed by QPCR.

Table : One-Way Analysis Of Variance (ANOVA) for concentration grouping 1.5X. ANOVA is a statistical test, which determines if the observed variance of mean concentrations within independent samples is significantly different. Statistical significance was determined by evaluating the associated p value to the hypothesis.

```
Source  DF      SS      MS      F      P
Factor   2  1.5192  0.7596  19.74  0.001
Error    9  0.3464  0.0385
Total   11  1.8656

S = 0.1962   R-Sq = 81.43%   R-Sq(adj) = 77.31%


                          Individual 95% CIs For Mean Based on
                          Pooled StDev
Level   N    Mean   StDev  --+---------+---------+---------+-------
1.5x-1  4  0.4513  0.2003          (-----*-----)
1.5x-2  4  1.0225  0.2594                              (-----*------)
1.5x-3  4  0.1668  0.0899  (------*-----)
                          --+---------+---------+---------+-------
                          0.00      0.35      0.70      1.05

Pooled StDev = 0.1962


Grouping Information Using Tukey Method

        N    Mean  Grouping
1.5x-2  4  1.0225  A
1.5x-1  4  0.4513    B
1.5x-3  4  0.1668    B

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 97.91%


1.5x-1 subtracted from:

          Lower   Center   Upper  --------+---------+---------+---------+-
1.5x-2   0.1837   0.5712  0.9586                       (----*-----)
1.5x-3  -0.6720  -0.2845  0.1030          (-----*----)
                                 --------+---------+---------+---------+-
                                      -0.70      0.00      0.70      1.40


1.5x-2 subtracted from:

          Lower   Center    Upper  --------+---------+---------+---------+-
1.5x-3  -1.2432  -0.8557  -0.4682  (-----*----)
                                  --------+---------+---------+---------+-
                                       -0.70      0.00      0.70      1.40
```

Sample 1x

Table : Minitab descriptive statistics for sample for concentration grouping 1X. Descriptive statistics quantitatively report the fundamental structures used in statistical analyses. The summaries of sample size, mean concentrations and standard deviations were used in our research.

Variable	N	N*	Mean	SE Mean	StDev	CoefVar	Minimum	Median	Q3
1x-1	4	0	0.4150	0.0294	0.0588	14.17	0.3551	0.4076	0.4750
1x-2	4	0	0.5765	0.0654	0.1309	22.71	0.4568	0.5759	0.6934
1x-3	4	0	0.3615	0.0673	0.1346	37.22	0.1753	0.3879	0.4719

Variable	Maximum
1x-1	0.4896
1x-2	0.6972
1x-3	0.4948

Figure : Mean concentration quantification for sample grouping 1X. The group was sampled in triplicate, with four independent QPCR analysis for each independent sample. Each bar represents a sample within the 1X grouping that was analyzed via QPCR. The horizontal line within each bar represents the mean concentration among the four independent samples analyzed by QPCR.

Table : One-Way Analysis Of Variance (ANOVA) for concentration grouping 1X. ANOVA is a statistical test, which determines if the observed variance of mean concentrations within independent samples is significantly different. Statistical significance was determined by evaluating the associated p value to the hypothesis.

```
Source  DF      SS      MS     F      P
Factor   2  0.1002  0.0501  3.88  0.061
Error    9  0.1161  0.0129
Total   11  0.2163

S = 0.1136   R-Sq = 46.33%   R-Sq(adj) = 34.40%

                           Individual 95% CIs For Mean Based on Pooled StDev
Level  N    Mean   StDev   -+---------+---------+---------+--------
1x-1   4  0.4150  0.0588        (----------*---------)
1x-2   4  0.5765  0.1309                   (----------*----------)
1x-3   4  0.3615  0.1346   (----------*----------)
                           -+---------+---------+---------+--------
                          0.24      0.36      0.48      0.60

Pooled StDev = 0.1136


Grouping Information Using Tukey Method

      N    Mean  Grouping
1x-2  4  0.5765  A
1x-1  4  0.4150  A
1x-3  4  0.3615  A

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 97.91%


1x-1 subtracted from:

        Lower   Center   Upper  --------+---------+---------+---------+-
1x-2  -0.0629   0.1614  0.3858                 (--------*--------)
1x-3  -0.2778  -0.0535  0.1708         (--------*--------)
                                --------+---------+---------+---------+-
                                     -0.25      0.00      0.25      0.50


1x-2 subtracted from:

        Lower   Center   Upper  --------+---------+---------+---------+-
1x-3  -0.4393  -0.2150  0.0093  (--------*--------)
                                --------+---------+---------+---------+-
                                     -0.25      0.00      0.25      0.50
```